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(54) **Title:** SINGLE-CHAIN TNF LIGAND FAMILY MOLECULES AND FUSION PROTEINS AND DERIVATIVES THEREOF

(57) **Abstract:** The present invention relates to a polypeptide comprising a component A with at least three tumor necrosis factor (TNF) homology domains of TNF-ligand family members (THD) in which C-terminal and N-terminal reference points are defined by consensus sequences and which are connected through short stretches of additional C-terminal and/or N-terminal amino acids of the THD or variants thereof, and a component B comprising a dimerization domain. Further, the present invention relates to a polypeptide comprising at least three THDs in which C-terminal and N-terminal reference points are defined by consensus sequences and which are connected through short stretches of additional C-terminal and/or N-terminal amino acids of the THD or variants thereof. Further, the invention relates to a nucleic acid comprising said polypeptides, a vector comprising said nucleic acid and a pharmaceutical composition comprising said polypeptides, or said nucleic acids or said vector. Further, the present invention relates to said polypeptides, said nucleic acid or said vector for the use as a medicament or for the use in the diagnosis, prophylaxis or treatment of hyperproliferative disorders and inflammatory disorders.



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SINGLE-CHAIN TNF LIGAND FAMILY MOLECULES AND FUSION PROTEINS AND DERIVATIVES
THEREOF

The present invention relates to a polypeptide comprising a component A with at least three tumor necrosis factor (TNF) homology domains of TNF-ligand family members (THD) in which C-terminal and N-terminal reference points are defined by consensus sequences and which are connected through short stretches of additional C-terminal and/or N-terminal amino acids of the THD or variants thereof, and a component B comprising a dimerization domain. Further, the present invention relates to a polypeptide comprising at least three THDs in which C-terminal and N-terminal reference points are defined by consensus sequences and which are connected through short stretches of additional C-terminal and/or N-terminal amino acids of the THD or variants thereof. Further, the invention relates to a nucleic acid comprising said polypeptides, a vector comprising said nucleic acid and a pharmaceutical composition comprising said polypeptides, or said nucleic acids or said vector. Further, the present invention relates to said polypeptides, said nucleic acid or said vector for the use as a medicament or for the use in the diagnosis, prophylaxis or treatment of hyperproliferative disorders and inflammatory disorder.

Background of the Invention

The cytokine TRAIL is capable of specifically inducing apoptosis in tumor cells without affecting non-transformed cells and, thus is considered as a valuable effector molecule for therapy of cancer and other diseases. Unlike other members of the tumor necrosis factor (TNF) superfamily, e.g. TNF or CD95L, TRAIL is well tolerated upon systemic application, which facilitates the development of TRAIL-based biopharmaceuticals. TRAIL activates the extrinsic apoptotic pathway via binding to death receptors (DR) 4 and 5 which leads to cell death of tumor cells. Nevertheless, effective tumor eradication upon triggering of death receptors is hampered by intracellular resistance mechanisms and low bioactivity of conventional TRAIL biopharmaceuticals. Different therapeutic strategies dealing with DR activation have been developed. Agonistic monoclonal antibodies directed against either DR4, such as Mapatumumab or DR5, e.g. Conatumumab and Lexatumumab have been tested in phase I or phase II clinical studies with ambiguous results (for review see Holland *et al.* 2014; Micheau *et al.*, 2013). Furthermore, soluble forms of TRAIL such as dulanermin (Herbst *et al.*, 2010 a, b) and a circularly permuted TRAIL (CPT, Chen *et al.*, 2012a) have been studied in several clinical trials. Although the combination of recombinant TRAIL with chemotherapeutics, e.g. thalidomide (Chen *et al.*, 2012b) has led to more promising results than the monotherapy with the recombinant product, current forms of TRAIL are still associated with disadvantages,

particularly a short half-life in the blood circulation, which does not exceed one hour. Thus, as a first step to overcome TRAIL-associated limitations, a single-chain format featuring three TRAIL modules (amino acid residues 95-281) connected by Gly/Ser peptides of 16, respectively 8 residues was developed, which has been shown to be more stable compared with soluble TRAIL (unpublished data; Schneider *et al.*, 2010; Siegemund *et al.*, 2012). Moreover, single-chain TRAIL can also be used for generation of antibody TRAIL fusion proteins. The homotrimeric TRAIL is naturally expressed as a membrane protein, which can also be present in a proteolytically cleaved, soluble form. This soluble form can induce apoptosis in tumor cells mainly via triggering the death receptor 4 (DR4) mediated extrinsic pathway, while a full activation of the apoptosis machinery involving an activation of death receptor 5 (DR5) still demands an oligomeric presence or localization of TRAIL at the plasma membrane. Since a therapeutic application of TRAIL should be based on a soluble protein, additional strategies are needed to mimic the bioactivity of membrane-localized TRAIL. The fusion of TRAIL or single-chain TRAIL to recombinant antibody formats, e.g. scFvs or diabodies, is an appropriate way to achieve a cell surface targeting, taking tumor specific markers into account (for review see de Bruyn *et al.*, 2013)). Such TRAIL fusion proteins have been shown to express higher tumor specificity and an increased serum half-life compared with soluble TRAIL (Schneider *et al.*, 2010). However, even the single-chain variants of TRAIL described so far exhibit a rather low thermal stability, with melting points of approximately 46-47 °C as determined by dynamic light scattering, which can affect therapeutic activity and stability during production and storage. In particular, evidence has been found that higher protein thermostability can directly correlate with an increased serum half-life time, actually representing one of the most requested properties in view of therapeutic applications for recombinant proteins (Gao *et al.*, 2009). Therefore, the development of derivatives of single-chain THDs (scTHDs), in particular single chain TRAILS (scTRAILS) with improved thermal stability and solubility is currently challenging. It has surprisingly been shown by the present inventors that the use of THDs with additional N- and/or C-terminal deletions and distinct mutations at the N- and C-terminus of the THD, in particular TRAIL provides several advantages including *inter alia* increased solubility of the proteins, increased thermal stability, higher recombinant production rate and higher molecular integrity, e.g. less degradation/chain termination. The use of these improved scTHDs in the diabody-scTHD format, in particular diabody-scTRAIL format provides further benefits for the therapeutic suitability of this format. This format has been further enhanced by including redesigned connecting peptides between V_H and V_L of the diabody as well as improving the connecting peptides between the diabody and the improved scTHD variants, in particular scTRAIL variants.

Summary of the Invention

In a first aspect the present invention provides a polypeptide comprising:

(i) component A comprising:

at least three THDs, wherein the C-terminus of the first and second THD,
5 respectively, which is in each case defined by the C-terminal consensus sequence

-S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1),

is linked to the N-terminus of the second and third THD, respectively, which is in
each case defined by the N-terminal consensus sequence

X₂-V/A/F-A-H-V/L/I/Y (SEQ ID NO: 2)

10 or

X₃-V/W/F/C-A/L-E/Y/Q/H-L (SEQ ID NO: 3),

through a peptide X_a, which is in each case independently selected and has a length
of 3 to 8 amino acids,

wherein X₁ is a non-polar/hydrophob or polar/neutral amino acid, preferably selected
15 from the group consisting of F, V, Q, A, I, L, and Y;

wherein X₂ is selected from the group consisting of P, K, V, I, and A;

wherein X₃ is selected from the group consisting of D, S, M, and I;

(ii) component B comprising

a dimerization domain consisting of an antibody V_L and V_H region linked directly to
20 each other with a peptide that has a length of between 7 and 11 amino acids.

In a second aspect the present invention provides a polypeptide comprising:

component C comprising:

at least three THDs, wherein the C-terminus of the first and second THD, respectively,
which is in each case defined by the C-terminal consensus sequence

25 -S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1),

is linked to the N-terminus of the second and third THD, respectively, which is in each
case defined by the N-terminal consensus sequence

X₂-V/A/F-A-H-V/L/I/Y (SEQ ID NO: 2)

or

30 X₃-V/W/F/C-A/L-E/Y/Q/H-L (SEQ ID NO: 3),

through a peptide X_c, which is in each case independently selected and has a length of 3 to
5 amino acids,

wherein X₁ is a non-polar/hydrophob or neutral/polar, amino acid, preferably selected from
the group consisting of F, V, Q, A, I, L, and Y;

35 wherein X₂ is selected from the group consisting of P, K, V, I, and A; and

wherein X_3 is selected from the group consisting of D, S, M, and I.

In a third aspect the present invention provides a nucleic acid encoding the polypeptides of the first and second aspect of the present invention.

In a fourth aspect the present invention provides a vector comprising the nucleic acids of the third aspect of the present invention.

In a fifth aspect the present invention provides a pharmaceutical composition comprising the polypeptides of the first or second aspect of the present invention or the nucleic acid of the third aspect of the present invention or the vector of the fourth aspect of the present invention.

In a sixth aspect the present invention relates to a polypeptide of the first or the second aspect, a nucleic acid of the third aspect or a vector of the fourth aspect for use as a medicament.

In a seventh aspect the present invention relates to a polypeptide of the first or the second aspect, a nucleic acid of the third aspect or a vector of the fourth aspect for use in the diagnosis, prophylaxis or treatment of proliferative disorders and anti-inflammatory disorders.

List of Figures

In the following, the content of the figures comprised in this specification is described. In this context please also refer to the detailed description of the invention above and/or below.

Figure 1: ScTRAIL molecules of this invention

Schematic representations of scTRAIL molecules described in the present application. With the exception of scTRAIL-95L8, THDs of human TRAIL spanning the amino acid positions 122 to 278 are depicted as boxes. Amino acid substitutions within the THDs are underlined.

Figure. 2: Western Blot analysis of selected scTRAIL molecules

The solubility of selected scTRAIL molecules with crucial amino acid deletions or substitutions at (A) positions 280, 281 and 122 or (B) positions 279 and 122 was tested in an anti-FLAG western blot. To this, HEK293 cells were transiently transfected with equal amounts of plasmid DNA and cultivated for three days in OptiMEM I medium supplemented with 50 μ M zinc chloride, followed by collecting of supernatants (S) and cell pellet samples (P) for reducing SDS-PAGE/western blotting. Underlined constructs produced soluble protein by the majority and were used for further characterization.

Figure 3: Melting point analysis of scTRAILS

The melting points of scTRAIL molecules were analyzed by dynamic light scattering using a zetasizer instrument.

Figure 4: Size exclusion chromatography of scTRAILS

Affinity-purified scTRAIL molecules were analyzed by size exclusion chromatography for their molecular constitution. The retention times of the standard proteins thyroglobulin (669 kDa), beta-amylase (200 kDa), bovine serum albumin (67 kDa), carboanhydrase (29 kDa) and FLAG peptide are indicated by lines.

5 **Figure 5: Binding studies of scTRAILS**

Selected scTRAIL molecules were tested for their binding to either (A) Colo205 cells using flow cytometry, or to (B) immobilized DR5-Fc fusion protein using an ELISA approach. Data points were fitted with curves and values of half-maximum binding (EC50) were determined.

Figure 6: Cytotoxicity test

10 The cytotoxic activity of the scTRAIL molecules was assayed in vitro on Colo205 colon cancer cells in presence of 250 ng/ml Bortezomib. The percentage of surviving cells was measured by crystal violet staining. Data points: mean $\pm$ S.E.M (n=3).

Figure 7: SEC and cytotoxicity test of scTRAIL₃-FLVGGVA

15 Purified scTRAIL₃-FLVGGVA was separated via size exclusion chromatography and fractions of the eluate were collected in order to separate the putative dimeric/aggregate (D) from the monomeric (M) form. After measuring the protein content of the fractions, equimolar concentrations of both species were subjected to a cytotoxicity assay using the cell lines Colo205, NCI-H460 and HT1080 with or without Bortezomib sensitization. Dependent on the specific cell line, the bioactivities of monomeric and dimeric scTRAIL were found to differ
20 significantly.

Figure 8: SEC of Db-scTRAIL-95L8

Db-scTRAIL-95L8 was separated by SEC (A) and fractions were blotted followed by detection with anti-FLAG M2 antibody (B). The peak represented by the fractions 6 and 7 is due to intact dimeric fusion protein. The peak involving the fractions 9 to 11 is characterized by signals from
25 a full-length polypeptide chain and a ~40 kDa fragment comprising FLAG tag, VH, VL and a part of the first TRAIL monomer. This fragment likely forms a dimer with a full-length polypeptide chain yielding a partially fragmented dimer.

Figure 9: SEC and bioactivity analysis of Db-scTRAIL variants

(A) Different molecular forms of Db-scTRAIL-95L8 and Db-Glyco-scTRAIL₃-FAVSGAA
30 were separated preparatively by SEC. (B) The molecular forms "A" and "B" were analyzed for their bioactivity in a cell viability assay on HCT-116, HT1080 and NCI-H460 cells with the indicated concentrations of the proteasome inhibitor Bortezomib.

Figure 10: Comparison of scTRAIL variants

Previously generated scTRAIL-95L8 (Siegemund *et al.*, 2012) and new scTRAIL₃-FAVSGAA (SEQ ID NO: 227) were compared by SEC. In addition, scFv or diabody fusion proteins comprising both versions of scTRAIL, respectively scTRAIL₃ are shown.

Figure 11: SEC of scTRAIL variants

5 Db-scTRAIL₃-FAVSGAA (upper left), Db-Glyco-scTRAIL₃-FAVSGAA (upper right), Db10-scTRAIL₃-FAVSGAA (lower left) and Db10-Glyco-scTRAIL₃-FAVSGAA (lower right) were analyzed by size exclusion chromatography (SEC) to demonstrate the effect of the glycosylated connecting peptide on protein quality.

Figure 12: Dimerization analysis of Db-scTRAIL₃ variants

10 (A) SEC analysis revealed the occurrence of a second molecular form in preparations of several Db-scTRAIL variants which could be identified as a dimer with a truncation in one polypeptide chain (arrow). Additionally, aggregates were observed for example in a preparation of Db-scTRAIL₃-FAVSGAA. (B) A combination of a glycosylated peptide between the V_L domain and the scTRAIL moiety together with an extension of the diabody connecting peptide to 8 or 10
15 residues has been proven to successfully reduce aggregation and fragmentation of the protein. The SEC analysis confirmed the quantitative dimerization of Db-Glyco-scTRAIL₃-FAVSGAA, Db8-Glyco-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA. (C) SEC analysis of Db-Glyco-scTRAIL₃-FAVSGIA.

20

Figure 13: Thermal stability of Db-scTRAIL₃ variants

Purified Db-scTRAIL-95L8, Db-Glyco-scTRAIL-95L8, Db-scTRAIL₃-FAVSGAA, Db-Glyco-scTRAIL₃-FAVSGAA, Db-Glyco-scTRAIL₃-FAVSGIA, Db10-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA were analyzed for their melting temperatures (T_m) by
25 dynamic light scattering.

Figure 14: Binding to cells

(A) Db-scTRAIL-95L8, Db-Glyco-scTRAIL-95L8, Db-Glyco-scTRAIL₃-FAVSGAA, Db-Glyco-scTRAIL₃-FAVSGIA, Db8-Glyco-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA were analyzed for their binding abilities to EGFR+ TRAILR+ HT1080 cells using
30 flow cytometry. The differences in the EC₅₀ values between the different Db variants are statistically not significant (P = 0.11, n = 3).

Figure 15: Cell viability assay in Colo 205 cells

Preparations of Db-scTRAIL-95L8, Db-Glyco-scTRAIL₃-FAVSGAA, Db-Glyco-scTRAIL₃-FAVSGIA, Db8-Glyco-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA were
35 analyzed for their bioactivity in a cell viability assay using Colo 205 sensitized with 250 ng/ml

of the proteasome inhibitor Bortezomib (mean $\pm$ SEM, n = 4). Where indicated, 10 μ g/ml of the anti-EGFR antibody Cetuximab was added prior to fusion protein incubation for EGFR competition studies.

Figure 16: Cell viability assay in HCT-116 cells

5 Preparations of Db-scTRAIL-95L8, Db-Glyco-scTRAIL₃-FAVSGAA, Db-Glyco-scTRAIL₃-FAVSGIA, Db8-Glyco-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA were analyzed for their bioactivity in a cell viability assay using HCT-116 sensitized with 5 ng/ml of the proteasome inhibitor Bortezomib (mean $\pm$ SEM, n = 2). Where indicated, 10 μ g/ml of the anti-EGFR antibody Cetuximab was added prior to fusion protein incubation for EGFR
10 competition studies.

Figure 17: Cell viability assay in HT 1080 cells

Preparations of Db-scTRAIL-95L8, Db-Glyco-scTRAIL₃-FAVSGAA, Db-Glyco-scTRAIL₃-FAVSGIA, Db8-Glyco-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA were analyzed for their bioactivity in a cell viability assay using HT1080 sensitized with 10 ng/ml of
15 the proteasome inhibitor Bortezomib (mean $\pm$ SEM, n = 2). Where indicated, 10 μ g/ml of the anti-EGFR antibody Cetuximab was added prior to fusion protein incubation for EGFR competition studies.

Figure 18: SDS-PAGE analysis

SDS-PAGE analysis (8% PAA, Coomassie stained) of Db-scTRAIL₃-FLVGGGPQRVA, dsDb-scTRAIL₃-FLVGGGPQRVA, scFv-EHD2-scTRAIL₃-FLVGGGPQRVA and scFv-Fc-scTRAIL₃-FLVGGGPQRVA under reducing (1, 2, 3, 4) and non-reducing (5, 6, 7, 8) conditions for fusion proteins targeting EGFR (A), HER2 (B), and HER3 (C). D) SDS-PAGE analysis of non-targeted constructs scTRAIL₃-FLVGGGPQRVA, EHD2-scTRAIL₃-FLVGGGPQRVA and Fc-scTRAIL₃-FLVGGGPQRVA under reducing (9, 10, 11) and non-reducing conditions (12,
20 13, 14).

Figure 19: SEC of EGFR-scTRAIL fusion protein

Size exclusion chromatography of EGFR-targeting dimeric scTRAIL₃-FLVGGGPQRVA fusion proteins.

Figure 20: Thermal stability analysis of Dbhu225-scTRAIL₃

30 Melting point of Dbhu225-scTRAIL₃-FLVGGGPQRVA was determined by dynamic light scattering.

Figure 21: Target antigen binding analysis (ELISA)

Binding of targeted dimeric scTRAIL₃-FLVGGGPQRVA fusion proteins to EGFR-Fc (A), HER2-Fc (B), and HER3-Fc (C) was analyzed by ELISA. Optical density was measured at 450
35 nm. Data are represented as mean $\pm$ S.D. (n=3).

Figure 22: TRAILR2 binding studies (ELISA)

Binding of scTRAIL₃-FLVGGGPQRVA fusion proteins targeting EGFR (A), HER2 (B), HER3 (C) and non-targeted constructs (D) to TRAIL-R2-Fc was analyzed by ELISA. Optical density was measured at 450 nm. Data are represented as mean $\pm$ S.D. (n=3).

Figure 23: Binding studies to intact Colo205 cells

Binding of scTRAIL₃-FLVGGGPQRVA fusion proteins targeting EGFR (A), HER2 (B), HER3 (C) and non-targeted constructs (D) to Colo205 cells was analyzed by flow cytometry. Data are represented as mean $\pm$ S.D. (n=3).

Figure 24: Cytotoxicity induction in Colo205 cells by TRAIL variants: effects of sensitizers and EGFR targeting

Induction of cell death of EGFR-targeting (A) and non-targeted (B) scTRAIL₃-FLVGGGPQRVA fusion proteins on Colo205 cells was analyzed in the absence and presence of Bortezomib (650 nM, 250 ng/ml). Effects of targeting were investigated by preincubation with Cetuximab (200-fold molar excess). Data are represented as mean $\pm$ S.D. (n=3).

Figure 25: Cytotoxicity induction in Colo205 cells upon HER2 and HER3 targeting

Induction of cell death of HER2- (A) and HER3-targeting (B) scTRAIL₃-FLVGGGPQRVA fusion proteins on Colo205 cells was analyzed in the absence and presence of Bortezomib (650 nM, 250 ng/ml). Data are represented as mean $\pm$ S.D. (n=3).

Figure 26: Cytotoxicity assay with HCT-116 cells

Induction of cell death of EGFR- (A), HER2- (B), and HER3-targeting (C) and non-targeted (D) scTRAIL₃-FLVGGGPQRVA constructs on HCT-116 cells was analyzed in the absence and presence of Bortezomib (650 nM, 250 ng/ml). Data are represented as mean $\pm$ S.D. (n=3).

Figure 27: Pharmacokinetic analyses

Pharmacokinetic properties of EGFR-targeting scTRAIL₃-FLVGGGPQRVA fusion proteins were analyzed in CD1 mice. 25 μ g protein was i.v. injected into the tail vein. Protein concentrations in blood samples were determined at time points indicated by ELISA and normalized to the 3 min value. Data are represented as mean $\pm$ S.D. (n=3).

Figure 28: Generation of IgG-scTRAIL₃ fusion proteins

Anti-EGFR IgG and anti-EGFR IgG-scTRAIL₃ fusion proteins were affinity purified from tissue culture supernatant obtained from transiently transfected HEK293 cells using anti-FLAG M2 agarose, and analyzed by reducing SDS-PAGE and Coomassie staining. Samples representing IgG-LC/HC-scTRAIL₃ (lane 1), IgG-heavy chain (HC)-scTRAIL₃ (lane 2), hu225 (anti-EGFR) IgG (lane 3) and IgG-light chain (LC)-scTRAIL₃ (lane 4) are shown.

Figure 29: Time-dependent caspase-8 (A) and -3/7 (B) activation in Colo205 cells

Caspase-8 (A) and -3/7 (B) activity was analyzed after treatment of the cells with scFvhu225-Fc-scTRAIL₃-FLVGGGPQRVA, Fc-scTRAIL₃-FLVGGGPQRVA, and scTRAIL₃-FLVGGGPQRVA at concentrations of 100 pM and 1 nM scTRAIL units in combination with bortezomib (BZB, 650 nM) or alone for 1 h, 2 h, 3 h, 5 h, 8 h, and 20 h. As controls, cells were treated either with bortezomib or with medium alone.

Figure 30: Effects of EGFR-targeting scTRAIL₃-FLVGGGPQRVA fusion proteins in Colo205 xenograft models

NMRI nu/nu mice bearing Colo205 tumors with a size of ~100 mm³ received six treatments with scTRAIL₃-FLVGGGPQRVA constructs in combination with bortezomib (5 µg BZB per treatment) or BZB alone. Points of treatment are indicated with dotted lines. A) 0.5 nmol dimeric EGFR-targeting scTRAIL₃-FLVGGGPQRVA molecules of different formats were analyzed. B) Effects of 0.1 nmol and 0.3 nmol scFvhu225-Fc-scTRAIL₃-FLVGGGPQRVA were compared.

Figure 31: Size exclusion chromatography and melting point of IgG-scTRAIL₃

(A) Affinity-purified preparations of anti-EGFR IgG, anti-EGFR IgG LC-scTRAIL₃-FAVSGAA, anti-EGFR IgG HC-scTRAIL₃-FAVSGAA and anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA were analyzed by size exclusion chromatography for their molecular constitution. The retention times of the standard proteins thyroglobulin (669 kDa), beta-amylase (200 kDa), bovine serum albumin (66 kDa), carboanhydrase (29 kDa) and FLAG peptide (1 kDa) are indicated by lines. (B) The melting point of anti-EGFR IgG HC-scTRAIL₃-FAVSGAA was analyzed by dynamic light scattering using a zetasizer instrument.

Figure 32: EGFR-specific binding of IgG-scTRAIL₃ fusion proteins

(A) The anti-EGFR human IgG1 scaffold antibody for IgG scTRAIL₃ fusion proteins was tested for binding to EGFR+ tumor cell lines Colo205 (mean ± SEM, n = 3) and HCT116 (mean ± SEM, n = 2) via flow cytometry, compared with the parental antibody cetuximab. (B) anti-EGFR IgG LC-scTRAIL₃-FAVSGAA, anti-EGFR IgG HC-scTRAIL₃-FAVSGAA and anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA were tested for binding to Colo205 and HCT116 cells via flow cytometry together with the scaffold antibody anti-EGFR IgG serving as a reference (mean ± SEM, n = 3). (C) Anti-EGFR IgG LC-scTRAIL₃-FAVSGAA, anti-EGFR IgG HC-scTRAIL₃-FAVSGAA and anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA were tested for binding to purified EGFR-Fc via ELISA together with the scaffold antibody anti-EGFR IgG serving as a reference (mean ± SEM, n = 3).

Figure 33: Cell viability assay in HCT116 cells applying IgG-scTRAIL₃ fusion proteins

Preparations of anti-EGFR IgG LC-scTRAIL₃-FAVSGAA, anti-EGFR IgG HC-scTRAIL₃-FAVSGAA and anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA were analyzed for their

bioactivity in a cell viability assay using HCT116 cells sensitized with 5 ng/ml of the proteasome inhibitor bortezomib or without sensitization (mean $\pm$ SEM, n = 3). Where indicated, 10 μ g/ml of the anti-EGFR antibody cetuximab was added prior to fusion protein incubation for EGFR competition studies.

5 **Figure 34: Cell viability assay in Colo205 cells applying IgG-scTRAIL₃ fusion proteins**

Preparations of anti-EGFR IgG LC-scTRAIL₃-FAVSGAA, anti-EGFR IgG HC-scTRAIL₃-FAVSGAA and anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA were analyzed for their bioactivity in a cell viability assay using Colo205 cells sensitized with 250 ng/ml of the proteasome inhibitor bortezomib or without sensitization (mean $\pm$ SEM, n = 2). Where indicated, 10 μ g/ml of the anti-EGFR antibody cetuximab was added prior to fusion protein incubation for EGFR competition studies.

10 **Figure 35: Cell viability assay in HT1080 cells applying IgG-scTRAIL₃ fusion proteins**

Preparations of anti-EGFR IgG LC-scTRAIL₃-FAVSGAA, anti-EGFR IgG HC-scTRAIL₃-FAVSGAA and anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA were analyzed for their bioactivity in a cell viability assay using HT1080 cells sensitized with 10 ng/ml of the proteasome inhibitor bortezomib or without sensitization (mean $\pm$ SEM, n = 2). Where indicated, 10 μ g/ml of the anti-EGFR antibody cetuximab was added prior to fusion protein incubation for EGFR competition studies.

15 **Figure 36: Pharmacokinetics of anti-EGFR IgG HC-scTRAIL₃-FAVSGAA**

Pharmacokinetic properties of EGFR-targeting IgG HC-scTRAIL₃-FAVSGAA were analyzed in CD1 mice. Data points from Db-scTRAIL₃-FLVGGGPQRVA were plotted for reasons of comparison. 25 μ g protein was i.v. injected into the tail vein. Protein concentrations in blood samples were determined at time points indicated by ELISA and normalized to the 3 min value. Data are represented as mean $\pm$ S.D. (n = 3).

20 **Figure 37: In vivo antitumor activity, pharmacokinetics and compatibility of Db10-Glyco-scTRAIL₃-FAVSGAA**

(A) Colo205-bearing nude mice received eight intravenous (i.v.) injections of the Db10-Glyco-scTRAIL₃-FAVSGAA fusion protein (0.1 nmol, 0.3 nmol, or 1.0 nmol) in combination with eight intraperitoneal (i.p.) injections of bortezomib (Brt; 5 μ g per injection) every day indicated by dots. Mean $\pm$ 95 % CI (n = 12 tumors per group). The 1.0 nmol-treated group received an identical regime of Db10-Glyco-scTRAIL₃-FAVSGAA and bortezomib after regrowth of tumors (volume approximately 100 mm³). Therefore, animals were divided into two subgroups of fast (I) and slow (II) regrowth. (B, C) Db10-Glyco-scTRAIL₃-FAVSGAA (168 μ g per animal in B /

25 µg per animal in C) was injected i.v. into Colo205-bearing nude mice (B) or CD-1 mice (C). The serum concentrations of the fusion protein were analyzed via ELISA. Mean ± SD (n = 3). (D, E) Activity of alanine aminotransferase (ALT, D) and α-amylase (E) was measured after 4 hours (only for D), 1 day, and 9 days after the first of total eight injections (every day) of Db10-
 5 Glyco-scTRAIL₃-FAVSGAA (0.1 nmol, 0.3 nmol, or 1.0 nmol; i.v.) and/or bortezomib (Brt, 5 µg; i.p.). Control mice were non-treated. Mean ± SD (n = 3).

Figure 38: scTRAIL₃-FAVSGAA fusion proteins with Fc comprising a glycosylated peptide linker

(A) Affinity-purified preparations of Fc-scTRAIL₃-FAVSGAA (SEQ ID NO: 246), scTRAIL₃-FAVSGAA-Fc (SEQ ID NO: 245) and scTRAIL₃-FAVSGAA-Fc-scTRAIL₃-FAVSGAA (SEQ ID NO: 247) were analyzed by size exclusion chromatography. (B) The same proteins were tested in ELISA for binding to TRAIL R1-Fc (n = 1) and TRAIL R2-Fc (mean ± S.D., n = 4). Monomeric scTRAIL₃-FLVGGGGPQRVA was used as a reference. (C) Fc-scTRAIL₃-FAVSGAA, scTRAIL₃-FAVSGAA-Fc and scTRAIL₃-FAVSGAA-Fc-scTRAIL₃-FAVSGAA
 15 were analyzed for their cytotoxic activity on Colo205 cells in presence or without 250 ng/ml bortezomib (Brt). Monomeric scTRAIL₃-FLVGGGGPQRVA served as a reference (mean ± S.D., n = 3).

Figure 39: scTRAIL₃-FAVSGAA fusion proteins with Fc comprising a glycine/serine, glycosylated or alpha-helical peptide linker

(A) 1.3×10⁶ HEK293 cells per 6-well were transiently transfected with 4 µg plasmid DNA and 12 µg polyethylene imine for expression of the listed scTRAIL₃-FAVSGAA Fc fusion proteins. 24 h after transfection, medium was replaced by 3 ml OptiMEM I + 50 µM zinc chloride and cells were cultivated for three days at 37 °C, 5 % CO₂. The yields of produced protein in supernatants were assayed by TRAIL ELISA (BD Biosciences) (mean ± S.D., n = 3). (B)
 25 Affinity-purified preparations of the indicated scTRAIL₃-FAVSGAA Fc fusion proteins were analyzed by size exclusion chromatography. (C) Melting points of scTRAIL₃-FAVSGAA fusion proteins with Fc were determined by dynamic light scattering.

Figure 40: Comparison of binding properties of scTRAIL₃ fusion proteins with Fc comprising a glycine/serine, glycosylated or alpha-helical peptide linker

(A) Binding of scTRAIL₃-FAVSGAA-20 G/S-Fc (SEQ ID NO: 252), scTRAIL₃-FAVSGAA-25 G/S-Fc (SEQ ID NO: 253), scTRAIL₃-FAVSGAA-“W”-Fc (SEQ ID NO: 254), scTRAIL₃-FAVSGAA-Fc (SEQ ID NO: 245), Fc-scTRAIL₃-FAVSGAA (SEQ ID NO: 246) and Fc-scTRAIL₃-FLVGGGGPQRVA to TRAIL-R1-Fc (left) or TRAIL-R2-Fc (right) in ELISA (mean ± S.D., n = 3). (B) Binding of scTRAIL₃-FAVSGAA-20 G/S-Fc, scTRAIL₃-FAVSGAA-25 G/S-Fc, scTRAIL₃-FAVSGAA-“W”-Fc, scTRAIL₃-FAVSGAA-Fc, Fc-scTRAIL₃-FAVSGAA and
 35

Fc-scTRAIL₃-FLVGGGPQRVA to Colo205 and HCT116 cells in flow cytometry (mean $\pm$ S.D., n = 3).

Figure 41: Cell viability assay in Colo205 cells applying scTRAIL₃ Fc fusion proteins

Preparations of scTRAIL₃-FAVSGAA-20 G/S-Fc, scTRAIL₃-FAVSGAA-25 G/S-Fc, scTRAIL₃-FAVSGAA-“W”-Fc, scTRAIL₃-FAVSGAA-Fc, Fc-scTRAIL₃-FAVSGAA and Fc-scTRAIL₃-FLVGGGPQRVA were analyzed for their bioactivity in a cell viability assay using Colo205 cells sensitized with 250 ng/ml of the proteasome inhibitor bortezomib (BZB) or without sensitization (mean $\pm$ S.D., n = 3).

Figure 42: Cell viability assay in HCT116 cells applying scTRAIL₃ Fc fusion proteins

Preparations of scTRAIL₃-FAVSGAA-20 G/S-Fc, scTRAIL₃-FAVSGAA-25 G/S-Fc, scTRAIL₃-FAVSGAA-“W”-Fc, scTRAIL₃-FAVSGAA-Fc, Fc-scTRAIL₃-FAVSGAA and Fc-scTRAIL₃-FLVGGGPQRVA were analyzed for their bioactivity in a cell viability assay using HCT116 cells sensitized with 5 ng/ml of the proteasome inhibitor bortezomib (BZB) or without sensitization (mean $\pm$ S.D., n = 3).

Figure 43: Biochemical properties and target-dependent bioactivity of Db α FAP-Glyco-scTRAIL₃-FAVSGAA

(A) Size exclusion chromatogram of Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA (SEQ ID NO: 255). (B) Melting point of Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA as detected by dynamic light scattering. (C) Binding of Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA to HT1080 tumor cells transfected with FAP in comparison to HT1080 wt (control) (mean $\pm$ S.D., n = 2). (D) Bioactivity of Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA on HT1080 FAP⁺ cells in presence or absence of competing anti-FAP IgG antibody *in vitro* (Brt, bortezomib) (mean $\pm$ S.D., n = 4).

Figure 44: Cell viability assay in HT1080 FAP⁺ cells applying Fc-scTRAIL and scFv36-Fc-scTRAIL₃ fusion proteins

Preparation of Fc-scTRAIL₃-FLVGGGPQRVA and scFv36-Fc-scTRAIL₃-FLVGGGPQRVA were analyzed for their bioactivity in a cell viability assay using stably transfected HT1080 FAP⁺ cells in the absence (A) or presence (B) of bortezomib (13 nM final concentration) (mean $\pm$ SD).

Figure 45: Binding of scTRAIL₃ fusion proteins to human TRAIL receptors via ELISA

Preparations of scFhu225-Fc-scTRAIL₃-FLVGGGPQRVA, Fc-scTRAIL₃-FLVGGGPQRVA, and Db10hu225-Glyco-scTRAIL₃-FAVSGAA were analyzed in ELISA for binding to human DR4-Fc (TRAIL-R1-Fc), DR5-Fc (TRAIL-R2-Fc), DcR1-Fc (TRAIL-R3-Fc), DcR2-Fc (TRAIL-R4-Fc), and OPG-Fc (mean $\pm$ SD).

Detailed Description of the Invention

Before the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodology, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions etc.), whether supra or infra, is hereby incorporated by reference in its entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention. Some of the documents cited herein are characterized as being "*incorporated by reference*". In the event of a conflict between the definitions or teachings of such incorporated references and definitions or teachings recited in the present specification, the text of the present specification takes precedence.

In the following, the elements of the present invention will be described. These elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and encompass embodiments which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements in this application should be considered disclosed by the description of the present application unless the context indicates otherwise.

Definitions

In the following, some definitions of terms frequently used in this specification are provided. These terms will, in each instance of its use, in the remainder of the specification have the respectively defined meaning and preferred meanings.

As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents, unless the content clearly dictates otherwise.

The term "polypeptide" refers to any peptide-bond-linked polymer of amino acids. A polypeptide can be one chain or may be composed of more than one chain, which are held together by covalent bonds, e.g. disulphide bonds and/or non-covalent bonds. Modifications of the peptide bonds or of side chains residues are possible, provided the activity of the resulting chemical entity (e.g. component A linked to component B) is not totally lost. The term shall not be construed as limiting the length of the polypeptide.

In the context of the present invention, the term "peptide" refers to a short polymer of amino acids linked by peptide bonds. It has the same chemical (peptide) bonds as proteins but is commonly shorter in length. The shortest peptide is a dipptide consisting of two amino acids joined by a peptide bond. There can also be tripeptides, tetrapeptides, pentapeptides etc. A peptide has an amino end and a carboxyl end, unless it is a cyclic peptide. Peptides usable in the present invention (including peptide derivatives, peptide variants, peptide fragments, peptide segments, peptide epitopes and peptide domains) can be further modified by chemical modification. This means such a chemically modified peptide may comprise other chemical groups than the 20 naturally occurring proteinogenic amino acids. Examples of such other chemical groups include without limitation glycosylated amino acids and phosphorylated amino acids. Chemical modifications of a peptide may provide advantageous properties as compared to the parent peptide, e.g. one or more of enhanced stability, increased biological half-life, or increased solubility.

The term "protein" as used in the context of the present specification refers to a molecule comprising one or more polypeptides that resume a secondary and tertiary structure and additionally refers to a protein that is made up of several polypeptides, i.e. several subunits, forming quaternary structures. The protein has sometimes non-peptide groups attached, which can be called prosthetic groups or cofactors.

The term "C-terminus" (also known as the carboxyl-terminus, carboxy-terminus, C-terminal tail, C-terminal end, or COOH-terminus) as referred to within the context of the present invention is the end of an amino acid chain (protein or polypeptide), terminated by a free carboxyl group (-COOH). When the protein is translated from messenger RNA, it is created from N-terminus to C-terminus. The term "N-terminus" (also known as the amino-terminus, NH₂-terminus, N-terminal end or amine-terminus) refers to the start of a protein or polypeptide terminated by an amino acid with a free amine group (-NH₂). The convention for writing peptide sequences is to put the N-terminus on the left and write the sequence from N- to C-terminus.

The term "TNF homology domain of TNF-ligand family member proteins" (THD) as used in the present specification refers to a protein domain shared by all tumor necrosis factor (TNF, formerly known as TNF α or TNF alpha) ligand family members. Homology implies

evolutionary lineage from a common ancestor. A homology domain is a conserved part of a given protein sequence and (tertiary) structure that can evolve, function, and exist independently of the rest of the protein chain. It is a structural feature shared by all members of a certain protein family. Each domain forms a compact three-dimensional structure and often can be independently stable, folded and critical for biological activity. The C-terminus of a THD within the meaning of the present invention is defined by the C-terminal consensus sequence: -S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1) and the N-terminus is defined by one of the two N-terminal consensus sequences: X₂-V/A/F-A-H-V/L/I/Y (SEQ ID NO: 2) or X₃-V/W/F/C-A/L-E/Y/Q/H-L (SEQ ID NO: 3), wherein X₁ is a non-polar/hydrophobic or polar/neutral amino acid, preferably selected from the group consisting of F, V, Q, A, I, L, and Y; X₂ is selected from the group consisting of P, K, V, I, and A; and X₃ is selected from the group consisting of D, S, M, and I. On the basis of a given TNF-ligand family member protein sequence and using above defined C-terminal and N-terminal homology sequences the skilled person can determine for the given TNF-ligand family member protein the THD. Among the members of the TNF family, the position and length of individual THDs vary considerably, but can be defined by the occurrence of conserved amino acid residues as identified by multiple sequence alignments using appropriate software tools (Bodmer *et al.*, 2002). More importantly, crystal structures can reveal distinct interactions between amino acid residues involved in, for example, homotrimerization of TNF family ligands. Informations of such kind can be helpful to refine THDs for given members of the TNF superfamily as described in Bodmer *et al.*, 2002. Furthermore, functional aspects like protein solubility or bioactivity, such as receptor binding and activation, of engineered protein variants can provide important hints regarding crucial amino acid residues or the minimal length of individual THDs. The term THDs comprises polypeptides based on naturally occurring TNF-ligand family member protein sequences as well as variants thereof, which retain the ability to bind specifically to the receptor of the respective TNF-ligand family member. Preferably such THD variants have an affinity of at least 50% of the wild type THD, more preferably at least 60%, 70%, 80%, 90% and most preferably at least 99%.

TNF-ligand family member proteins comprise a group of multifunctional cytokines that can cause, e.g. programmed cell death (apoptosis), differentiation, cell survival, and immune regulation. TNF is a monocyte-derived cytokine that has been implicated in tumor regression, septic shock, and cachexia which is recognized by its specific receptor. Nineteen proteins have been identified as part of the TNF-ligand family on the basis of sequence, functional, and structural similarities. All these cytokines seem to form homotrimeric (or heterotrimeric in the case of LT-alpha/beta) complexes that are recognized by their specific receptors. The following proteins are members of the TNF-ligand family: TNF-related apoptosis inducing ligand (TRAIL;

TNFSF10), a cytokine that induces apoptosis; CD40L (TNFSF5 = tumor necrosis factor superfamily member 5), a cytokine that seems to be important in B-cell development and activation; CD27L (TNFSF7), a cytokine that plays a role in T-cell activation which induces the proliferation of co-stimulated T cells and enhances the generation of cytolytic T cells; CD30L (TNFSF8), a cytokine that induces proliferation of T cells; FasL (TNFSF6), a cell surface protein involved in cell death; 4-1BBL (TNFSF9), an inducible T cell surface molecule that contributes to T-cell stimulation; OX40L (TNFSF4), a cell surface protein that co-stimulates T cell proliferation and cytokine production. Further members of the TNF-ligand family members comprise EDA; LTA (TNFSF1); LTB (TNFSF3); CD153 (TNFSF8); RANKL (TNFSF11); TWEAK (TNFSF12); APRIL (TNFSF13); BAFF (TNFSF13B); LIGHT (TNFSF14); VEGI (TNFSF15); GITRL (TNFSF18). More information about the sequences of TNF-ligand family members may be obtained for example from publicly accessible databases such as Genbank. TNF-ligand family members interact with their cognate receptors, e.g. TNF with TNFR1 and TNFR2, TRAIL with TRAILR1 (DR4), TRAILR2 (DR5), TRAILR3 (DcR1), TRAILR4 (DcR2) and OPG. The ligands mediate oligomerization and activation of their respective receptors. The interaction of members of the TNF receptor family with its ligands is characterized by binding of the receptors at the space between two of the three TNF-ligand family member protein monomers of the TNF-ligand family member protein homotrimer, the biological active form of TNF and other members of the TNF-ligand family.

The apoptosis-inducing cytokine TRAIL is expressed on many cells of the innate and adaptive immune system in a stimulus dependent manner. TRAIL exerts its function via high affinity binding of multiple receptors. Two of these receptors (DR4 and DR5) recruit adapter proteins via death domain interactions and initiate the formation of the death inducing signaling complex (DISC) leading in turn to the induction of apoptosis. Binding of homotrimeric TRAIL to DR4 and DR5 induces oligomerization of the receptors and initiation of a caspase-mediated pathway. Besides the induction of apoptosis, TRAIL is also capable of weakly activating NFκB and MAP kinase pathways. TRAIL has shown activity against primary tumor explants derived from patient pancreatic and colorectal cancer. Anti-tumor activity of TRAIL may be enhanced when administered in combination with other chemotherapeutics. Thus, agonizing TRAIL-death receptors is a promising strategy for therapy of hyperproliferative disorders and autoimmune disorders.

The term “consensus sequence” as used within this specification refers to a calculated order of most frequent residues, either nucleotide or amino acid, found at each position in a sequence alignment between two or more sequences. It represents the results of a multiple sequence alignment in which related sequences are compared to each other and similar sequence

motifs are calculated. Conserved sequence motifs are depicted as consensus sequences, which indicate identical amino acids, i.e. amino acids identical among the compared sequences, conserved amino acids, i.e. amino acids which vary among the compared amino acid sequence but wherein all amino acids belong to a certain functional or structural group of amino acids, e.g. polar or neutral, and variable amino acids, i.e. amino acids which show no apparent relatedness among the compared sequence.

The consensus sequence of the C-terminus and N-terminus of the THD is a sequence that is located within the TNF-ligand family member sequence, respectively, and is particularly conserved among TNF-ligand family members. These sequences delineate the part of the TNF-ligand family member participating in the trimerization. Accordingly, the two consensus sequences serve as C-terminal and N-terminal reference points within a given TNF-ligand family member, which may comprise additional N- or C-terminal amino acids that may not be present in other TNF-ligand family members. Thus, the use of consensus sequences allows to refer to the same region of different TNF-ligand family member without referring to a specific position as the N-terminal and C-terminal end of the fragment of the TNF-ligand family member present in the polypeptides of the invention. Human TRAIL, for example, comprises the following amino acid sequence spanning amino acid positions 121 to 125: VAAHI (SEQ ID NO: 25). Accordingly, the skilled person can determine the absolute amino acid position of the amino acids of the C-terminal and N-terminal consensus sequence by determining its absolute position within the full-length amino acid sequence of a given TNF-ligand family member. For example X₂ of the N-terminal consensus sequence corresponds to the amino acid at position 122 of human TRAIL, and is preferably valine. X₂ corresponds to position 145 in human FasL and is preferably lysine. It corresponds to amino acid position 88 in human TNF and is preferably proline. It is immediately apparent to the skilled person that the different lengths of N-terminal amino acids preceding the N-terminal consensus sequence in different TNF-ligand family members requires a definition of the C- and N-terminal reference point that is independent of the absolute position of the THD within the respective TNF-ligand family member.

The term “dimerization domain” as used herein refers to a protein or polypeptide, a fragment or part of a protein or polypeptide which mediates a close proximity between two identical or different protein or polypeptide molecules (monomers) of the invention and thus, enables protein-protein interaction which allows dimerization of two structurally similar or different monomers joined by non-covalent or covalent bonds. The dimerization leads to the formation of a macromolecular complex formed by two, covalently or non-covalently bound, macromolecules such as proteins.

In the context of the present invention the term "half-life-extension domain" refers to a binding moiety which prolongs the serum/plasma half-life of a pharmaceutically active moiety, i.e. a pharmaceutically active moiety exhibits a prolonged serum/plasma half-life when being part of the half-life-extension domain. The binding moiety may be but is not limited to a polypeptide or protein.

The terms "target specific binding domain" or "ligand" are used interchangeably in the context of the present invention and refer to a binding moiety comprising a structural unit which facilitates or improves specific binding of the polypeptide of the second aspect of the present invention to its target. This domain may be a natural ligand, a carbohydrate, a protein or a peptide, for example isolated by display technologies from ligand libraries (Sergeeva 2006). The binding of such a target specific binding domain is considered specific to a given target if it binds with the highest affinity to the respective target and only with lower affinity, e.g. at least 10-fold lower, preferably at least 100-fold lower affinity to other targets even to targets with a related amino acid sequence.

The term "target" or "target molecule" as used in the present invention refers to a natural existing cellular or molecular structure towards which other molecules have a certain binding affinity or to which other molecules specifically bind. "Specific binding" means that a binding moiety (e.g. an antibody) binds stronger to a target, such as an epitope, for which it is specific compared to the binding to another target if it binds to the first target with a dissociation constant (K_d) which is lower than the dissociation constant for the second target. Targets can be recognized by their ligands which bind with a certain affinity to their targets and thus, the ligand binding to its respective target results in a biological effect. Preferably, the binding is both specific and occurs with a high affinity, preferably with K_d of less than 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} M or less. Such affinity is preferably measured at 37°C Suitable assays include surface plasmon resonance measurements (e.g. Biacore), quartz crystal microbalance measurements (e.g. Attana), and competition assays.

The terms " V_L region" and " V_H region" refers to V_L and V_H regions of an antibody; i.e. the N-terminal variable region of the light chain of an immunoglobulin and the N-terminal variable region of the heavy chain of an immunoglobulin, respectively. The individual V_L and V_H regions are each composed of three hypervariable regions (complementary determining region (CDR)1, CDR2 and CDR3) and four framework regions (framework (FR) region 1, FR2, FR3, FR4). Identifying the respective subregions within a given sequence is routine in the art and may for example be accomplished by IgBlast of the NCBI. The variable regions of the heavy and the light chain form together the binding region of an antibody. In immunoglobulins, the V_L and the V_H regions are located on different polypeptide chains, but they can be located on the same

chain in recombinant antibody derivatives. Interactions of a V_L and a V_H region allows the polypeptide of the present invention to interact with its respective target antigen.

The term “antigen” according to the present invention refers to any structure recognized by molecules of the immune response, e.g. antibodies, T cell receptors (TCRs) and the like. An antigen may be foreign or toxic to the body or may be a cellular protein that is associated with a particular disease. Antigens are recognized by highly variable antigen receptors (B-cell receptor or T-cell receptor) of the adaptive immune system and may elicit a humoral or cellular immune response. Antigens that elicit such a response are also referred to as immunogen. A fraction of the proteins inside cells, irrespective of whether they are foreign or cellular, are processed into smaller peptides and presented to by the major histocompatibility complex (MHC). A cellular immune response is elicited, if the small peptide fragment is bound by a T-cell receptor. Cell surface antigens can be selected from the group of cytokine receptors, integrins, cell adhesion molecules, cell type-specific cell surface antigen, tissue-specific cell surface antigen, cell surface-expressed tumor-associated antigen, cluster of differentiation antigens, or carbohydrates.

“Antibodies” as used in the context of the present invention are glycoproteins belonging to the immunoglobulin superfamily; the terms antibody and immunoglobulin are often used interchangeably. An antibody refers to a protein molecule produced by plasma cells and is used by the immune system to identify and neutralize foreign objects such as bacteria and viruses. The antibody recognizes a unique part of the foreign target, its antigen.

The term “antibody fragment” as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. Examples of binding fragments encompassed within the term “antibody fragment” include a fragment antigen binding (Fab) fragment, a Fab’ fragment, a $F(ab')_2$ fragment, a heavy chain antibody, a single-domain antibody (sdAb), a single-chain fragment variable (scFv), a fragment variable (Fv), a V_H domain, a V_L domain, a single domain antibody, a nanobody, an IgNAR (immunoglobulin new antigen receptor), a di-scFv, a bispecific T-cell engager (BITEs), a dual affinity re-targeting (DART) molecule, a triple body, a diabody, a single-chain diabody, an alternative scaffold protein, and a fusion protein thereof.

The term “diabody” as used within this specification refers to a fusion protein or a bivalent antibody which can bind different antigens. A diabody is composed of two single protein chains which comprise fragments of an antibody, namely variable fragments. Diabodies comprise a heavy chain variable domain (V_H) connected to a light-chain variable domain (V_L) on the same polypeptide chain (V_H - V_L , or V_L - V_H). By using a short peptide connecting the two variable domains, the domains are forced to pair with the complementary domain of another

chain and thus, create two antigen-binding sites. Diabodies can target the same (monospecific) or different antigens (bispecific).

A “single domain antibody”, refers to antibody fragments consisting of a single, monomeric variable domain of an antibody. Simply, they only comprise the monomeric heavy chain variable regions of heavy chain antibodies produced by camelids or cartilaginous fish. Due to their different origins they are also referred to VHH or VNAR (variable new antigen receptor)-fragments. Alternatively, single-domain antibodies can be obtained by monomerization of variable domains of conventional mouse or human antibodies by the use of genetic engineering. They show a molecular mass of approximately 12-15 kDa and thus, are the smallest antibody fragments capable of antigen recognition. Further examples include nanobodies or nanoantibodies.

The term “antibody mimetic” as used within the context of the present specification refers to compounds which can specifically bind antigens, similar to an antibody, but are not structurally related to antibodies. Usually, antibody mimetics are artificial peptides or proteins with a molar mass of about 3 to 20 kDa which comprise one, two or more exposed domains specifically binding to an antigen. Examples include inter alia the LACI-D1 (lipoprotein-associated coagulation inhibitor); affilins, e.g. human- γ B crystalline or human ubiquitin; cystatin; Sac7D from *Sulfolobus acidocaldarius*; lipocalin and anticalins derived from lipocalins; DARPs (designed ankyrin repeat domains); SH3 domain of Fyn; Kunits domain of protease inhibitors; monobodies, e.g. the 10th type III domain of fibronectin; adnectins: knottins (cysteine knot miniproteins); atrimers; evibodies, e.g. CTLA4-based binders, affibodies, e.g. three-helix bundle from Z-domain of protein A from *Staphylococcus aureus*; Trans-bodies, e.g. human transferrin; tetranectins, e.g. monomeric or trimeric human C-type lectin domain; microbodies, e.g. trypsin-inhibitor-II; affilins; armadillo repeat proteins. Nucleic acids and small molecules are sometimes considered antibody mimetics as well (aptamers), but not artificial antibodies, antibody fragments and fusion proteins composed from these. Common advantages over antibodies are better solubility, tissue penetration, stability towards heat and enzymes, and comparatively low production costs.

As used herein, the term “variant” is to be understood as a peptide or protein which differs in comparison to the peptide or protein from which it is derived by one or more changes in its length or sequence. The polypeptide from which a protein variant is derived is also known as the parent or parental polypeptide. The term “variant” comprises “fragments” or “derivatives” of the parent molecule. Typically, “fragments” are smaller in length or size than the parent molecule, whilst “derivatives” exhibit one or more differences in their sequence in comparison to the parent molecule. Also encompassed are posttranslational modifications of the parent proteins

(e.g. glycosylation, biotinylation, phosphorylation, ubiquitinylation, palmitoylation, or proteolysis). Typically, a variant is constructed artificially, preferably by gene-technological means whilst the parent polypeptide or polynucleotide is a wild-type protein or polynucleotide. However, also naturally occurring variants are to be understood to be encompassed by the term "variant" as used herein. Further, the variants usable in the present invention may also be derived from homologs, orthologs, or paralogues of the parent molecule or from artificially constructed variant, provided that the variant exhibits at least one biological activity of the parent molecule, i.e. is functionally active.

As used in this specification the term "nucleic acid" comprises polymeric or oligomeric macromolecules, or large biological molecules, essential for all known forms of life. Nucleic acids, which include DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), are made from monomers known as nucleotides. Most naturally occurring DNA molecules consist of two complementary biopolymer strands coiled around each other to form a double helix. The DNA strand is also known as polynucleotides consisting of nucleotides. Each nucleotide is composed of a nitrogen-containing nucleobase as well as a monosaccharide sugar called deoxyribose or ribose and a phosphate group. Naturally occurring nucleobases comprise guanine (G), adenine (A), thymine (T), uracil (U) or cytosine (C). The nucleotides are joined to one another in a chain by covalent bonds between the sugar of one nucleotide and the phosphate of the next, resulting in an alternating sugar-phosphate backbone. If the sugar is desoxyribose, the polymer is DNA. If the sugar is ribose, the polymer is RNA. Typically, a polynucleotide is formed through phosphodiester bonds between the individual nucleotide monomers. In the context of the present invention the term "nucleic acid" includes but is not limited to ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and mixtures thereof such as e.g. RNA-DNA hybrids (within one strand), as well as cDNA, genomic DNA, recombinant DNA, crRNA and mRNA. A nucleic acid may consist of an entire gene, or a portion thereof, the nucleic acid may also be a miRNA, siRNA, or a piRNA.

As used in this specification the term "vector", also referred to as an expression construct, is usually a plasmid or virus designed for protein expression in cells. The vector is used to introduce a specific gene into a target cell, and can use the cell's mechanism for protein synthesis to produce the protein encoded by the gene. The expression vector is engineered to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the gene carried on the expression vector. The goal of a well-designed expression vector is the production of significant amount of stable messenger RNA, and therefore proteins. Examples of suitable vectors include but are not limited to plasmids, cosmids, phages, viruses or artificial chromosomes. An example for a commonly used expression vector is pGEX-4T2.

The term "pharmaceutical composition" as used in the present application refers to a substance and/or a combination of substances being used for the identification, prevention or treatment of a tissue status or disease. The pharmaceutical composition is formulated to be suitable for administration to a patient in order to prevent and/ or treat disease. Further a pharmaceutical composition refers to the combination of an active agent with a carrier, inert or active, making the composition suitable for therapeutic use. Pharmaceutical compositions can be formulated for oral, parenteral, topical, inhalative, rectal, sublingual, transdermal, subcutaneous or vaginal application routes according to their chemical and physical properties. Pharmaceutical compositions comprise solid, semisolid, liquid, transdermal therapeutic systems (TTS). Solid compositions are selected from the group consisting of tablets, coated tablets, powder, granulate, pellets, capsules, effervescent tablets or transdermal therapeutic systems. Also comprised are liquid compositions, selected from the group consisting of solutions, syrups, infusions, extracts, solutions for intravenous application, solutions for infusion or solutions of the carrier systems of the present invention. Semisolid compositions that can be used in the context of the invention comprise emulsion, suspension, creams, lotions, gels, globules, buccal tablets and suppositories.

The term "active agent" refers to the substance in a pharmaceutical composition or formulation that is biologically active, i.e. that provides pharmaceutical value. A pharmaceutical composition may comprise one or more active agents which may act in conjunction with or independently of each other. The active agent can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as but not limited to those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The term "disease" and "disorder" are used interchangeably herein, referring to an abnormal condition, especially an abnormal medical condition such as an illness or injury, wherein a cell, a tissue, an organ, or an individual is not able to efficiently fulfil its function anymore. Typically, but not necessarily, a disease is associated with specific symptoms or signs indicating the presence of such disease. The presence of such symptoms or signs may thus, be indicative for a cell, a tissue, an organ, or an individual suffering from a disease. An alteration of these symptoms or signs may be indicative for the progression of such a disease. A progression of a disease is typically characterised by an increase or decrease of such symptoms or signs which may indicate a "worsening" or "bettering" of the disease. The "worsening" of a disease is characterised by a decreasing ability of a cell, tissue, organ or individual/patient to fulfil its

function efficiently, whereas the "bettering" of a disease is typically characterised by an increase in the ability of a cell, tissue, an organ or an individual/patient to fulfil its function efficiently.

The term "hyperproliferative disorder" as used in the present application refers to disorders wherein the cell division of the cells is increased in relation to normal tissue. Such disorders are characterized by an abnormal proliferation (production) i.e. overproduction of cells. Hyperproliferative disorders comprise tumor diseases. Tumor diseases may comprise benign or malignant tumors wherein malignant tumor diseases are referred to as cancer. The term hyperproliferative disorder comprises cancers as well as pre-cancerous disorders. Cancer comprises proliferative disorders of mesenchymal origin, i.e. connective tissue (sarcomas) and of epithelial tissues (carcinomas). Common examples of sarcomas are osteosarcoma, chondrosarcoma, liposarcoma, leiomyosarcoma, angiosarcoma and fibrosarcoma and sarcomas of the gastrointestinal tract (GIST). Examples for carcinomas are carcinomas of the skin, testis, liver, gastrointestinal tract such as esophagus, stomach, pancreas, and colon, nasopharynx, bladder, cervix, ovarian, urethra, bladder; prostate and other genitourinary carcinomas, lung, kidney, endocrine tissues such as thyroid and pituitary gland, teratocarcinomas, carcinomas of the brain. Malignancies of the hematologic system are classified as lymphoma or leukemia. Inflammation orchestrates the microenvironment around tumors, contributing to proliferation, survival and migration of cancer cells, thus potentially promoting malignant disease.

Inflammation is in principle a protective immunovascular response that involves immune cells, blood vessels, and a plethora of molecular mediators. The purpose of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair. The term "inflammatory disorder" as used in the context of the present invention refers to a situation wherein a physiological inflammatory response turns into a potentially harmful effect for the body. Inflammatory disorders causing damage to normal tissues comprise but are not limited to autoimmune disorders and neurodegenerative diseases.

Embodiments

In the following passages different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous. In the work leading to the present invention, it was surprisingly shown that the polypeptides of the invention display improved stability and half-life and further can be used for the generation of antibody fusion proteins.

Based on these results the present invention in a first aspect relates to a polypeptide comprising:

- (i) component A comprising, consisting essentially or consisting of:
at least three THDs, wherein the C-terminus of the first and second THD,
respectively, which is in each case defined by the C-terminal consensus sequence

-S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1),

- 5 is linked to the N-terminus of the second and third THD, respectively, which is in
each case defined by the N-terminal consensus sequence

X₂-V/A/F-A-H-V/L/I/Y (SEQ ID NO: 2)

or

X₃-V/W/F/C-A/L-E/Y/Q/H-L (SEQ ID NO: 3),

- 10 through a peptide X_a, which is in each case independently selected and has a length
of 3 to 8 amino acids,

wherein X₁ is a non-polar/hydrophobic or polar/neutral amino acid, preferably
selected from the group consisting of F, V, Q, A, I, L, and Y;

wherein X₂ is selected from the group consisting of P, K, V, I, and A;

- 15 wherein X₃ is selected from the group consisting of D, S, M, and I;

- (ii) component B comprising

a dimerization domain consisting of an antibody V_L and V_H region linked directly to
each other with a peptide that has a length of between 7 and 11 amino acids.

Component A of the polypeptides of the present invention, preferably has a thermal
20 stability of at least 48°C, more preferably of at least 49°C, more preferably of at least 50°C, more
preferably of at least 51°C, more preferably of at least 52°C, more preferably of at least 53°C,
more preferably of at least 54°C, more preferably of at least 55°C, more preferably of at least
56°C and most preferably of at least 57°C. Preferably, the polypeptide of the first aspect
comprising component A has at least the above indicated thermal stabilities. The skilled person
25 is well aware how to measure thermal stabilities of polypeptides. Exemplary methods are taught
herein below.

Accordingly, it is preferred that component A comprises, essentially consists or consists of
the following arrangement of polypeptide elements from N- to C-terminus, wherein “THD1” is
an abbreviation of the “first THD”, “THD2 is an abbreviation for the “second THD” etc.: THD1-
30 X_a-THD2-X_a-THD3.

As outlined above the C-terminal and N-terminal consensus sequences serve the purpose of
defining the C- and N-terminal end of the region of the THD of TNF-family members that is
necessarily included in the fragment of the respective TNF-ligand family member. The basis for
this is the observation of the present inventors that the inclusion of a THD defined by these C-
35 and N-terminal endpoints in the context of a connecting sequence X_a of the indicated length

leads to a single chain TNF-ligand polypeptide which provides the above noted advantages. In the following the determination of a THD and its sequence on the basis of the C-terminal and N-terminal consensus sequences is illustrated exemplary for the preferred human TNF-ligand family member TRAIL. The sequence of human TRAIL is indicated in SEQ ID NO: 5. It comprises the amino acid sequence "VAAHI" spanning amino acid positions 122 to 125. This sequence conforms to the N-terminal consensus sequence X_2 -V/A/F-A-H-V/L/I/Y, when X_2 is V (the other consensus positions are highlighted by bold print). V is located at amino acid position 122 of human TRAIL. Thus, the N-terminal end of a THD derived from human TRAIL is at amino acid position 122. Similarly, human TRAIL comprises the sequence S-F-F-G-A-F (SEQ ID NO: 6) spanning amino acid positions 273 to 278. This sequence conforms to the C-terminal consensus sequence -S/T/V-F/Y/S-F-G-A/L/V/I- X_1 , when X_1 is F (the other consensus positions are highlighted by bold print). Thus, the C-terminal end of a THD derived from human TRAIL is at amino acid position 278. Taken together, the THD of human TRAIL spans amino acid positions 122 to 278. A similar analysis for human TNF (see SEQ ID NO: 11) indicates that the THD of human TNF spans amino acid positions 88 to 228. It is noted that the actual N- and C-terminal sequence of THD1, THD2, THD3, etc comprised in component A does not need to be identical to the wild-type amino sequence of the respective TNF-ligand family member from which the THD is derived. It is sufficient if the amino acids intervening the N- and C-terminal consensus sequence or a variant thereof are included and a N- and C-terminal sequence that conforms to the C-terminal consensus sequence and to the respective N-terminal consensus sequence used for the determination of the N-terminal end. This is illustrated exemplary for human TRAIL: The N-terminal sequence of a THD based on human TRAIL is X_2 -V/A/F-A-H-V/L/I/Y, wherein X_2 has one of the meanings indicated above, this sequence is followed by amino acids 126 to 272 of SEQ ID NO: 1 and the C-terminal sequence is S/T/V-F/Y/S-F-G-A/L/V/I- X_1 , wherein X_1 has one of the meanings indicated above.

Taken together the C-terminal end of the first THD, the N-terminal and C-terminal end of the second THD and the N-terminal end of the third THD are defined. It is apparent to the skilled person that the definition of the C-terminal end and N-terminal end of the THDs comprised in component A is required in as far as these two ends are connected via peptides X_a . It is preferred, however, that also the first THD comprises the N-terminal consensus sequence and that the respective last THD, preferably the third THD comprises the C-terminal consensus sequence.

In a preferred embodiment of the first aspect of the present invention, component A comprises at least three identical THDs.

Preferably, the TNF-ligand family members from which the THDs are derived are selected from the group consisting of TNF, CD95L (FasL), LT alpha, LT beta, CD40L, TWEAK,

LIGHT, CD27L, BAFF, TRAIL. Preferred are the human TNF ligand family members human TNF, human CD95L (FasL), human LT α , human LT β , human CD40L, human TWEAK, human LIGHT, human CD27L, human BAFF, human TRAIL. More preferably, component A comprises at least three THDs of TRAIL, even more preferred of human TRAIL.

5 In another preferred embodiment the sequence of the TNF-ligand family member from which the THD is derived is selected from the group consisting of SEQ ID NO: 5 (TRAIL), SEQ ID NO: 7 (FasL), SEQ ID NO: 8 (LIGHT), SEQ ID NO: 9 (Lt α), SEQ ID NO: 10 (Lt β), SEQ ID NO: 11 (TNF), SEQ ID NO: 12 (CD40L), SEQ ID NO: 13 (TWEAK), SEQ ID NO: 14 (CD27L), SEQ ID NO: 15 (BAFF).

10 As outlined above component A comprises at least three TNF homology domains of TNF-ligand family member proteins (THD), wherein the C-terminus of the first and second THD, respectively, and optionally of the third and further THDs is in each case defined by the C-terminal consensus sequence

-S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1)

15 wherein X₁ is a non-polar/hydrophob or polar/neutral amino acid, preferably selected from the group consisting of F, V, Q, A, I, L, and Y;

Preferably, the C-terminal amino acid sequence of the first and second THD, respectively, and optionally the third and all further THDs is selected from the group consisting of the sequences S-F-F-G-A-F (SEQ ID NO: 6), T-F-F-G-L-Y (SEQ ID NO: 16), S-Y-F-G-A-F (SEQ
20 ID NO: 17), V-F-F-G-A-F (SEQ ID NO: 18), T-F-F-G-A-V (SEQ ID NO: 19), V-Y-F-G-I-I (SEQ ID NO: 20), T-S-F-G-L-L (SEQ ID NO: 21), T-Y-F-G-L-F (SEQ ID NO: 22), T-F-F-G-V-Q (SEQ ID NO: 23), and T-F-F-G-A-L (SEQ ID NO: 24). Preferably, the C-terminal amino acid sequence of the first and second THD is S-F-F-G-A-F (SEQ ID NO: 6).

It is preferred that the C-terminal amino acid sequence and N-terminal sequence,
25 respectively, of the THD is closely related to the amino acid sequence of the TNF-ligand family member from which the THD is derived. Thus, the C-terminal amino acid sequence of a THD derived from human TRAIL is preferably S-F-F-G-A-F (SEQ ID NO: 6) or a closely related sequence. For THDs derived from human TRAIL X₁ is preferably F. Similarly, for THDs derived from human FasL the C-terminal amino acid sequence is preferably T-F-F-G-L-Y (SEQ
30 ID NO: 16) or a closely related sequence. For THDs derived from human FasL X₁ is preferably Y. For THDs derived from human LIGHT the C-terminal amino acid sequence is preferably S-Y-F-G-A-F (SEQ ID NO: 17) or a closely related sequence. For THDs derived from human LIGHT X₁ is preferably F. For THDs derived from human Lt α the C-terminal amino acid sequence is preferably V-F-F-G-A-F (SEQ ID NO: 18) or a closely related sequence. For THDs
35 derived from human LT β X₁ is preferably F. For THDs derived from human LT β the C-terminal

amino acid sequence is preferably T-F-F-G-A-V (SEQ ID NO: 19) or a closely related sequence. For THDs derived from human LT β X₁ is preferably V. For THDs derived from human TNF the C-terminal amino acid sequence is preferably V-Y-F-G-I-I (SEQ ID NO: 20) or a closely related sequence. For THDs derived from human TNF X₁ is preferably I. For THDs derived from human CD40L the C-terminal amino acid sequence is preferably T-S-F-G-L-L (SEQ ID NO: 21) or a closely related sequence. For THDs derived from human CD40L X₁ is preferably L. For THDs derived from human TWEAK the C-terminal amino acid sequence is preferably T-Y-F-G-L-F (SEQ ID NO: 22) or a closely related sequence. For THDs derived from human TWEAK X₁ is preferably F. For THDs derived from human CD27L the C-terminal amino acid sequence is preferably T-F-F-G-V-G (SEQ ID NO: 23) or a closely related sequence. For THDs derived from human CD27L X₁ is preferably G. For THDs derived from human BAFF the C-terminal amino acid sequence is preferably T-F-F-G-A-L (SEQ ID NO: 24) or a closely related sequence. For THDs derived from human BAFF X₁ is preferably L.

In the context of defining the N-terminal and C-terminal amino acid sequences the phrase “closely related” refers to variants which comprise an amino acid alteration in one or two, preferably one of the six and five amino acids, respectively, which still fit(s) the C-terminal and N-terminal consensus sequence, respectively.

The C-terminus of the first and second THD, respectively, or optionally of the third and further THDs as defined above and is linked to the N-terminus of the second and third THD, respectively which is defined by the N-terminal consensus sequence



wherein X₂ is selected from the group consisting of P, K, V, I, and A.

Preferably, the N-terminus of the second and third THD, respectively is selected from the group consisting of the sequences V-A-A-H-I (SEQ ID NO: 25), K-V-A-H-L (SEQ ID NO: 26), P-A-A-H-L (SEQ ID NO: 27), P-V-A-H-V (SEQ ID NO: 28), I-A-A-H-V (SEQ ID NO: 29), and I-A-A-H-Y (SEQ ID NO: 30). Preferably, the N-terminus of the second and third THD is V-A-A-H-I-T (SEQ ID NO: 25).

It is preferred that the N-terminal amino acid sequence actually comprised in the THD is closely related to the amino acid sequence of the TNF-ligand family member from which the THD is derived. Accordingly, the N-terminal amino acid sequence of a THD derived from human TRAIL is preferably V-A-A-H-I (SEQ ID NO: 25) or a closely related sequence. For THDs derived from human TRAIL X₂ is preferably V, A, or I, most preferably V. The N-terminal amino acid sequence of a THD derived from human FasL is preferably K-V-A-H-L (SEQ ID NO: 26) or a closely related sequence. For THDs derived from human FasL X₂ is preferably K. The N-terminal amino acid sequence of a THD derived from human LIGTH, LT α

or LT β is preferably P-A-A-H-L (SEQ ID NO: 27) or a closely related sequence. For THDs derived from human LIGHT, LT α or LT β . X₂ is preferably P. The N-terminal amino acid sequence of a THD derived from human TNF preferably P-V-A-H-V (SEQ ID NO: 28) or a closely related sequence. For THDs derived from human TNF X₂ is preferably P. The N-terminal amino acid sequence of a THD derived from human CD40L preferably I-A-A-H-V (SEQ ID NO: 29) or a closely related sequence. For THDs derived from human CD40L X₂ is preferably I. The N-terminal amino acid sequence of a THD derived from human TWEAK preferably I-A-A-H-Y (SEQ ID NO: 30) or a closely related sequence. For THDs derived from human TWEAK X₂ is preferably I.

As outlined above component A comprises the C-terminus of the first and second THD, respectively, which is defined above and is linked to the N-terminus of the second and third THD which is defined by the N-terminal consensus sequence

X₃-V/W/F/C-A/L-E/Y/Q/H-L (SEQ ID NO: 3)

wherein X₃ is selected from the group consisting of D, S, M, and I. This N-terminal consensus sequence is preferably present, if one or more THDs are derived from CD27L or BAFF.

Preferably the N-terminus of the second and third THD, respectively is selected from the group consisting of the sequences D-V-A-E-L (SEQ ID NO: 31), D-W-A-E-L (SEQ ID NO: 32), D-F-A-E-L (SEQ ID NO: 33), D-C-A-E-L, (SEQ ID NO: 34), D-V-L-E-L (SEQ ID NO: 35), D-V-A-Y-L (SEQ ID NO: 36), D-V-L-Y-L (SEQ ID NO: 37), D-V-A-Q-L (SEQ ID NO: 38), D-V-L-Q-L (SEQ ID NO: 39), D-V-A-H-L (SEQ ID NO: 40), D-V-L-H-L (SEQ ID NO: 41), D-W-L-E-L (SEQ ID NO: 42), D-W-A-Y-L (SEQ ID NO: 43), D-W-L-Y-L (SEQ ID NO: 44), D-W-A-Q-L (SEQ ID NO: 45), D-W-L-Q-L (SEQ ID NO: 46), D-W-A-H-L (SEQ ID NO: 47), D-W-L-H-L (SEQ ID NO: 48), D-F-L-E-L (SEQ ID NO: 49), D-F-A-Y-L (SEQ ID NO: 50), D-F-L-Y-L (SEQ ID NO: 51), D-F-A-Q-L (SEQ ID NO: 52), D-F-L-Q-L (SEQ ID NO: 53), D-F-A-H-L (SEQ ID NO: 54), D-F-L-H-L (SEQ ID NO: 55), D-C-L-E-L (SEQ ID NO: 56), D-C-A-Y-L (SEQ ID NO: 57), D-C-L-Y-L (SEQ ID NO: 58), D-C-A-Q-L (SEQ ID NO: 59), D-C-L-Q-L (SEQ ID NO: 60), D-C-A-H-L (SEQ ID NO: 61), and D-C-L-H-L (SEQ ID NO: 62). Preferably, the N-terminus of the second and third THD, respectively is selected from the group consisting of the sequences S-V-A-E-L (SEQ ID NO: 63), S-W-A-E-L (SEQ ID NO: 64), S-F-A-E-L (SEQ ID NO: 65), S-C-A-E-L, (SEQ ID NO: 66), S-V-L-E-L (SEQ ID NO: 67), S-V-A-Y-L (SEQ ID NO: 68), S-V-L-Y-L (SEQ ID NO: 69), S-V-A-Q-L (SEQ ID NO: 70), S-V-L-Q-L (SEQ ID NO: 71), S-V-A-H-L (SEQ ID NO: 72), S-V-L-H-L (SEQ ID NO: 73), S-W-L-E-L (SEQ ID NO: 74), S-W-A-Y-L (SEQ ID NO: 75), S-W-L-Y-L (SEQ ID NO: 76), S-W-A-Q-L (SEQ ID NO: 77), S-W-L-Q-L (SEQ ID NO: 78), S-W-A-H-L (SEQ ID NO: 79), S-W-L-H-L (SEQ ID NO: 80), S-F-L-E-L (SEQ ID NO: 81), S-F-A-Y-L (SEQ ID NO: 82), S-F-L-Y-L (SEQ ID NO:

83), S-F-A-Q-L (SEQ ID NO: 84), S-F-L-Q-L (SEQ ID NO: 85), S-F-A-H-L (SEQ ID NO: 86), S-F-L-H-L (SEQ ID NO: 87), S-C-L-E-L (SEQ ID NO: 88), S-C-A-Y-L (SEQ ID NO: 89), S-C-L-Y-L (SEQ ID NO: 90), S-C-A-Q-L (SEQ ID NO: 91), S-C-L-Q-L (SEQ ID NO: 92), S-C-A-H-L (SEQ ID NO: 93), and S-C-L-H-L (SEQ ID NO: 94). Preferably, the N-terminus of the

5 second and third THD, respectively is selected from the group consisting of the sequences M-V-A-E-L (SEQ ID NO: 95), M-W-A-E-L (SEQ ID NO: 96), M-F-A-E-L (SEQ ID NO: 97), M-C-A-E-L, (SEQ ID NO: 98), M-V-L-E-L (SEQ ID NO: 99), M-V-A-Y-L (SEQ ID NO: 100), M-V-L-Y-L (SEQ ID NO: 101), M-V-A-Q-L (SEQ ID NO: 102), M-V-L-Q-L (SEQ ID NO: 103), M-V-A-H-L (SEQ ID NO: 104), M-V-L-H-L (SEQ ID NO: 105), M-W-L-E-L (SEQ ID NO: 106),

10 M-W-A-Y-L (SEQ ID NO: 107), M-W-L-Y-L (SEQ ID NO: 108), M-W-A-Q-L (SEQ ID NO: 109), M-W-L-Q-L (SEQ ID NO: 110), M-W-A-H-L (SEQ ID NO: 111), M-W-L-H-L (SEQ ID NO: 112), M-F-L-E-L (SEQ ID NO: 113), M-F-A-Y-L (SEQ ID NO: 114), M-F-L-Y-L (SEQ ID NO: 115), M-F-A-Q-L (SEQ ID NO: 116), M-F-L-Q-L (SEQ ID NO: 117), M-F-A-H-L (SEQ ID NO: 118), M-F-L-H-L (SEQ ID NO: 119), M-C-L-E-L (SEQ ID NO: 120), M-C-A-Y-

15 L (SEQ ID NO: 121), M-C-L-Y-L (SEQ ID NO: 122), M-C-A-Q-L (SEQ ID NO: 123), M-C-L-Q-L (SEQ ID NO: 124), M-C-A-H-L (SEQ ID NO: 125), M-C-L-H-L (SEQ ID NO: 126). Preferably the N-terminus of the second and third THD, respectively is selected from the group consisting of the sequences I-V-A-E-L (SEQ ID NO: 127), I-W-A-E-L (SEQ ID NO: 128), I-F-A-E-L (SEQ ID NO: 129), I-C-A-E-L, (SEQ ID NO: 130), I-V-L-E-L (SEQ ID NO: 131), I-V-

20 A-Y-L (SEQ ID NO: 132), I-V-L-Y-L (SEQ ID NO: 133), I-V-A-Q-L (SEQ ID NO: 134), I-V-L-Q-L (SEQ ID NO: 135), I-V-A-H-L (SEQ ID NO: 136), I-V-L-H-L (SEQ ID NO: 137), I-W-L-E-L (SEQ ID NO: 138), I-W-A-Y-L (SEQ ID NO: 139), I-W-L-Y-L (SEQ ID NO: 140), I-W-A-Q-L (SEQ ID NO: 131), I-W-L-Q-L (SEQ ID NO: 142), I-W-A-H-L (SEQ ID NO: 143), I-W-L-H-L (SEQ ID NO: 144), I-F-L-E-L (SEQ ID NO: 145), I-F-A-Y-L (SEQ ID NO: 146), I-F-L-

25 Y-L (SEQ ID NO: 147), I-F-A-Q-L (SEQ ID NO: 148), I-F-L-Q-L (SEQ ID NO: 149), I-F-A-H-L (SEQ ID NO: 150), I-F-L-H-L (SEQ ID NO: 151), I-C-L-E-L (SEQ ID NO: 152), I-C-A-Y-L (SEQ ID NO: 153), I-C-L-Y-L (SEQ ID NO: 154), I-C-A-Q-L (SEQ ID NO: 155), I-C-L-Q-L (SEQ ID NO: 156), I-C-A-H-L (SEQ ID NO: 157), and I-C-L-H-L (SEQ ID NO: 158).

The N-terminal amino acid sequence of a THD derived from human CD27L is preferably

30 D-V-A-E-L (SEQ ID NO: 31) or a closely related sequence. For THDs derived from human CD27L X₃ is preferably D. The N-terminal amino acid sequence of a THD derived from human BAFF is preferably D-C-L-Q-L (SEQ ID NO: 60) or a closely related sequence. For THDs derived from human BAFF X₃ is preferably D.

Preferably, component A comprises the C-terminal sequences of the first and second THD

35 selected from the group consisting of S-F-F-G-A-F (SEQ ID NO: 6), T-F-F-G-L-Y (SEQ ID NO:

16), S-Y-F-G-A-F (SEQ ID NO: 17), V-F-F-G-A-F (SEQ ID NO: 18), T-F-F-G-A-V (SEQ ID NO: 19), V-Y-F-G-I-I (SEQ ID NO: 20), T-S-F-G-L-L (SEQ ID NO: 21), T-Y-F-G-L-F (SEQ ID NO: 22), T-F-F-G-V-Q (SEQ ID NO: 23), T-F-F-G-A-L (SEQ ID NO: 24) or a closely related sequence thereof and the N-terminal sequences of the second and third THD selected from the group V-A-A-H-I-T (SEQ ID NO: 25), K-V-A-H (SEQ ID NO: 26), P-A-A-H-L (SEQ ID NO: 27), P-V-A-H-V (SEQ ID NO: 28), I-A-A-H-V (SEQ ID NO: 29), I-A-A-H-Y (SEQ ID NO: 30), D-V-A-E-L (SEQ ID NO: 31) and D-C-L-Q-L (SEQ ID NO: 60) or a closely related sequences thereof.

It is preferred that the C-terminal amino acid sequence of a THD derived from human TRAIL is preferably S-F-F-G-A-F (SEQ ID NO: 6) or a closely related sequence and the N-terminal amino acid sequence is preferably V-A-A-H-I (SEQ ID NO: 25) or a closely related sequence. For THDs derived from human TRAIL X₁ is preferably F and X₂ is preferably V, A, or I, most preferably V.

Similarly, for THDs derived from human FasL the C-terminal amino acid sequence is preferably T-F-F-G-L-Y (SEQ ID NO: 16) or a closely related sequence and the N-terminal amino acid sequence is preferably K-V-A-H-L (SEQ ID NO: 26) or a closely related sequence. For THDs derived from human FasL X₁ is preferably Y and X₂ is preferably K.

For THDs derived from human LIGHT the C-terminal amino acid sequence is preferably S-Y-F-G-A-F (SEQ ID NO: 17) or a closely related sequence and the N-terminal amino acid sequence is preferably P-A-A-H-L (SEQ ID NO: 27) or a closely related sequence. For THDs derived from human LIGHT X₁ is preferably F and X₂ is preferably P.

For THDs derived from human LT α the C-terminal amino acid sequence is preferably V-F-F-G-A-F (SEQ ID NO: 18) or a closely related sequence and the N-terminal amino acid sequence is preferably P-A-A-H-L (SEQ ID NO: 27) or a closely related sequence. For THDs derived from human LT α X₁ is preferably F and X₂ is preferably P.

For THDs derived from human LT β the C-terminal amino acid sequence is preferably T-F-F-G-A-V (SEQ ID NO: 19) or a closely related sequence and the N-terminal amino acid sequence is preferably P-A-A-H-L (SEQ ID NO: 27) or a closely related sequence. For THDs derived from human LT β X₁ is preferably V and X₂ is preferably P.

For THDs derived from human TNF the C-terminal amino acid sequence is preferably V-Y-F-G-I-I (SEQ ID NO: 20) or a closely related sequence and the N-terminal amino acid sequence is preferably P-V-A-H-V (SEQ ID NO: 28) or a closely related sequence. For THDs derived from human TNF X₁ is preferably I and X₂ is preferably P.

For THDs derived from human CD40L the C-terminal amino acid sequence is preferably T-S-F-G-L-L (SEQ ID NO: 21) or a closely related sequence and the N-terminal amino acid

sequence is preferably I-A-A-H-V (SEQ ID NO: 29) or a closely related sequence. For THDs derived from human CD40L X₁ is preferably L and X₂ is preferably I.

For THDs derived from human TWEAK the C-terminal amino acid sequence is preferably T-Y-F-G-L-F (SEQ ID NO: 22) or a closely related sequence and the N-terminal amino acid sequence is preferably I-A-A-H-Y (SEQ ID NO: 30) or a closely related sequence. For THDs derived from human TWEAK X₁ is preferably F and X₂ is preferably I.

For THDs derived from human CD27L the C-terminal amino acid sequence is preferably T-F-F-G-V-G (SEQ ID NO: 23) or a closely related sequence and the N-terminal amino acid sequence of a THD derived from human CD27L is preferably D-V-A-E-L (SEQ ID NO: 31) or a closely related sequence. For THDs derived from human CD27L X₁ is preferably G and X₃ is preferably D.

For THDs derived from human BAFF the C-terminal amino acid sequence is preferably T-F-F-G-A-L (SEQ ID NO: 24) or a closely related sequence and the N-terminal amino acid sequence is preferably D-C-L-Q-L (SEQ ID NO: 60) or a closely related sequence. For THDs derived from human BAFF X₁ is preferably L and X₃ is preferably D.

Preferred are combinations of the C-terminal sequence of the first and second THD and N-terminal sequences of the second and third THD as follows: T-F-F-G-L-Y (SEQ ID NO: 16) and K-V-A-H (SEQ ID NO: 26), S-Y-F-G-A-F (SEQ ID NO: 17) and P-A-A-H-L (SEQ ID NO: 26), V-F-F-G-A-F (SEQ ID NO: 18) and P-A-A-H-L (SEQ ID NO: 27), T-F-F-G-A-V (SEQ ID NO: 19) and P-A-A-H-L (SEQ ID NO: 27), V-Y-F-G-I-I (SEQ ID NO: 20) and P-V-A-H-V (SEQ ID NO: 27), T-S-F-G-L-L (SEQ ID NO: 21) and I-A-A-H-V (SEQ ID NO: 29), T-Y-F-G-L-F (SEQ ID NO: 22) and I-A-A-H-Y (SEQ ID NO: 29), T-F-F-G-V-Q (SEQ ID NO: 23) and D-V-A-E-L (SEQ ID NO: 31), T-F-F-G-A-L (SEQ ID NO: 24) and D-C-L-Q-L (SEQ ID NO: 60).

Most preferred is the combination S-F-F-G-A-F (SEQ ID NO: 6) and V-A-A-H-I (SEQ ID NO: 25).

It is understood that in each of the preceding cases each of the respective THDs also comprise the amino acid sequence of the respective TNF-ligand family member or variant thereof intervening the respective N-terminal and C-terminal sequence.

The at least three THDs are connected to each other by at least two intervening peptides X_a, wherein the first THD (THD1) is connected with one peptide X_a to the second THD (THD2), which is connected with one peptide X_a to the third THD (THD3) and so forth; if more than three THDs are comprised. The peptide X_a may in each case be different or all peptides X_a may be identical. It is preferred that the peptides X_a are identical. The peptide X_a preferably comprises amino acid sequences N-terminal to the N-terminal consensus sequence of the respective THD or C-terminal of the C-terminal consensus sequence. In each case these sequences may comprise

mutations. Preferably, such peptides have an amino acid length in the range of 3 to 8 amino acids. The peptides can have a length of 3, 4, 5, 6, 7, or 8 amino acids, particularly preferred lengths are 4 or 5, most preferably 4 amino acids.

As outlined above component A comprises the C-terminus of the first and second THD, respectively, which is defined by a consensus sequence as described above and is linked to the N-terminus of the second and third THD, respectively, defined by a consensus sequence as described above through a peptide X_a, which is in each case independently selected and has a length of 3 to 8 amino acids.

Preferably, the peptide X_a consists of

10 X_e-X_f-X_g

wherein X_e is selected from the group consisting of L, L-V, L-V-G, L-V-G-G (SEQ ID NO: 159), L-V-G-G-I (SEQ ID NO: 160), L-V-S, L-V-S-G (SEQ ID NO: 161), A, A-V, A-V-S, A-V-G, A-V-G-G (SEQ ID NO: 162), A-V-S-G (SEQ ID NO: 163), I-V, I-V-S, I-V-G, I-V-G-G (SEQ ID NO: 164), I-V-S-G (SEQ ID NO: 165), K, K-L, M, M-V, A, A-L, M-V-G, M-V-Q, G, G-V, G-V-H, W, W-V, W-V-R, W-V-R-P ((SEQ ID NO: 167), K-L-L, I, A-V, Q, Q-V, and Q-V-H;

X_f is absent or selected from the group consisting of G, S, G-G, S-G, G-S, S-S, G-G-G, G-G-S, G-S-G, S-G-G, G-S-S, S-G-S and S-S-S; and

X_g is absent or selected from the group consisting of R, Q-R, P-Q-R, G-P-Q-R (SEQ ID NO: 166), L-R, N, V-N, K, L-K, L, G-L, K, D-K, Q, P-Q, A, R-A, W, G-W, Q, and T-Q.

Preferably,

- (i) X_e is selected from L, L-V, L-V-G, L-V-G-G (SEQ ID NO: 159), L-V-S, L-V-S-G (SEQ ID NO: 161), A, A-V, A-V-S, A-V-G, A-V-G-G, A-V-S-G (SEQ ID NO: 163), I-V, I-V-S, I-V-G, I-V-G-G, (SEQ ID NO: 164) and I-V-S-G (SEQ ID NO: 165); X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from R, Q-R, P-Q-R, and G-P-Q-R (SEQ ID NO: 166);
- (ii) X_e is selected from K, and K-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from R, and L-R,
- (iii) X_e is selected from M, M-V; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from N and V-N;
- (iv) X_e is selected from A, and A-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from K and L-K;
- (v) X_e is selected from M, M-V, and M-V-G; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from L, and G-L;

- (vi) X_e is selected from A, and A-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from K, and D-K;
- (vii) X_e is selected from K, and K-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from Q, and P-Q;
- 5 (viii) X_e is selected from Q, Q-V, and Q-V-H; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from A, and R-A;
- (ix) X_e is selected from W, W-V, W-V-R, and W-V-R-P; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from W, and G-W; or
- 10 (x) X_e is selected from K, K-L, and K-L-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from Q, and T-Q.

More preferably, the at least three THDs of component A are connected by the peptide X_a which is selected from the group consisting of I-V-S-G (SEQ ID NO: 165), A-V-S-G (SEQ ID NO: 163), I-V-G-G (SEQ ID NO: 164), A-V-G-G (SEQ ID NO: 162), L-V-S-G (SEQ ID NO: 161), L-V-G-G (SEQ ID NO: 159), L-V-G-G-R (SEQ ID NO: 168), L-V-G-G-G (SEQ ID NO: 169), L-V-G-G-P (SEQ ID NO: 170), L-V-G-G-A (SEQ ID NO: 171), L-V-G-G-P-Q (SEQ ID NO: 172), L-V-G-G-P-Q-R (SEQ ID NO: 173), L-V-G-G-G-P-Q-R (SEQ ID NO: 174), and L-V-G-G-G-G-P-Q (SEQ ID NO: 175), more preferably I-V-S-G (SEQ ID NO: 165), A-V-S-G (SEQ ID: 163), L-V-S-G (SEQ ID NO: 161) or L-V-G-G (SEQ ID NO: 159), even more preferably L-V-G-G (SEQ ID NO: 159) or A-V-S-G (SEQ ID: 163) and most preferably A-V-S-G (SEQ ID: 163). These amino acid sequences are preferred for X_a , if the THDs are derived from TRAIL, in particular human TRAIL.

In one embodiment the arginine at position 121 of TRAIL, preferably of huTRAIL, if comprised in X_a , is preferably replaced with glycine. This replacement will not decrease thermal stability. The replacement with lysine is less preferred.

25 In one embodiment the glycine at position 281 of TRAIL, preferably of huTRAIL, if comprised in X_a , can be replaced with serine. This replacement decreases aggregate formation.

Preferably, the amino acid sequence of the junction between two THDs, i.e. the C-terminal sequence- X_a -N-terminal sequence", preferably at the junction of the first and second and the second and third or subsequent THD domains of component A is selected from the group consisting of (X_a is in each case underlined): S-F-F-G-A-F-L-V-G-G-G-P-Q-R-V-A-A-H-I (SEQ ID NO: 176); S-F-F-G-A-F-L-V-G-G-P-Q-R-V-A-A-H-I (SEQ ID NO: 177); S-F-F-G-A-F-L-V-S-G-I-A-A-H-I (SEQ ID NO: 178); S-F-F-G-A-F-A-V-G-G-A-A-A-H-I (SEQ ID NO: 179); S-F-F-G-A-F-A-V-G-G-I-A-A-H-I (SEQ ID NO: 180); S-F-F-G-A-F-I-V-G-G-I-A-A-H-I (SEQ ID NO: 181); S-F-F-G-A-F-A-V-S-G-I-A-A-H-I (SEQ ID NO: 182) and S-F-F-G-A-F-I-V-S-G-I-A-A-H-I (SEQ ID NO: 183). These amino acid sequences are preferred for C-terminal

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sequence- X_a -N-terminal sequence junctions of two THDs, if the THDs are derived from TRAIL, in particular human TRAIL.

It is most preferred that the amino acid sequence of the C-terminal sequence- X_a -N-terminal sequence that are at the junction of the first and second, and second and third or subsequent THD domains of component A is selected from the group consisting of (X_a is in each case underlined): S-F-F-G-A-L-V-G-G-V-A-A-H-I (SEQ ID NO: 184); S-F-F-G-A-F-L-V-G-G-R-V-A-A-H-I (SEQ ID NO: 185); S-F-F-G-A-F-L-V-G-G-I-A-A-H-I (SEQ ID NO: 186); S-F-F-G-A-F-L-V-G-G-A-A-A-H-I (SEQ ID NO: 187) or S-F-F-G-A-F-A-V-S-G-A-A-A-H-I (SEQ ID NO: 188). These amino acid sequences are preferred for C-terminal sequence- X_a -N-terminal sequence junctions of two THDs, if the THDs are derived from TRAIL, in particular human TRAIL.

In another preferred embodiment the at least three THDs of component A are identical which is indicated by subscript indices of the respective TNF-ligand family member THD, i.e. scTRAIL₃. This symbolizes three linked TRAIL THD molecules.

In another preferred embodiment the polypeptide of the first aspect of the present invention comprises a component B comprising a dimerization domain consisting of an antibody V_L and V_H region linked directly to each other with a peptide that has a length of between 7 and 11 amino acids.

Preferably, the V_L and V_H region of an antibody specifically bind to a target molecule on the cell surface. Preferably, the target molecule is selected from the group consisting of tyrosine-kinase-receptors (EGFR, HER2, HER3, HER4), VEGFRs, heteromeric integrin α - or β -receptor family, tumor stroma markers like fibroblast activation protein (FAP) endoglyx-1, or endosialin, galectin, EpCAM, CEA, CD44 and tumor specific variants thereof and other tumor selective cell surface markers, CD2, CD5, CD7, CD19, CD20, CD21, CD22, CD24, CD25, CD30, CD33, CD38, CD40, CD52, CD56, CD71, CD72, CD73, CD105, CD117, CD123, CD133, c-Met, PDGFR, IGF1-R, HMW-MAA, TAG-72, GD2, GD3, GM2, folate receptor, Lgr5, Ley, Muc-1, Muc-2, PSMA, PSCA and uPAR. More preferably, the target molecule is FAP, EGFR, HER2 or HER3.

Preferably the V_L region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 4 and/or the V_H region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 237, if the target antigen is EGFR. Preferably the V_L region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 238 and/or the V_H region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 239, if the target antigen is HER-2. Preferably the V_L region comprises, essentially consists or consists of the amino acid sequence

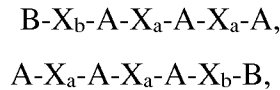
according to SEQ ID NO: 240 and/or the V_H region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 241, if the target antigen is HER-3.

Preferably, the V_H and V_L region are directly linked to each other by a peptide of 7, 8, 9, 10 or 11 amino acids in length. This peptide is preferably a flexible amino acid stretch. Particularly preferred is that the V_H and V_L region are directly linked to each other by a peptide of 5, 8 or 10 amino acids in length which is selected from the group consisting of G-G-G-G-S (SEQ ID NO: 189), G-G-G-G-S-G-G-G: (SEQ ID NO: 190) or G-G-G-G-S-G-G-G-G-S (SEQ ID NO: 199).

In another preferred embodiment component A is linked to component B by a peptide X_b. The peptide X_b preferably has a length of between 5 to 35 amino acids, more preferably between 10 to 30, more preferably 12 to 25, i.e. 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35, preferably 16 amino acids. It is preferred that the peptide X_b is selected from glycine and/or serine rich peptides. A peptide is considered glycine and/or serine rich, if at least 70% of the amino acids are selected from glycine and/or serine, preferably more than 75%, more than 80%, more than 90%. Other amino acids that may be present comprise are preferably those that do not result in structural rigidity, e.g. proline, thus preferably small amino acids are comprised in addition to serine and/or glycine. Preferred examples of such small amino acids are alanine or threonine. Preferred examples of peptides are selected from (GGS)_n, (GGSGG)_m, (SEQ ID NO: 257), wherein n is an integer between 1 to 12, preferably between 3 to 7 and m is an integer between 1 to 7, preferably between 3 to 5. Further preferred peptides are selected from the group consisting of SEQ ID NO 192 (GGSGGASSGG), SEQ ID NO: 193 (GGSGGGSSGG), SEQ ID NO 194 (AAAGNGTSNGTSEFG) SEQ ID NO: 258 (GSGNGTSNGTSGSSGG) and SEQ ID NO: 259 (GSGNGTSNGTSGSSRT). It is further preferred that X_b comprises at least one glycosylation motif. Glycosylation motifs comprise for instance nitrogen atoms in asparagine chains. Even more preferably, X_b has a length of 16 amino acids and has two glycosylation motifs. (SEQ ID NO: 194, A-A-A-G-N-G-T-S-N-G-T-S-E-F-G-G). The glycosylation motif is preferably inserted in one of the above outlined preferred peptides. Preferably, at or close to the middle of the peptide.

It is preferred that peptide X_b may be selected independently of any peptide selected for linking the at least three THD of component A.

Preferably, the structure of the polypeptide according to the first aspect of the present invention comprises, essentially consists or consists of (from N- to C-terminus, A: component A; B: component B; X_a: peptide linking the at least three THDs of component A; X_b: linking component A to component B; it is noted that below structures do not exclude the possibility that additional peptides are attached N- and/or C-terminally)



or may be



As outlined above the polypeptide of the first aspect may comprise more than three components A, e.g. 4, 5, 6, 7, 8, or 9, more preferably 6 or 9. These may all be arranged consecutively and component B is then position N- and/or C terminally or one or more component B may be interspersed between component A. Preferred structures of the polypeptide according to the first aspect of the present invention comprises, essentially consists or consists of (from N- to C-terminus):

15 $B-X_b-A-X_a-A-X_a-A-X_a-A-X_a-A; A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_b-B, B-X_b-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_b-B; A-X_a-A-X_a-A-X_b-B-X_b-A-X_a-A-X_a-A, B-X_b-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A; A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_b-B, B-X_b-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_b-B; A-X_a-A-X_a-A-X_b-B-X_b-A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A, or A-X_a-A-X_a-A-X_a-A-X_a-A-X_a-A-X_b-B-X_b-A-X_a-A-X_a-A.$

In another preferred embodiment the polypeptide comprising component A linked to component B is selected from the group consisting of Db-scTRAIL₃-FAVSGAA (SEQ ID NO: 195), dsDb3M6-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 196), Db10-scTRAIL₃-FAVSGGA (SEQ ID NO: 197), Db-glyco-scTRAIL₃-FAVSGAA (SEQ ID NO: 198), Db-glyco-scTRAIL₃-FAVSGIA (SEQ ID NO: 199). More preferably, the polypeptide comprising component A linked to component B is selected from the group consisting of Db8-glyco-scTRAIL₃-FAVSGGA (SEQ ID NO: 200), Db10-glyco-scTRAIL₃-FAVSGGA (SEQ ID NO: 201).

Another optional element which may or may not be present in a polypeptide according to the first aspect of the present invention is a tag allowing for example the detection and or purification of a polypeptide of the first aspect of the present invention. Examples for such tags are His-tag, FLAG-tag, HA-tag, STREP-tag, myc-tag or GST (see Arnau *et al.*, 2006). Preferably, such tag is positioned outside the region comprising the at least three THDs of component A and component B. It is also possible to position a protease cleavage site (e.g. a Thrombin cleavage site) adjacent to the tag, in this context it is preferred that the tags are positioned directly at the C-terminus or N-terminus of the tag allowing to remove the tag after purification. Preferably, the tag is N-terminally to be cleaved after detection or purification.

In a second aspect the present invention relates to a polypeptide comprising component C comprising: at least three THDs, wherein the C-terminus of the first and second THD, respectively, which is in each case defined by the consensus sequence

-S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1),

is linked to the N-terminus of the second and third THD, respectively, which is in each case defined by the consensus sequence

X₂-V/A/F-A-H-V/L/I/Y (SEQ ID NO: 2)

5

or

X₃-V/W/F/C-A/L-E/Y/Q/H-L (SEQ ID NO: 3),

through a peptide X_c, which is in each case independently selected and has a length of 3 to 5 amino acids,

wherein X₁ is a non-polar/hydrophob or polar/neutral amino acid, preferably selected from the group consisting of F, V, Q, A, I, L, and Y;

wherein X₂ is selected from the group consisting of P, K, V, I, and A; and

wherein X₃ is selected from the group consisting of D, S, M, and I.

In a preferred embodiment component C comprises at least three TNF homology domains of TNF-ligand family member proteins (THD), wherein the C-terminus of the first and second THD, respectively, in each case defined by the consensus sequence

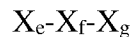
-S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1)

wherein X₁ is a non-polar/hydrophob or polar/neutral amino acid, preferably selected from the group consisting of F, V, Q, A, I, L, and Y. Component C is identical to component A described above regarding the first aspect of the invention but for the fact that the peptide X_c connecting the the THDs in component C has a length of between 3 to 5 amino acids rather than between 3 to 8 amino acids as peptide X_a. This also applies to all preferred and particular preferred embodiments of component A outlined in detail above.

Component C of the polypeptide of the present invention, preferably has a thermal stability of at least 48°C, more preferably of at least 49°C, more preferably of at least 50°C, more preferably of at least 51°C, more preferably of at least 52°C, more preferably of at least 53°C, more preferably of at least 54°C, more preferably of at least 55°C, more preferably of at least 56°C, more preferably of at least 57°C, more preferably of at least 58°C, more preferably of at least 59°C and most preferably at least 60°C. Preferably, the polypeptide of the second aspect comprising component C has at least the above indicated thermal stabilities. The skilled person is well aware how to measure thermal stabilities of polypeptides. Exemplary methods are taught herein below.

As outlined above component C comprises the C-terminus of the first and second THD, respectively, which is defined by a consensus sequence as described above and is connected to the N-terminus of the second and third THD, respectively, defined by a consensus sequence as

described above through a peptide X_c , which is in each case independently selected and has a length of 3 to 5 amino acids, preferably 4 amino acids. Preferably, the peptide X_c consists of



wherein X_e is selected from the group consisting of L, L-V, L-V-G, L-V-G-G, L-V-S, L-V-S-G, A, A-V, A-V-S, A-V-G, A-V-G-G, A-V-S-G, I-V, I-V-S, I-V-G, I-V-G-G, I-V-S-G, K, K-L, M, M-V, A, A-L, M-V-G, M-V-Q, G, G-V, G-V-H, W, W-V, W-V-R, W-V-R-P, K-L-L, I, A-V, Q, Q-V, and Q-V-H;

X_f is absent or selected from the group consisting of G, S, G-G, S-G, G-S, S-S, G-G-G, G-G-S, G-S-G, S-G-G, G-S-S, S-G-S and S-S-S; and

X_g is absent or selected from the group consisting of R, Q-R, P-Q-R, G-P-Q-R, L-R, N, V-N, K, L-K, L, G-L, K, D-K, Q, P-Q, A, R-A, W, G-W, Q, and T-Q.

Preferably,

(i) X_e is selected from L, L-V, L-V-G, L-V-G-G, L-V-S, L-V-S-G, A, A-V, A-V-S, A-V-G, A-V-G-G, A-V-S-G, I-V, I-V-S, I-V-G, I-V-G-G, and I-V-S-G; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from R, Q-R, P-Q-R, and G-P-Q-R;

(ii) X_e is selected from K, and K-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from R, and L-R,

(iii) X_e is selected from M, M-V; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from N and V-N;

(iv) X_e is selected from A, and A-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from K and L-K;

(v) X_e is selected from M, M-V, and M-V-G; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from L, and G-L;

(vi) X_e is selected from A, and A-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from K, and D-K;

(vii) X_e is selected from K, and K-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from Q, and P-Q;

(viii) X_e is selected from Q, Q-V, and Q-V-H; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from A, and R-A;

(ix) X_e is selected from W, W-V, W-V-R, and W-V-R-P; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from W, and G-W; or

(x) X_e is selected from K, K-L, and K-L-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from Q, and T-Q.

More preferably, the at least three THD domains of component C are linked by the peptide X_c which is selected from the group consisting of L-V-G, I-V-S-G, A-V-S-G, A-V-S-G-A, I-V-G-G, A-V-G-G, A-V-G-G-A, L-V-S-G, L-V-G-G, L-V-G-G-R, L-V-G-G-G, L-V-G-G-P, L-V-G-G-A.

5 More preferably, the at least three THDs of component A are connected by the peptide X_a which is selected from the group consisting of I-V-S-G (SEQ ID NO: 165), A-V-S-G (SEQ ID NO: 163), I-V-G-G (SEQ ID NO: 164), A-V-G-G (SEQ ID NO: 162), L-V-S-G (SEQ ID NO: 161), L-V-G-G (SEQ ID NO: 159), L-V-G-G-R (SEQ ID NO: 168), L-V-G-G-G (SEQ ID NO: 169), L-V-G-G-P (SEQ ID NO: 170), L-V-G-G-A (SEQ ID NO: 171), L-V-G-G-P-Q (SEQ ID NO: 172), L-V-G-G-P-Q-R (SEQ ID NO: 173), L-V-G-G-G-P-Q-R (SEQ ID NO: 174), and L-V-G-G-G-G-P-Q (SEQ ID NO: 175), more preferably I-V-S-G (SEQ ID NO: 165), A-V-S-G (SEQ ID: 163), L-V-S-G (SEQ ID NO: 161) or L-V-G-G (SEQ ID NO: 159), even more preferably L-V-G-G (SEQ ID NO: 159) or A-V-S-G (SEQ ID: 163) and most preferably A-V-S-G (SEQ ID: 163). These amino acid sequences are preferred for X_a, if the THDs are derived from
15 TRAIL, in particular human TRAIL.

In one embodiment the arginine at position 121 of TRAIL, preferably of huTRAIL, if comprised in X_a, is preferably replaced with glycine. This replacement will not decrease thermal stability. The replacement with lysine is less preferred.

20 In one embodiment the glycine at position 281 of TRAIL, preferably of huTRAIL, if comprised in X_a, can be replaced with serine. This replacement decreases aggregate formation.

Preferably, the amino acid sequence of the C-terminal sequence-X_c-N-terminal sequence that are at the junction of the first and second, and second and third or subsequent THD domains of component A is selected from the group consisting of (X_c is in each case underlined): S-F-F-G-A-F-L-V-S-G-I-A-A-H-I (SEQ ID NO: 178); S-F-F-G-A-F-A-V-G-G-A-A-A-H-I (SEQ ID NO: 179); S-F-F-G-A-F-A-V-G-G-I-A-A-H-I (SEQ ID NO: 180); S-F-F-G-A-F-I-V-G-G-I-A-A-H-I (SEQ ID NO: 181); S-F-F-G-A-F-A-V-S-G-I-A-A-H-I (SEQ ID NO: 182) and S-F-F-G-A-F-I-V-S-G-I-A-A-H-I (SEQ ID NO: 183). These amino acid sequences are preferred for C-terminal sequence-X_c-N-terminal sequence that are the THD junction, if the THDs are derived from TRAIL, in particular human TRAIL.

30 It is most preferred that, the amino acid sequence of the C-terminal sequence-X_c-N-terminal sequence that are at the junction of the first and second, and second and third or subsequent THD domains of component A is selected from the group consisting of (X_c is in each case underlined): S-F-F-G-A- L-V-G-G-V-A-A-H-I (SEQ ID NO: 184); S-F-F-G-A-F-L-V-G-G-R-V-A-A-H-I (SEQ ID NO: 185); S-F-F-G-A-F-L-V-G-G-I-A-A-H-I (SEQ ID NO: 186); S-F-F-G-A-F-L-V-G-G-A-A-A-H-I (SEQ ID NO: 187) or S-F-F-G-A-F-A-V-S-G-A-A-A-H-I (SEQ
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ID NO: 188). These amino acid sequences are preferred for C-terminal sequence-X-N-terminal sequence that are the THD junction, if the THDs are derived from TRAIL, in particular human TRAIL.

In another preferred embodiment the polypeptide of the second aspect of the present invention comprises a component D which is selected from the group consisting of a dimerization or multimerization domain, a half-life extension domain, a target specific binding domain or combinations thereof. Preferred components D possess both a dimerization or multimerization domain and a target specific binding domain.

It is preferred that the dimerization or multimerization or the target specific binding domains are proteins or parts thereof coupled to the polypeptide of the second aspect of the present invention. Dimerization domains are dimerization domains from an antibody, e.g. an Fc region, a CH3 domain of IgG, IgA, or IgD, a CH2 domain of IgE or IgM, a CH4 domain of IgE or IgM, and the CH1 and CL domain. A preferred dimerization domain from an antibody, is the CH2 domain of IgE (EHD2), variants or fragments thereof. Other dimerization or multimerization domains include barnase-barstar, C4bp, CD59, peptides derived from collagen, GST, the α and β subunits of inactive human chorionic gonadotropin, maltose-binding protein (MBP), p53 and fragments thereof, phosphatase, streptavidin, surfactant protein D, tenascin, tetranectin, dock-and-lock (DNL) motifs, and uteroglobin.

They are preferably combined with a target specific binding domain which may be an antibody or a target specific binding fragment thereof, an antibody light chain or a target specific binding fragment thereof, an antibody heavy chain or a target specific binding fragment thereof, a single-chain fragment variable (scFv), a diabody, a single-chain diabody a single domain antibody or an antibody mimetic.

If component D comprises, essentially consists or consists an antibody heavy chain or a target specific binding fragment thereof, it is preferred that a further polypeptide according to the second aspect of the invention is provided in which component D comprises, essentially consists or consists of the antibody light chain or a target specific binding fragment thereof. Preferably, both the antibody heavy and light chain or the target specific binding fragments thereof, bind to the same target.

In another preferred embodiment component D comprises, essentially consists or consists of a dimerization and one or more target-specific binding domains (ts-domain). Preferably, this ts-domain is a scFv, diabody, IgG, heavy or light chain or target specific binding fragment thereof. Preferred examples are antibody Fc fragments, variants or fragments thereof, e.g. an EHD2, combined with an antibody or target specific binding fragment thereof, single-chain fragment variable (scFv), a diabody, a single-chain diabody, a single domain antibody or an

antibody mimetic. Particular preferred components D are selected from the group consisting of EHD2 (SEQ ID NO: 202), scFvhu225-EHD2 (SEQ ID NO: 203), scFv4D5-EHD2 (SEQ ID NO: 204), and scFv3M6-EHD2 (SEQ ID NO: 206). The combination of dimerization domain and one or more target specific binding domain, specifically binding to a particular cell type, leads to constructs inducing rapid apoptosis of that cell type.

In another preferred embodiment component D comprises, essentially consists or consists of a target specific binding domain which is preferably an antibody fragment or an antibody mimetic. The antibody fragment is preferably selected from the group consisting of a fragment antigen binding (Fab) fragment, a Fab' fragment, a heavy chain antibody, a single-domain antibody (sdAb), a single-chain variable fragment (scFv), a V_H domain, a V_L domain, a single domain antibody, a nanobody, an IgNAR (immunoglobulin new antigen receptor), a di-scFv, a bispecific T-cell engager (BITEs), a dual affinity re-targeting (DART) molecule, single-chain diabody, an alternative scaffold protein. Antibody-mimetics are selected from the group consisting of LACI-D1 (lipoprotein-associated coagulation inhibitor); affilins, e.g. human- γ B crystalline or human ubiquitin; cystatin; Sac7D from *Sulfolobus acidocaldarius*; lipocalin and anticalins derived from lipocalins; DARPins (designed ankyrin repeat domains); a domain of a membrane receptor; SH3 domain of Fyn; Kunits domain of protease inhibitors; monobodies, e.g. the 10th type III domain of fibronectin; adnectins; knottins (cysteine knot miniproteins); fynomers; atrimers; evibodies, e.g. CTLA4-based binders, affibodies, e.g. three-helix bundle from Z-domain of protein A from *Staphylococcus aureus*; Trans-bodies, e.g. human transferrin; tetranectins, e.g. monomeric α -human C-type lectin domain; microbodies, e.g. trypsin-inhibitor-II; affilins and armadillo repeat proteins. Most preferably the target specific binding domain is a Fab-fragment or a single-chain variable fragment (scFv).

It is further preferred that the target-specific binding domain is a complete antibody, a heavy chain of an antibody or a light chain of an antibody or a target specific binding fragment thereof, a diabody, a single chain antibody or an antibody mimetic. More preferably, the target specific binding domain is a diabody. Such a fusion protein or a bivalent antibody can bind different antigens and is composed of two single protein chains which comprise parts of an antibody, namely single-chain variable (scFv) fragments. Fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) on the same polypeptide chain (V_H-V_L). Preferably the V_L and V_H region of an antibody specifically bind to a target molecule on the cell surface.

Target molecules that are preferably targeted with the target specific binding domain are a receptor molecule or a membrane molecule specific to tumor cells. Preferably, the target molecule is selected from the group consisting of tyrosine-kinase-receptors (EGFR, HER2,

HER3, HER4), VEGFRs, heteromeric integrin α - or β -receptor family, tumor stroma markers like fibroblast activation protein (FAP) endoglyx-1, or endosialin, galectin, EpCAM, CEA, CD44 and tumor specific variants thereof and other tumor selective cell surface markers, CD2, CD5, CD7, CD19, CD20, CD21, CD22, CD24, CD25, CD30, CD33, CD38, CD40, CD52, CD56, CD71, CD72, CD73, CD105, CD117, CD123, CD133, c-Met, PDGFR, IGF1-R, HMW-MAA, TAG-72, GD2, GD3, GM2, folate receptor, Lgr5, Ley, Muc-1, Muc-2, PSMA, PSCA and uPAR. More preferably, the target molecule is FAP, EGFR, HER2 or HER3.

Preferably, the diabody consists of an antibody V_L and V_H region linked directly to each other with a peptide that has a length of between 0 and 15, 4 to 13, preferably 7 to 11, most preferably 8 to 10 amino acids. Preferably, the diabody consists of a V_L and V_H domain linked directly to each other with a peptide of between 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids. Preferably, the V_H and V_L region are directly linked to each other by a peptide of 7, 8, 9, 10 or 11 amino acids in length. Surprisingly such long peptides still provided stable diabodies in the context of component C. This peptide is preferably a flexible amino acid stretch. More preferably, the V_H and V_L region are directly linked to each other by a peptide of 4 to 15 glycine and/or serine residues or 5, 8 or 10 amino acids in length, preferably by one of the sequences selected from the group consisting of SEQ ID NO: 260 (GGGGS), SEQ ID NO: 207 (8 aa: GGGSGGG) and SEQ ID NO: 208 (10 aa: GGGSGGGGS).

Preferably the V_L region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 4 and/or the V_H region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 237, if the target antigen is EGFR. Preferably the V_L region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 238 and/or the V_H region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 239, if the target antigen is HER-2. Preferably the V_L region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 240 and/or the V_H region comprises, essentially consists or consists of the amino acid sequence according to SEQ ID NO: 241, if the target antigen is HER-3.

Even more preferably, the polypeptide comprising component C linked to component D is selected from the group consisting of Db-scTRAIL₃-FAVSGAA (SEQ ID NO: 195), dsDb3M6-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 196), Db10-scTRAIL₃-FAVSGGA (SEQ ID NO: 197), Db-glyco-scTRAIL₃-FAVSGAA (SEQ ID NO: 198), Db-glyco-scTRAIL₃-FAVSGIA (SEQ ID NO: 199).

Preferably, the target specific binding domain is an antibody or fragment. Preferably, the fragment is selected from the group consisting of a complete antibody, a fragment antigen binding (Fab) fragment, a Fab' fragment, a F(ab')₂ fragment, a heavy chain antibody, a single-

domain antibody (sdAb), a single-chain variable fragment (scFv), or a part thereof, a di-scFv, a bispecific T-cell engager (BITEs), a dual affinity re-targeting (DART) molecule, a triple body, an alternative scaffold protein, and a fusion protein thereof, and combinations thereof. More preferably, the target specific binding domain comprises a Fc region and a single chain variable fragment (scFv). Even more preferably, the target specific binding domain linked to component C comprises Fc region parts. Even more preferably, the polypeptide comprising component C and component D is selected from the group consisting of scFvhu225-Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 209), Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 210) or scFv4D5-Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 211).

It is particularly preferred that the target specific binding domain is a single chain variable fragment and the dimerization or multimerization domain is either a Fc fragment or EHD2.

In another preferred embodiment component C is linked to component D by a peptide X_d. The peptide X_d preferably has a length of between 5 to 35 amino acids, preferably 10 to 30 amino acids, more preferably 15 to 25, most preferably 18 to 22 amino acids, i.e. 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 and 35 amino acids, preferably 19, 20 or 21 amino acids, most preferably 20 amino acids. It is preferred that the peptide X_d is a glycine and/or serine rich peptides. A peptide is considered glycine and/or serine rich, if at least 70% of the amino acids are selected from glycine and/or serine, preferably more than 75%, more than 80%, more than 90%. Other amino acids that may be present comprise are preferably those that do not result in structural rigidity, e.g. proline, thus preferably small amino acids are comprised in addition to serine and/or glycine. Preferred examples of such small amino acids are alanine or threonine. Preferred examples of peptides are selected from (GGG)_n, (GGSGG)_m, (SEQ ID NO: 257), wherein n is an integer between 1 to 12, preferably between 3 to 7 and m is an integer between 1 to 7, preferably between 3 to 5. Further preferred peptides are selected from the group consisting of SEQ ID NO 192 GGSGGASSGG), SEQ ID NO: 193 (GGSGGGSSGG) SEQ ID NO: 194 (AAGNGTSNGTSEFGG) SEQ ID NO: 258 (GSGNGTSNGTSGSSGG) and SEQ ID NO: 259 (GSGNGTSNGTSGSSRT). It is further preferred that X_d comprises at least one glycosylation motif. Glycosylation motifs comprise for instance nitrogen atoms in asparagine chain. Even more preferably, X_d has a length of 16 amino acids and has two glycosylation motifs (SEQ ID NO: 194, A-A-A-G-N-G-T-S-N-G-T-S-E-F-G-G). The glycosylation motif is preferably inserted in one of the above outlined preferred peptides. Preferably, at or close to the middle of the peptide.

Peptide X_d preferably has a length of between 18 to 22, if the dimerization is either a Fc fragment or EHD2, preferably a Fc fragment. Preferably, the structure of the polypeptide according to the second aspect of the present invention linked to a component D comprises (from

N- to C-terminus, D: component D; X_c: peptide linking the at least three THDs of component C; X_d: linking component C to component D)

5 THD-X_c-THD-X_c-THD-X_d-D,
D-X_d-THD-X_c-THD-X_c-THD, or
D-X_d-THD-X_c-THD-X_c-THD-X_d-D,

10 As outlined above the polypeptide of the second aspect may comprise more than three components C, e.g. 4, 5, 6, 7, 8, or 9, more preferably 6 or 9. Such constructs show an even larger heat stability than those only comprising 3 components C. The components C may all be arranged consecutively and component D is then position N- and/or C terminally or one or more component D may be interspersed between component C. Preferred structures of the polypeptide according to the first aspect of the present invention comprises, essentially consists or consists of (from N- to C-terminus):

15 D-X_d-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C; C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_dD, D-X_d-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_d-D; C-X_c-C-X_c-C-X_d-D-X_b-C-X_c-C-X_c-C, D-X_d-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C; C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_d-D, D-X_d-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_d-D; C-X_c-C-X_c-C-X_d-D-X_b-C-X_c-C-X_c-C-X_c-C-X_c-C, or C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_c-C-X_d-D-X_b-C-X_c-C-X_c-C.

20 Another optional element which may or may not be present in a polypeptide according to the first aspect of the present invention is a tag allowing for example the detection and or purification of a polypeptide of the first aspect of the present invention. Examples for such tags are His-tag, FLAG-tag, HA-tag, STREP-tag, myc-tag or GST. Preferably, such tag is positioned outside the region comprising the at least three THDs of component C and component D. It is also possible to position a protease cleavage site (e.g. a Thrombin cleavage site) adjacent to the tag, e.g. directly C-terminally of an N-terminal tag allowing to remove the tag after purification. In this context it is preferred that the tags are positioned directly at the C-terminus or N-terminus of the polypeptide allowing to remove the tag after purification. Preferably, the tag is N-terminally to be cleaved after detection or purification.

30 The polypeptide of the first and second aspect of the present invention relate also to polypeptide complexes of the polypeptides of the first and second aspect of the present invention, e.g. homodimeric, and/or homotrimeric complexes of component A of the polypeptide of the first aspect of the present invention and the polypeptide according to the second aspect of the present invention. Thus, the polypeptides of the first and second aspect of the present invention are capable of forming multimers, such as dimers, trimers, tetramers etc. It is preferred

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that component A of the polypeptide of the first aspect of the present invention and the polypeptide of the second aspect of the present invention form trimers. It is preferred that component B of the first aspect and component D of the second aspect of the present invention are capable of forming multimers such as dimers, trimers, tetramers etc. In a particularly preferred embodiment component B of the first aspect and component D of the second aspect of the present invention are selected such that multimerization, such as dimerization of the polypeptide of the first aspect of the present invention and the polypeptide of the second aspect of the present invention comprising component C linked to component D, is possible. More preferably, the polypeptide of the first aspect of the present invention and the polypeptide of the second aspect of the present invention comprising component C linked to component D exhibit a multimeric form, such as a dimeric, trimeric, tetrameric etc. form, most preferably a dimeric form. Accordingly, in a particular preferred embodiment the polypeptide of the first aspect of the present invention and the polypeptide of the second aspect of the present invention comprising component C linked to component D is dimeric.

In a third aspect the present invention provides a nucleic acid encoding the polypeptides according to the first and second aspect of the present invention. The nucleic acid may be RNA or DNA or a hybrid thereof. Preferably, the nucleic acid also comprises sequences allowing for the expression of the polypeptide according the first and second aspect of the present invention in a suitable expression system. The nucleic acid can be codon optimized for the respective expression system.

In a fourth aspect the present invention provides a vector comprising the nucleic acid of the third aspect of the present invention. It is preferred that the genes of interest encoded by the introduced polynucleotide are expressed within the cell upon introduction of the vector or vectors. Preferably, the vector provides for transcription and expression of the polypeptide encoded by the nucleic acid in a suitable host cell system. Preferably, the expression vector is selected from the group consisting of a bacterial, yeast, baculovirus, plant and mammalian expression vector, more preferably the expression vector is a bacterial expression vector or a cell-free expression vector.

In a fifth aspect the present invention provides a pharmaceutical composition comprising the polypeptide of the first or the second aspect of the present invention, or the nucleic acid of the third aspect, or the vector of the fourth aspect and pharmaceutical acceptable carriers and/or suitable excipients. The pharmaceutical composition is selected from the group consisting of solid, liquid, semi-solid or transdermal therapeutic systems. It is envisioned that the pharmaceutical compositions of the invention comprise one or more polypeptides of the first and/or second aspect of the invention.

The present inventors have noted that the apoptosis inducing activity of the polypeptides of the first and/or second aspect of the present invention can be further enhanced by including in the pharmaceutical composition one or more proteasome inhibitor. Proteasome inhibitors are drugs that block the action of proteasomes, cellular complexes that break down proteins.

5 Preferred proteasome inhibitors are selected from the group comprising bortezomib, lactacystin, disulfiram, epigallocatechin-3-gallate, salinosporamide A, carfilzomib, epoxomicin, ixazomib, ONX 0912, CEP-18770, and MLN9708, preferably bortezomib.

It is also envisioned that the pharmaceutical composition comprises a combination of one polypeptide of the present invention in combination with a chemotherapeutic agent, e.g.
10 gemcitabine, capecitabine, doxorubicin, methotrexate, 5-fluorouracil, azathioprine, cytarabin, fludarabin, tegafur, 6-thioguanine, cisplatin, carboplatin, paclitaxel, docetaxel, vinorelbin, vincristin, etoposid, teniposid, irinotecan, rituximab, panitumumab, bortezomib, vorinostat, ganitumumab, cetuximab, or sorafenib.

The pharmaceutical composition may also comprise both one or more proteasome
15 inhibitors and one or more chemotherapeutic agents.

In a sixth aspect the present invention relates to a polypeptide of the first or the second aspect, a nucleic acid of the third aspect or a vector of the fourth aspect for use as a medicament.

In a seventh aspect the present invention relates to a polypeptide of the first or the second aspect, a nucleic acid of the third aspect or a vector of the fourth aspect or the pharmaceutical
20 composition of the fifth aspect for use in the diagnosis, prophylaxis or treatment of hyperproliferative disorders and inflammatory disorders.

Preferred hyperproliferative diseases are selected from the group consisting of precancerosis; dysplasia; metaplasia; and cancer.

Particular preferred cancers to be treated by the polypeptides of the present invention are
25 carcinomas of the gastrointestinal tract, liver, kidney, bladder, prostate, endometrium, ovary, testes, skin, invasive oral cancers, small cell and non-small cell lung carcinomas, hormone-dependent breast cancers, hormone-independent breast cancers, transitional and squamous cell cancers, neurological malignancies including neuroblastoma, gliomas, astrocytomas, osteosarcomas, soft tissue sarcomas, hemangioas, endocrinological tumors, hematologic
30 neoplasias including leukemias, lymphomas, and other myeloproliferative and lymphoproliferative diseases, carcinomas in situ, hyperplastic lesions, adenomas, fibromas, histiocytosis, chronic inflammatory proliferative diseases, vascular proliferative diseases and virus-induced proliferative diseases, skin diseases characterized by hyperproliferation of keratinocytes and/or T cells. Particular preferred diseases treatable with the compounds of the

present invention are solid tumors, in particular lung, breast, pancreas, colorectal, ovarian, prostatic and gastric cancers and adenocarcinomas.

The precancerosis treatable with the polypeptides of the present invention are preferably selected from the group consisting of precancerosis of the skin, in particular actinic keratosis, cutaneous horn, actinic cheilitis, tar keratosis, arsenic keratosis, x-ray keratosis, Bowen's disease, bowenoid papulosis, lentigo maligna, lichen sclerosus, and lichen rubber mucosae; precancerosis of the digestive tract, in particular erythroplakia, leukoplakia, Barrett's esophagus, Plummer-Vinson syndrome, crural ulcer, gastropathia hypertrophica gigantea, borderline carcinoma, neoplastic intestinal polyp, rectal polyp, porcelain gallbladder; gynaecological precancerosis, in particular carcinoma ductale in situ (CDIS), cervical intraepithelial neoplasia (CIN), leukoplakia, endometrial hyperplasia (grade III), vulvar dystrophy, vulvar intraepithelial neoplasia (VIN), hydatidiform mole; urologic precancerosis, in particular bladder papillomatosis, Queyrat's erythroplasia, testicular intraepithelial neoplasia (TIN), leukoplakia; carcinoma in situ (CIS); precancerosis caused by chronic inflammation, in particular pyoderma, osteomyelitis, acne conglobata, lupus vulgaris, and fistula.

Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exist chronic irritation or inflammation. Dysplastic disorders which can be treated with the compounds of the present invention include, but are not limited to, anhidrotic ectodermal dysplasia, anterofacial dysplasia, asphyxiating thoracic dysplasia, atriadigital dysplasia, bronchopulmonary dysplasia, cerebral dysplasia, cervical dysplasia, chondroectodermal dysplasia, cleidocranial dysplasia, congenital ectodermal dysplasia, craniodiaphysial dysplasia, craniocarpotarsal dysplasia, craniometaphysial dysplasia, dentin dysplasia, diaphysial dysplasia, ectodermal dysplasia, enamel dysplasia, encephalo-ophthalmic dysplasia, dysplasia epiphysialis heminelia, dysplasia epiphysialis multiplex, dysplasia epiphysialis punctata, epithelial dysplasia, faciodigitogenital dysplasia, familial fibrous dysplasia of jaws, familial white folded dysplasia, fibromuscular dysplasia, fibrous dysplasia of bone, florid osseous dysplasia, hereditary renal-retinal dysplasia hidrotic ectodermal dysplasia, hypohidrotic ectodermal dysplasia, lymphopenic thymic dysplasia, mammary dysplasia, mandibulofacial dysplasia, metaphysical dysplasia, Mondini dysplasia, monostotic fibrous dysplasia, mucoepithelial dysplasia, multiple epiphysial dysplasia, oculoauriculovertebral dysplasia, oculodentodigital dysplasia, oculovertbral dysplasia, odontogenic dysplasia, ophthalmomandibulomelic dysplasia, periapical cemental dysplasia, polyostotic fibrous

dysplasia, pseudoachondroplastic spondyloepiphyseal dysplasia, retinal dysplasia, septo-optic dysplasia, spondyloepiphyseal dysplasia, and ventriculoradial dysplasia.

Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplastic disorders, which are treatable are preferably selected from the group consisting of agnogenic myeloid metaplasia, apocrine metaplasia, atypical metaplasia, autoparenchymatous metaplasia, connective tissue metaplasia, epithelial metaplasia, intestinal metaplasia, metaplastic anemia, metaplastic ossification, metaplastic polyps, myeloid metaplasia, primary myeloid metaplasia, secondary myeloid metaplasia, squamous metaplasia, squamous metaplasia of amnion, symptomatic myeloid metaplasia and regenerative metaplasia.

Many skin diseases are characterized by hyperproliferation of keratinocytes and/or T cells. Examples of such diseases which are treatable with the compounds of the present invention comprise without limitations psoriasis in particular psoriasis vulgaris, psoriasis capitis, psoriasis guttata, psoriasis inversa; neurodermatitis; ichthyosises; alopecia areata; alopecia totalis; alopecia subtotalis; alopecia universalis; alopecia diffusa; atopic dermatitis; lupus erythematoses of the skin; dermatomyositis of the skin; atopic eczema; morphea; scleroderma; alopecia areata Ophiasis type; androgenic alopecia; allergic contact dermatitis; irritative contact dermatitis; contact dermatitis; pemphigus vulgaris; pemphigus foliaceus; pemphigus vegetans; scarring mucous membrane pemphigoid; bullous pemphigoid; mucous membrane pemphigoid; dermatitis; dermatitis herpetiformis Duhring; urticaria; necrobiosis lipoidica; erythema nodosum; prurigo simplex; prurigo nodularis; prurigo acuta; linear IgA dermatosis; polymorphic light dermatosis; erythema solaris; exanthema of the skin; drug exanthema; purpura chronica progressiva; dihydrotic eczema; eczema; fixed drug exanthema; photoallergic skin reaction; and periorale dermatitis.

Inflammatory disorders that can be treated with the polypeptides of the invention include but are not limited to Acute disseminated encephalomyelitis (ADEM), Addison's disease, Agammaglobulinemia, Alopecia areata, Amyotrophic lateral sclerosis (Also Lou Gehrig's disease; Motor Neuron Disease), Ankylosing Spondylitis, Antiphospholipid syndrome, Antisynthetase syndrome, Atopic allergy, Atopic dermatitis, Autoimmune aplastic anemia, Autoimmune cardiomyopathy, Autoimmune enteropathy, Autoimmune hemolytic anemia, Autoimmune hepatitis, Autoimmune inner ear disease, Autoimmune lymphoproliferative syndrome, Autoimmune pancreatitis, Autoimmune peripheral neuropathy, Autoimmune polyendocrine syndrome, Autoimmune progesterone dermatitis, Autoimmune thrombocytopenic purpura, Autoimmune urticarial, Autoimmune uveitis, Balo disease/Balo concentric sclerosis, Behçet's disease, Berger's disease, Bickerstaff's encephalitis, Blau syndrome, Bullous

pemphigoid, Cancer, Castleman's disease, Celiac disease, Chagas disease, Chronic inflammatory demyelinating polyneuropathy, Chronic inflammatory demyelinating polyneuropathy, Chronic obstructive pulmonary disease, Chronic recurrent multifocal osteomyelitis, Churg-Strauss syndrome, Cicatricial pemphigoid, Cogan syndrome, Cold agglutinin disease, Complement

5 component 2 deficiency, Contact dermatitis, Cranial arteritis, CREST syndrome, Crohn's disease, Cushing's Syndrome, Cutaneous leukocytoclastic angiitis, Degos's disease, Dercum's disease, Dermatitis herpetiformis, Dermatomyositis, Diabetes mellitus type 1, Diffuse cutaneous systemic sclerosis, Discoid lupus erythematosus, Dressler's syndrome, Drug-induced lupus, Eczema, Endometriosis, Enthesitis-related arthritis, Eosinophilic fasciitis, Eosinophilic gastroenteritis,

10 Eosinophilic pneumonia, Epidermolysis bullosa acquisita, Erythema nodosum, Erythroblastosis fetalis, Essential mixed cryoglobulinemia, Evan's syndrome, Fibrodysplasia ossificans progressive, Fibrosing alveolitis (or Idiopathic pulmonary fibrosis), Gastritis, Gastrointestinal pemphigoid, Glomerulonephritis, Goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome (GBS), Hashimoto's encephalopathy, Hashimoto's thyroiditis, Henoch-Schönlein

15 purpura, Herpes gestationis aka Gestational Pemphigoid, Hidradenitis suppurativa, Hughes-Stovin syndrome, Hypogammaglobulinemia, Idiopathic inflammatory demyelinating diseases, Idiopathic pulmonary fibrosis, Idiopathic thrombocytopenic purpura (See Autoimmune thrombocytopenic purpura), IgA nephropathy, Inclusion body myositis, Interstitial cystitis, Juvenile idiopathic arthritis aka Juvenile rheumatoid arthritis, Kawasaki's disease, Lambert-

20 Eaton myasthenic syndrome, Leukocytoclastic vasculitis, Lichen planus, Lichen sclerosus, Linear IgA disease (LAD), Lupoid hepatitis aka Autoimmune hepatitis, Lupus erythematosus, Majeeb syndrome, Microscopic colitis, Microscopic polyangiitis, Miller-Fisher syndrome, Mixed connective tissue disease, Morphea, Mucha-Habermann disease aka Pityriasis lichenoides et varioliformis acuta, Multiple sclerosis, Myasthenia gravis, Myositis, Ménière's disease,

25 Narcolepsy, Neuromyelitis optica (also Devic's disease), Neuromyotonia, Occular cicatricial pemphigoid, Opsoclonus, yoclonus syndrome, Ord's thyroiditis, Palindromic rheumatism, PANDAS (pediatric autoimmune neuropsychiatric disorders associated with streptococcus), Paraneoplastic cerebellar degeneration, Paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Pars planitis, Parsonage-Turner syndrome, Pemphigus vulgaris, Perivenous

30 encephalomyelitis, Pernicious anaemia, POEMS syndrome, Polyarteritis nodosa, Polymyalgia rheumatic, Polymyositis, Primary biliary cirrhosis, Primary sclerosing cholangitis, Progressive inflammatory neuropathy, Psoriasis, Psoriatic arthritis, Pure red cell aplasia, Pyoderma gangrenosum, Rasmussen's encephalitis, Raynaud phenomenon, Reiter's syndrome, Relapsing polychondritis, Restless leg syndrome, Retroperitoneal fibrosis, Rheumatic fever, Rheumatoid

35 arthritis, Sarcoidosis, Schizophrenia, Schmidt syndrome another form of APS, Schnitzler

syndrome, Scleritis, Scleroderma, Serum Sickness, Sjögren's syndrome, Spondyloarthritis, Stiff person syndrome, Still's disease see Juvenile Rheumatoid Arthritis, Subacute bacterial endocarditis (SBE), Susac's syndrome, Sweet's syndrome, Sydenham chorea, Sympathetic ophthalmia, Systemic lupus erythematosus see Lupus erythematosus, Takayasu's arteritis, 5 Temporal arteritis (also known as "giant cell arteritis"), Thrombocytopenia, Tolosa-Hunt syndrome, Transverse myelitis, Ulcerative colitis (one of two types of idiopathic inflammatory bowel disease "IBD"), undifferentiated connective tissue disease different from Mixed connective tissue disease, undifferentiated spondyloarthritis, Urticarial vasculitis, Vasculitis, Vitiligo, and Wegener's granulomatosis. Hypersensitivity includes but is not limited to allergy, 10 such as asthma, anaphylaxis or atopy; cytotoxic-antibody-dependent diseases such as autoimmune hemolytic anemia, thrombocytopenia, rheumatic heart diseases, erythroblastosis fetal, Goodpasture's syndrome, membranous nephropathy, Graves' disease, myasthenia gravis; immune complex diseases such as serum sickness, arthus reaction, rheumatoid arthritis, post streptococcal glomerulo nephritis, lupus nephritis systemic lupus erythematosus, extrinsic 15 allergic alveolitis (hypersensitivity pneumonitis), cell-mediated immune response such as contact dermatitis, Mantoux test, chronic transplant rejection, and multiple sclerosis.

Examples

Example 1: New derivatives of single-chain TRAIL produced in soluble form

20 A single-chain TRAIL molecule (scTRAIL) is composed of three TRAIL subunits connected by two peptides. A state-of-the-art scTRAIL molecule in which the individual subunits start with aa 95 connected by 8 amino acids ((GGGS)₂) is described as 95L8. To distinguish the molecules described in the following invention from state-of-the-art molecules, we use a nomenclature describing the characteristic amino acid sequence between the conserved C-terminal residue 25 Phe278 and the N-terminal residue Ala123 of the TNF homology domain of scTRAIL. These amino acids were defined as reference points within the TRAIL molecule only and can be followed by additional N- and/or C-terminal TRAIL sequences. The index "3" was added to the name of the molecule in case that three identical TRAIL subunits are joined. This nomenclature is used throughout the text to define the residues within the C- and N-terminal consensus 30 sequence as well as the connecting amino acid sequence, which are also designated X_a and X_c, respectively, for the polypeptides of the invention. For example, the C-terminal consensus sequence of TRAIL linked by an intervening peptide to the N-terminal consensus sequence of TRAIL in the polypeptide according to SEQ ID NO: 212 has the following sequence (bold amino acid sequences are used for naming the construct):

SFFGAF
C-terminal
Consensus

LVGGGGSVRERGPQR
intervening peptide

VAAHI
N-terminal
consensus

5

Accordingly, the scTRAIL construct is named: scTRAIL₃-FLVGGGGSVRERGPQRVA. Since the length of the intervening peptide does not fit the definition of X_a and X_c, respectively, this polypeptide is an exemplary prior art polypeptide but not a polypeptide according to the invention.

10

A scTRAIL variant comprising TRAIL subunits starting with residue 95 and ending with residue 281 (95L8), i.e. having the following composition: subunit-1 (aa 95-281 of TRAIL of SEQ ID NO: 5)-(GGGS)₂-subunit 2 (aa 95-281 of TRAIL of SEQ ID NO: 5)-(GGGS)₂-subunit 3 (aa 95-281 of TRAIL of SEQ ID NO: 5), was used as a starting point with the aim of generating a panel of new scTRAIL molecules with improved properties as outlined above. The new scTRAIL variants are characterized by shortened N-termini of the THD and unique configurations of the peptides connecting the THDs. In a first set, four new scTRAIL molecules with individual subunits starting from amino acid residue 114 and 120, respectively, were generated. Two different Gly/Ser polypeptides with a length of 4 (GGGS) or 8 residues (GGGS)₂ were used in these molecules, leading to the variants scTRAIL₃-FLVGGGGSVRERGPQRVA (SEQ ID NO: 212), scTRAIL₃-FLVGGGGSGGGSVRERGPQRVA (SEQ ID NO: 213), scTRAIL₃-FLVGGGGSQRVA (SEQ ID NO: 214), and scTRAIL₃-FLVGGGGSGGGSQRVA (SEQ ID NO: 215), (see Figure 1A, Table 1). Additionally, scTRAIL molecules starting from Gly118 were generated, whereby those molecules only comprised wild-type TRAIL sequences scTRAIL₃-FLVGGPQRVA (SEQ ID NO: 216), or comprised a single glycine substitution scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 217), or two glycine substitutions scTRAIL₃-FLVGGGGPQRVA (SEQ ID NO: 218), (see Table 1). Furthermore, we reduced the sequence length of THD and the intervening amino acid sequences, yielding the molecules scTRAIL₃-FLVGGQRVA (SEQ ID NO: 219), scTRAIL₃-FLVGGQVA (SEQ ID NO: 220), and scTRAIL₃-FLVGGVA (SEQ ID NO: 221), respectively (see Figure 1B). The scTRAIL₃ expression constructs were cloned using the previously described pIRESpuro-scTRAIL plasmid vector (Schneider *et al.*, 2010, Siegemund *et al.*, 2012). In a first step, individual DNA modules coding for each of the three scTRAIL₃ subunits were PCR-amplified by oligonucleotides comprising the described sequence modifications. Complementary regions at the ends of the DNA modules were used to assemble the entire scTRAIL₃ DNA construct in a multilevel PCR process prior to cloning into pIRESpuro-scTRAIL via EcoRI/ NotI. All TRAIL molecules contained an additional N-terminal FLAG tag for purification and detection. The scTRAIL variants were

expressed in HEK293 cells and purified by anti-FLAG affinity chromatography from the cell culture supernatant as published (Siegemund *et al.*, 2014). All these scTRAIL variants could be purified in soluble form from cell culture supernatants of transfected HEK293 cells. In contrast, a scTRAIL molecule with Val122 mutated to glycine, i.e. scTRAIL₃-FLVGGGA (SEQ ID NO: 222), as well as molecules with Leu279 mutated in addition to glycine, e.g. scTRAIL₃-FLGGGA (SEQ ID NO: 223), were found to yield insoluble proteins (see Figure 2A, Table 2).

Structural modeling of scTRAIL suggested that the C-terminal residues Leu279 and Val280 are involved in a hydrophobic interaction with the N-terminal Val122, which seems to be crucial for the structural integrity and solubility of the whole molecule. This was supported by the finding that mutation of Leu279 and/or Val122 to glycine resulted in insoluble protein. Therefore, we decided to introduce C-terminal and N-terminal mutations to substitute the pivotal positions 279 and 122 by other hydrophobic or small neutral amino acid residues (see Figure 1E) in order to improve stability and proper folding of these scTRAIL variants (see Table 2 and 3, Figure 2B). Three of these six variants were proved to be soluble and bioactive, namely scTRAIL₃-FLVGGIA (SEQ ID NO: 224), scTRAIL₃-FLVGGAA (SEQ ID NO: 225), and scTRAIL₃-FAVGGAA (SEQ ID NO: 226). In a second round of optimization, the sequence positions 279, 281 and 122 were varied with distinct combinations of isoleucine, alanine or serine residues (see Figure 1F). The molecules scTRAIL₃-FAVSGAA (SEQ ID NO: 227), scTRAIL₃-FAVGGIA (SEQ ID NO: 228), scTRAIL₃-FIVGGIA (SEQ ID NO: 229), scTRAIL₃-FLVSGIA (SEQ ID NO: 230), scTRAIL₃-FIVSGIA (SEQ ID NO: 231), and scTRAIL₃-FAVSGIA (SEQ ID NO: 232), whereby the TRAIL monomers are connected by a single glycine (G), could be expressed in a soluble, active form.

Example 2: Improved thermal stability of novel scTRAIL molecules

The thermal stability of all soluble scTRAIL molecules were determined by dynamic light scattering using a zetasizer nano (Malvern) and a protein concentration of 100 µg/ml in PBS. Starting with 35 °C, temperature was increased at 1 °C intervals with 2 min incubation for each temperature step. The melting point was defined as the temperature where a drastic increase in kcps values was observed (see Figure 3). A melting point of 46 °C was determined for soluble, homotrimeric TRAIL. Similar values were observed for variants with N-terminal start of the subunits at position 95 or 114, combined with peptides longer than 4 aa residues, such as scTRAIL-95L8 and scTRAIL₃-FLVGGGGSGGGSVRERGPQRVA. All other variants exhibited an increased thermal stability with the best variant scTRAIL₃-FLVGGVA (SEQ ID NO: 233), possessing a melting point of 57 °C (see Table 3, Figure 3). These findings indicated that a reduced length of the TRAIL monomers and shorter intervening peptide sequence correlate with

higher melting temperatures and therefore with better protein stability, presumably due to a reduced conformational flexibility of the molecule. Favorable versions of scTRAIL carrying N- as well as C-terminal substitutions of amino acids, e.g. scTRAIL₃-FAVSGAA, showed a 7 °C increase of the melting temperature, compared with the parental version scTRAIL-95L8, e.g., scTRAIL₃-FLVGGGGSQRVA. In general, all scTRAIL molecules comprising shortened N-terminal sequences starting at amino acid position 118 of human TRAIL or even shorter variants, when combined with short intervening peptide sequences (0-2 amino acids), were shown to have a higher protein stability compared to the published scTRAIL molecules, e.g. scTRAIL-95L8 and commercially available sTRAIL.

Example 3: Mutation of Gly281 to serine improves solubility and reduces aggregation propensity

To analyze expression levels of the newly designed molecules, equal cell numbers of stably transfected HEK293 pools were seeded, cultivated for three days in serum free medium and supernatants and cell pellet samples were tested by Western blot with an anti-FLAG antibody for secretion of soluble (Table 2) and total protein expression, respectively. Derivatives bearing a Gly281Ser mutation e.g. scTRAIL₃-FAVSGAA, scTRAIL₃-FIVSGIA, and scTRAIL₃-FAVSGIA showed improved expression compared to scTRAIL-95L8. This finding was unexpected since Gly281 is a surface-exposed amino acid residue not involved in any secondary structures (Hymowitz *et al.*, 1999; Hymowitz *et al.*, 2000). Our data suggest that this modification has a positive influence on the solubility of scTRAIL proteins. Furthermore, we checked the properties of the purified scTRAIL molecules by SDS-PAGE and size exclusion chromatography (SEC). Reducing SDS-PAGE followed by Coomassie staining revealed that the majority of each protein is present as a single band, corresponding to the respective calculated molecular masses. We found that new scTRAIL proteins show mostly a monomeric composition as analyzed by SEC (see Figure 4). Nonetheless, minor amounts of low-order multimers, putatively dimers, were detected in the samples. We quantified the ratio of these multimers relative to the total amount of protein by integration of SEC peaks. Preparations of newly developed scTRAIL variants comprising substitutions at positions 281, 121 and 122, e.g. scTRAIL₃-FLVSGIA, scTRAIL₃-FIVSGIA and scTRAIL₃-FAVSGIA, were found to have remarkably low percentages of multimers of 5 % of the total protein or less. A common characteristic of all these scTRAIL variants is the presence of the sequence motif “G281S-R121G-V122I”.

Example 4: The new scTRAIL molecules show unaltered TRAIL receptor binding activity

To evaluate functionality of the various TRAIL variants, specific binding to TRAILR-positive Colo205 cells was quantified by immunofluorescence flow cytometry. Cells (300.000 cells/well) were incubated with serial dilutions of scTRAIL variants starting at 30 µg/ml and bound molecules were detected with anti-FLAG and anti-mouse-FITC antibodies. Analyzed scTRAIL variants showed specific binding, with EC₅₀ values in the low nanomolar range. As an example, cell binding of three variants is shown in Fig. 5A, with slightly superior binding of the variants scTRAIL₃-FAVGGAA and scTRAIL₃-FLVGGAA compared to the variant scTRAIL₃-FLVGGVA. In addition, different scTRAIL variants for specific binding to DR5 were analyzed by ELISA (see Figure 5B). All tested scTRAIL molecules were shown to bind to DR5-Fc fusion with EC₅₀ values in the low nanomolar range, in accordance with the flow cytometry data.

Example 5: The new scTRAIL molecules efficiently induce apoptosis *in vitro*

The apoptosis-inducing activity of the new scTRAIL variants was tested in a cytotoxicity assay using Colo205 colon carcinoma cells sensitized with 250 ng/ml bortezomib. All soluble scTRAIL₃ molecules were shown to be bioactive (see Figure 6) with EC₅₀ values in the sub-nanomolar range. scTRAIL variants with low tendency to form dimers or higher order aggregates are preferable components for directed engineering of dimeric scTRAIL molecules. The relation between the molecular composition and the specific bioactivity was analyzed in detail for scTRAIL₃-FLVGGVA. To this, equimolar concentrations of SEC-separated dimers and monomers were analyzed for their apoptotic activity on the cancer cell lines Colo205, NCI-H460 and HT1080 (see Figure 7). Spontaneously formed dimers of scTRAIL₃-FLVGGVA were found to be more bioactive than the monomers on all tested cell lines. Depending on the cell line-specific intrinsic characteristics of the DR4- and DR5-triggered apoptosis, monomers of scTRAIL₃-FLVGGVA were either nearly inactive (Colo205) or were 50-fold (HT1080), respectively 13-fold (NCI-H460) less bioactive than the dimers. This result supports our rationale that an effective TRAIL-based protein therapeutic must comprise two trivalent TRAIL units, achieved in the scTRAIL₃ format which allows directed dimerization via genetic fusion to specific dimerization motifs and/or targeting antibody derivatives with intrinsic dimerization, such as diabodies. The latter format, diabody-scTRAIL, in addition ensures targeting of TRAIL to restrict activity, e.g. apoptosis induction, to a target positive cell, e.g. a tumor cell.

Example 6: Db-scTRAIL₃ fusion proteins comprising derivatives of scTRAIL

A previously described Db-scTRAIL (TRAIL module aa 95-281, linked by two peptides of 8 aa each (Db-scTRAIL-95L8, Siegemund *et al.*, 2012)) showed typically a heterogeneous molecular

composition in the size exclusion chromatography (see Figure 8A). Analyzing individual SEC fractions by SDS-PAGE and subsequent anti-FLAG Western blotting we found that the peak with the higher retention time comprises, in addition to full length protein, a truncated species (see Figure 8B) with a molecular mass between 35 and 40 kDa. Further, Db-scTRAIL-95L8 by chemical crosslinking using BS₃ was analyzed. The dimeric constitution of the molecule could be confirmed as seen by the presence of a major crosslinking product with a molecular mass of ~190 kDa. In addition we observed a side product (~130 kDa), which corresponded to a dimeric Db-scTRAIL with a truncation in one of its two polypeptide chains. This assumption could be confirmed by molecular crosslinking and Western blotting of SEC-separated fractions of the protein, in which fractions representing the smaller form showed an additional crosslinking product with the respective size of ~130 kDa. The partially truncated forms of Db-scTRAIL were shown to be less bioactive than the full-length protein on various cell lines (see Figure 9) supporting our hypothesis that a new molecular design is necessary to bypass structural and functional deficiencies of these TRAIL fusion proteins. The newly designed Db-scTRAIL fusion proteins comprise the optimized scTRAIL₃ derivatives described in Examples 1-5 (as well as modifications of peptides within the diabody and those connecting diabody to the scTRAIL variants). The molecules scTRAIL₃-FAVSGAA and scTRAIL₃-FAVSGIA as preferred derivatives for the generation of advanced Db-scTRAIL₃ fusion proteins were selected. ScTRAIL₃-FAVSGAA showed best expression properties among all scTRAIL₃ derivatives combined with a high thermal stability. The second variant scTRAIL₃-FAVSGIA was selected because of its lower aggregation tendency compared with other scTRAIL₃ derivatives. To yield advanced diabody fusion proteins comprising preferred scTRAIL₃ derivatives, we replaced state-of-the-art scTRAIL sequences from the existing vector construct pCR3-Db-scTRAIL (Siegemund *et al.*, 2012) by the new constructs using *EcoRI*/*XbaI* cloning, followed by stable HEK293 cell line generation. Recombinant products from cell culture supernatants were purified using anti-FLAG affinity chromatography and subjected to biochemical and functional analyses. In a first optimization approach, analyses of the new molecule Db-scTRAIL₃-FAVSGAA revealed by Coomassie staining a single band with a molecular mass of 86 kDa upon reducing SDS-PAGE. In SEC analysis of Db-scTRAIL₃-FAVSGAA we observed a smaller hydrodynamic radius and a strong reduction of truncated species compared to Db-scTRAIL-95L8 (see Figure 10, lower left). For potential further improvement, in a next step, introduction of glycosylation sites were investigated. A modified peptide for connecting the diabody with the scTRAIL moiety was defined by the amino acid residues AAAGNGTSTNGTSEFGG. This peptide comprises two N-glycosylation sites which were shown to be glycosylated upon recombinant expression of this newly designed molecule, Db-Glyco-scTRAIL₃-FAVSGAA. Surprisingly, Db-Glyco-scTRAIL₃-

FAVSGAA showed less aggregation than the corresponding Db-scTRAIL₃-FAVSGAA, while the presence of truncated species remained at a low level, comparable to Db-scTRAIL₃-FAVSGAA (see Figure 11, upper). As a consequence, the use of scTRAIL derivatives in combination with a new glycosylated peptide between the C-terminal scFv (diabody) domain and the N-terminus of scTRAIL led to molecules which are less prone to fragmentation and aggregation. Nevertheless, a minor proportion of aggregates and truncated forms were still detectable (see Figure 11, upper, Figure 12A).

In order to further optimize the molecular integrity and stability of Db-scTRAIL₃, variants with modified peptides connecting V_H and V_L, such that the intrinsic feature of spontaneous diabody formation is not limited were developed (see Figure 12B). Therefore, Db-Glyco-scTRAIL₃-FAVSGAA served as a model to analyze the impact of diabody connecting peptide lengths of 5 (GGGGS, state-of-the-art control) vs. 8 (GGGSGGG) and 10 amino acid residues (GGGSGGGGS), respectively. Unexpectedly, we found that expression of fusion proteins with the longest variant of the diabody connecting peptide Db10-Glyco-scTRAIL₃-FAVSGAA resulted in the lowest percentage of fragmented species, while dimerization, i.e. generation of a functionally intact diabody, was fully maintained (see Figure 12B, right). To prove the additional advantage of introduction of N-terminal glycosylation sites into the peptide between diabody and scTRAIL₃ also for this advanced diabody-scTRAIL₃ fusion protein format Db10-Glyco-scTRAIL₃-FAVSGAA, we designed and produced an unglycosylated variant Db10-scTRAIL₃-FAVSGAA for reasons of comparison. The former, glycosylated variant showed higher protein quality as revealed from SEC (see Figure 11, lower). A second selected scTRAIL derivative, scTRAIL₃-FAVSGIA, was used in the fusion protein Db-Glyco-scTRAIL₃-FAVSGIA. The protein quality of this derivative was found to be comparable to the variant Db10-Glyco-scTRAIL₃-FAVSGAA (see Figure 12B, C).

Example 7: Novel Db-scTRAIL₃ molecules show improved thermal stability

As for the scTRAIL derivatives, the thermal stability of all new Db-scTRAIL₃ molecules (see Figure 13) was determined. It was found that Db-scTRAIL fusion proteins comprising the new scTRAIL₃ derivatives scTRAIL₃-FAVSGAA and scTRAIL₃-FAVSGIA are characterized by a higher thermal stability than the previous state of the art molecules Db-scTRAIL-95L8 or Db-Glyco-scTRAIL-95L8, serving as reference. As an example, a melting point of 55 °C for the molecule Db-scTRAIL₃-FAVSGAA was measured, whereas 50 °C was determined for Db-scTRAIL-95L8. Similar protein stability was observed irrespectively of the presence of the glycosylated peptide, leading to the conclusion that the modified scTRAIL₃ module largely contributes to the increase in thermal stability. Surprisingly, the use of an elongated diabody

connecting peptide, as exemplified in the molecules Db10-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA, did not result in a decreased protein stability, compared to Db-Glyco-scTRAIL₃-FAVSGAA comprising the standard connecting peptide of 5 amino acids in length.

5 **Example 8: Receptor and target antigen binding of novel anti-EGFR Db-scTRAIL₃ fusion proteins**

The receptor affinity of the advanced anti-EGFR Db-scTRAIL₃ molecules was evaluated by flow cytometry using the fibrosarcoma cell line HT 1080 (see Figure 14). Similar to data obtained for the scTRAIL derivatives, the developed diabodies displayed EC₅₀ values in the nanomolar range.

10 For bifunctional fusion proteins, such as diabody-scTRAIL₃, the underlying binding mechanisms are more complex due to specific and simultaneous interaction with two different types of receptors, EGFR and TRAILR. Binding to respective target cells of the new Db-scTRAIL₃ variants was comparable to the references, Db-scTRAIL-95L8 and Db-Glyco-scTRAIL-95L8, with no significant difference in EC₅₀. Thus, the functionality of the scTRAIL derivatives with

15 respect to specific binding to cognate receptors is maintained in the new diabody fusion protein structure and the modifications of the connecting peptides, e.g. introduction of glycosylation sites and extended diabody, as well as in scTRAIL N- as well as C- terminal mutations, did not impact high affinity binding to the respective targets on the cell surface.

20 **Example 9: Improved bioactivity of novel anti-EGFR Db-scTRAIL₃ fusion proteins**

The apoptosis-inducing activity of the new Db-scTRAIL₃ molecules Db-Glyco-scTRAIL₃-FAVSGAA, Db-Glyco-scTRAIL₃-FAVSGIA, Db8-Glyco-scTRAIL₃-FAVSGAA and Db10-Glyco-scTRAIL₃-FAVSGAA was tested in cell death assays on Colo205, HCT116 (both colon carcinoma) and HT1080 (fibrosarcoma) cells. Therefore, 20,000 cells/well were seeded in 96-well plates. On the following day, cells were treated with the indicated concentrations of the

25 apoptosis sensitizer Bortezomib in combination with titrations of the TRAIL proteins in triplicates for 16 h. Cell death was assayed by crystal violet staining. All of the new Db-scTRAIL₃ molecules were shown to be highly bioactive (see Figures 15, 16, 17, Table 4), with superior cell death induction on several tumor cell lines, e.g. HCT116 and HT1080. Due to the unique biology of each cancer cell line, apparent differences in TRAIL sensitivity of different

30 tumor cells were expected for new Db-scTRAIL₃ molecules, too. Irrespective of these differences in TRAIL sensitivity of tumor cells and targeting dependence of apoptosis induction, the new Db-scTRAIL₃ molecules developed here display improved functional activity compared to the control Db-scTRAIL-95L8. The data presented in Figure 9 shows that a higher percentage of full-length protein, as evident in preparations of the advanced Db-scTRAIL₃ molecules,

35 directly correlates with the higher bioactivity observed *in vitro*. The contribution of targeting to

bioactivity could be demonstrated for all molecules by blocking studies in which EGFR binding of the diabody-scTRAIL₃ competed with EGFR specific antibody Cetuximab, added in excess to the test system (Figures 15, 16, 17, Table 4). It was shown that the advantages of structural improvement of the apoptosis-inducing ligand scTRAIL, resulting in e.g. higher protein stability and high specific bioactivity, are transferable to the construction of target-specific scTRAIL molecules in the diabody-scTRAIL fusion protein format. Additional inventive steps of protein design were introduced including glyco-engineering and length variation of connecting peptide sequences between distinct domains of the fusion proteins to yield considerable improvements with regard to production efficacy, product quality (molecular integrity, protein stability) and functional activity (PK and apoptosis inducing activity) of the new generation of target-specific Db-scTRAIL₃ molecules.

Example 10: Targeted dimeric scTRAIL₃ fusion proteins containing scTRAIL₃FLVGGGPQRVA

To further validate the concept of targeted dimeric scTRAIL fusion proteins, four different formats were generated, including, besides Db-scTRAIL₃ (SEQ ID NO: 234), a disulfide-stabilized version thereof (dsDb-scTRAIL₃) (SEQ ID NO: 235), an EHD2 fusion protein (scFv-EHD2-scTRAIL₃) (SEQ ID NO: 236), and an Fc construct (scFv-Fc-scTRAIL₃). All four different formats were developed with target specificities for EGFR, HER2 and HER3, thereby containing the same scTRAIL₃ variant (scTRAIL₃-FLVGGGPQRVA). Furthermore, corresponding non-targeted constructs were generated (scTRAIL₃, EHD2-scTRAIL₃, Fc-scTRAIL₃). All fusion proteins were produced with stably transfected HEK293 cells and purified from the supernatant via anti-FLAG affinity chromatography. SDS-PAGE analysis and size exclusion chromatography confirmed purity and integrity of all proteins (see Figures 18 and 19). Only minor amounts of low-order multimers were detected by SEC, demonstrating that targeted dimeric scTRAIL₃ molecules can be generated via fusion to diabodies, EHD2, or Fc parts. Exemplarily, the thermal stability of the Db-scTRAIL₃-FLVGGGPQRVA construct targeting EGFR was analyzed by dynamic light scattering (see Figure 20). The measured melting point of 54°C corresponds to those of the other novel fusion proteins and is thus higher compared to the previously described molecules Db-scTRAIL-95L8 or Db-Glyco-scTRAIL-95L8 (see Figure 13).

Example 11: Receptor and antigen binding of targeted dimeric scTRAIL₃-FLVGGGPQRVA fusion proteins

The new scTRAIL₃-FLVGGGPQRVA fusion proteins were evaluated concerning their ability to bind to their corresponding target antigen and TRAIL-R2 by ELISA as well as by IF flow cytometry using intact Colo 205 cells. Antigen binding was analyzed in ELISA using Fc fusion

proteins of EGFR, HER2 and HER3. All proteins showed specific, concentration-dependent binding to the corresponding antigen and all formats targeting the same antigen showed binding with similar EC₅₀ values (see Figure 21, Table 5). The EC₅₀ values of the EGFR-targeting fusion proteins thereby correspond to those of the above mentioned constructs. Further ELISA studies revealed similar binding of all proteins to TRAIL-R2-Fc, independent of their format or antigen specificity (see Figure 22, Table 5). The ELISA results were confirmed by flow cytometry studies with antigen- and TRAIL receptor-expressing Colo205 cells (see Figure 23, Table 5). These data show that all proteins possess full functionality concerning binding to their antigen and TRAIL-R2, independent of their format or target specificity.

Example 12: Bioactivity of novel targeted dimeric scTRAIL₃-FLVGGGPQRVA fusion proteins

Cell death induction of the novel targeted dimeric scTRAIL₃-FLVGGGPQRVA fusion proteins was analyzed using Colo205 and HCT-116 cells. One day before treatment, 50,000 Colo205 cells/well or 15,000 HCT-116 cells/well were seeded in 96-well plates. After pretreatment of the cells with the sensitizer Bortezomib (250 ng/ml, 650 nM) or medium as control for 30 min, cells were incubated with serial dilutions of the fusion proteins for 16 h. Cell death was analyzed by crystal violet staining. To evaluate the effect of targeting, Cetuximab (200-fold molar excess) was added simultaneously to pretreatment with either Bortezomib or medium for EGFR-targeting and non-targeted constructs. All formats of EGFR-targeting fusion proteins showed strong induction of cell death on Colo205 (see Figure 24A) and HCT-116 cells (Figure 26), which could be further enhanced in the presence of Bortezomib. By addition of Cetuximab, the effects of EGFR-targeting fusion proteins could be reduced to the level of the non-targeted constructs (see Figure 24, Table 6). Furthermore, the HER2- and HER3-targeting proteins showed potent cell death induction on Colo205 as well as HCT-116 cells in the absence and presence of Bortezomib (see Figure 25, 26). This confirms the suitability of our new fusion proteins of different formats and targeting specificities for effective induction of cell death in various cancer cell lines.

Example 13: Pharmacokinetics of novel dimeric scTRAIL₃-FLVGGGPQRVA fusion proteins targeting EGFR

One advantage of generating different formats of scTRAIL₃-FLVGGGPQRVA fusion proteins is the possibility to modify the pharmacokinetic properties. To evaluate the PK profiles, 25 µg protein were injected intravenously into the tail of female CD1 mice. After 3 min, 30 min, 1 h, 2 h, 6 h, 1 d, 3 d and 7 d serum samples were taken. Protein concentrations in the serum were quantified by ELISA and normalized to the 3 min value. The terminal half-lives of the fusion proteins were increased from about 2 h for the diabody constructs to 6.5 h for the EHD2 fusion

protein and to greater 17 h in case of scFv-Fc-scTRAIL₃ (see Figure 27, Table 7). Thus, the different formats offer a possibility to improve the pharmacokinetic properties, which is an important issue concerning therapeutic application of proteins.

Example 14: Genetic fusion of scTRAIL₃-FAVSGAA to the C-terminus of an IgG kappa light chain or heavy chain yielding an IgG-scTRAIL₃ fusion protein

In order to combine several advantageous principles in one molecule, i.e. dimeric configuration of scTRAIL₃, tumor associated antigen targeting by an IgG antibody and improvement of the pharmacokinetic profile by interaction of the IgG Fc moiety with FcRn, we fused scTRAIL₃-FAVSGAA to the C-terminus of either IgG kappa light chain or heavy chain separated by a 15 amino acid residues long peptide with two N-glycosylation sites. In the example shown, an EGFR-specific human IgG1 with mutated Fc domain affecting ADCC and CDC function was used as a scaffold for IgG-scTRAIL, but non-mutated Fc domain is equally suited. Expression cassettes for IgG heavy and light chains, with or without fusion to scTRAIL₃, were cloned into a double gene vector based on the GS System pEE vectors (Lonza), followed by transient transfection of HEK293 cells and production in serum free OptiMEM I medium supplemented with 50 µM ZnCl₂. Facilitated by N-terminal FLAG tags fused to the heavy chain, we were able to affinity-purify IgG and IgG-scTRAIL₃ fusion proteins. Reducing SDS-PAGE followed by Coomassie staining revealed the occurrence of gene products corresponding to heavy chain (51 kDa) (SEQ ID NO: 242), kappa light chain (24 kDa) (SEQ ID NO: 243, light chain-scTRAIL₃-FAVSGAA (80 kDa) (SEQ ID NO: 244) and heavy chain-scTRAIL₃-FAVSGAA (107 kDa) (SEQ ID NO: 205) (Figure 28).

Example 15: Induction of caspase-8 and -3/7 activity by scTRAIL₃-FLVGGGPQRVA fusion proteins

Since it has been shown previously that targeting and dimeric assembly of scTRAIL greatly improves apoptosis induction, three scTRAIL₃-FLVGGGPQRVA constructs were analyzed to investigate these effects on the level of caspase-8 and -3/7 activity. Colo205 cells (15,000/well) were cultivated for 24 h at 37 °C, 5% CO₂. After preincubation with bortezomib (650 nM final concentration) or medium for 30 min, cells were treated with scTRAIL₃-FLVGGGPQRVA constructs for different time periods. Levels of active caspase-8 and -3/7 were detected using Caspase-Glo[®] 8 Assay and Caspase-Glo[®] 3/7 Assay (Promega) according to the manufacturer's instructions. Bortezomib alone only marginally induced activation of caspase-8 and -3/7 at the analyzed concentration and treatment intervals (Figure 29 A, B). At a concentration of 100 pM scTRAIL, incubation of the cells with scTRAIL₃-FLVGGGPQRVA resulted in activation of caspase-8 and -3/7 detectable upon 20 h incubation. Compared to that the dimeric Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 210) showed faster activation kinetics with a marked caspase

activity already after 8 h. The dimeric EGFR-targeting fusion protein scFvhu225-Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 209) showed an even further accelerated activation reaching high activity already after 2 h and inducing considerable higher levels of active caspases compared to Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 210) and scTRAIL₃-FLVGGGPQRVA. Using a concentration of 1 nM scTRAIL, all constructs showed faster activation kinetics for initiator and executioner caspases. At this dose, both Fc fusion proteins displayed similar activation profiles reaching comparable levels of active caspase-8 and -3/7, however slightly delayed for the non-targeted molecule. Despite a faster induction of caspase activity at this higher concentration, scTRAIL₃-FLVGGGPQRVA was not able to induce activity levels as high as those measured for the dimeric constructs. In the absence of bortezomib, treatment with a concentration of 1 nM scTRAIL units induced caspase-8 and -3/7 activity with a similar kinetic as compared to that measured in the presence of bortezomib. However, slightly lower maximum activity levels were reached, especially for the executioner caspases. Thus, dimeric assembly of scTRAIL, e.g. via fusion to an Fc part, increases the activity of those molecules, which can be further enhanced by fusion to a targeting moiety, e.g. a scFv.

Example 16: *In vivo* anti-tumor activity of scTRAIL₃-FLVGGGPQRVA fusion proteins

To prove the anti-tumor activity of dimeric EGFR-targeting scTRAIL₃-FLVGGGPQRVA fusion proteins *in vivo*, a Colo205 xenograft model was used. In a first experiment, 3 x 10⁶ Colo205 cells were injected subcutaneously into the left and right flank of female NMRI nu/nu mice (10 weeks old). Treatment was started when tumors reached a volume of ~100 mm³. Mice received six treatments with 5 µg bortezomib (i.p.) and 0.5 nmol protein in 150 µl PBS or PBS as control (i.v.). After three treatments every other day, treatment intervals were gradually increased by one day. All analyzed formats showed similar anti-tumor effects inducing complete tumor regression (Figure 30A). This proves the suitability of all formats for *in vivo* therapy. A second experiment was performed using the same xenograft model. When tumors reached a size of ~100 mm³, mice received combinatorial injections of 5 µg bortezomib (i.p.) and 0.3 nmol or 0.1 nmol protein in 150 µl PBS (i.v.). Control mice were treated with bortezomib (i.p.) and PBS (i.v.). Mice received treatments twice a week (every fourth and third day, respectively) for three weeks. Similar to treatment with 0.5 nmol, a dose of 0.3 nmol scFvhu225-Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 209) induced complete tumor regression (Figure 30B). Even for a dose as low as 0.1 nmol, anti-tumor effects were observed demonstrating the high therapeutic activity of these molecules in combination with bortezomib.

Example 17: EGFR-specific binding is maintained in anti-EGFR IgG-scTRAIL-FAVSGAA fusion proteins

IgG fusion proteins comprising scTRAIL₃-FAVSGAA (SEQ ID NO: 227) at the C-terminus of either light chain (anti-EGFR IgG LC-scTRAIL₃-FAVSGAA), heavy chain (anti-EGFR IgG HC-scTRAIL₃-FAVSGAA) or both (anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA) of a human EGFR-specific IgG1 antibody were expressed in soluble form from transiently transfected HEK293 cells and affinity-purified by anti-FLAG chromatography. The size exclusion chromatography analysis of the anti-EGFR IgG-scTRAIL₃ fusion proteins and the scaffold anti-EGFR IgG revealed a correct molecular assembly of the proteins (Figure 31A). Exemplified here for anti-EGFR IgG HC-scTRAIL₃-FAVSGAA, a melting point of 56 °C was determined by dynamic light scattering (Figure 31B). Thus, anti-EGFR IgG HC-scTRAIL₃-FAVSGAA is characterized by the same thermal stability such as Db10-Glyco-scTRAIL₃-FAVSGAA, providing additional evidence that increased thermal stability of the scTRAIL₃ molecules is maintained in the context of fusion to an IgG.

The binding of anti-EGFR IgG-scTRAIL₃-FAVSGAA variants and the reference molecule anti-EGFR IgG was tested by flow cytometry on the EGFR-positive colon carcinoma cell lines Colo205 and HCT116, or via ELISA on purified EGFR-Fc. The IgG used here was derived from cetuximab by humanization of mouse V_H and V_L, followed by their insertion into a human IgG1 framework. As depicted in Figure 32A and Table 8, anti-EGFR IgG shows a binding behavior almost equal to the parental antibody cetuximab on Colo205 and HCT116 cells. Upon genetic fusion of anti-EGFR IgG with scTRAIL₃-FAVSGAA in the configurations described at the beginning, the resulting proteins maintained binding to the tested cell lines, albeit the EC₅₀ values of the fusion proteins were throughout higher than for anti-EGFR IgG (Figure 32B, C, Table 9). Interestingly, among the dimeric scTRAIL₃ fusion proteins, the configuration with fusion of scTRAIL₃ to the IgG heavy chain seems to interfere less with EGFR binding than the light chain fusion, proven by lower EC₅₀ values detected for anti-EGFR IgG HC-scTRAIL₃-FAVSGAA (Table 9).

Example 18: anti-EGFR IgG-scTRAIL₃-FAVSGAA fusion proteins show EGFR-dependent increase of scTRAIL₃ bioactivity on tumor cell lines

Bioactivities of the IgG-scTRAIL₃ fusion proteins were compared in presence or absence of a molar excess of cetuximab in order to demonstrate EGFR-dependent enhancement of cell death induction in the tumor cell lines HCT116, Colo205 (colon carcinoma) and HT1080 (fibrosarcoma) (Figures 33-35, Table 10). Additionally, bioactivities were tested in presence of the apoptosis sensitizer bortezomib. The dimeric fusion proteins anti-EGFR IgG LC-scTRAIL₃-

FAVSGAA and anti-EGFR IgG HC-scTRAIL₃-FAVSGAA were bioactive with EC₅₀ values in the low picomolar range, resembling roughly the bioactivity of Db10-Glyco-scTRAIL₃-FAVSGAA. Importantly, competition of EGFR binding by excessive amounts of cetuximab resulted in reduced bioactivities on all cell lines tested, providing evidence for increased tumor cell killing as a consequence of tumor antigen targeting. The tetrameric form anti-EGFR IgG LC/HC-scTRAIL₃-FAVSGAA was characterized by higher bioactivities than the dimeric formats throughout on all tested cell lines, suggesting a correlation between number of scTRAIL moieties within one molecule and bioactivity. Depending on the characteristics of each cell line, further enhanced cell killing was shown upon co-treatment with bortezomib for all anti-EGFR IgG-scTRAIL₃-FAVSGAA variants.

Example 19: anti-EGFR IgG HC-scTRAIL₃-FAVSGAA shows enhanced serum half-life

To shed light on the pharmacokinetics of the new IgG-scTRAIL₃ fusion proteins, we analyzed the serum half-life of anti-EGFR IgG HC-scTRAIL₃-FAVSGAA in CD1 mice and observed a terminal serum half-life $t_{1/2\beta}$ of 16.09 ± 2.62 h and an AUC of 488.83 ± 82.32 %h (Figure 36). As a reference, Db-scTRAIL₃-FLVGGGPQRVA was measured with a terminal serum half-life $t_{1/2\beta}$ of 2.57 ± 0.17 h and an AUC of 235.23 ± 48.04 %h, suggesting FcRn-mediated elongation of the serum half-life of IgG-scTRAIL₃-FAVSGAA molecules and therefore a high potential for sustained antitumor activity.

Example 20: In vivo study of Db10-Glyco-scTRAIL₃-FAVSGAA

The antitumor bioactivity of Db10-Glyco-scTRAIL₃-FAVSGAA was investigated using the established mouse xenograft model of the human colon carcinoma cell line Colo205. Db-scTRAIL₃ fusion was applied intravenously when tumors reached a volume of ~ 100 mm³. Three different doses of 0.1 nmol, 0.3 nmol and 1 nmol protein were applied in a daily regimen for 8 days in combination with intraperitoneal injection of clinical grade bortezomib. Whereas treatment with 0.1 nmol did not induce measurable antitumor effects, a transient, partial inhibition of tumor growth was observed for the 0.3 nmol group, which was, however, statistically not significant (Figure 37A). In contrast, a strong and rapid reduction of tumor volumes with macroscopically undetectable tumors in 9/12 cases at day 20 was observed for the 1 nmol dose. A re-growth of tumors was observed for a subpopulation around day 25 (subgroup I) and for a second subpopulation around day 42 (subgroup II). Both subgroups received a second, identical treatment cycle starting at day 28 (subgroup I) or day 43 (subgroup II) (Figure 37A). An antitumor response was again observed for both groups. At the end of the observation period (d109), 4/12 tumors stayed in complete macroscopic remission and 3 other tumors were in a stable, not actively growing state with volumes below 100 mm³.

Serum concentrations of Db10-Glyco-scTRAIL₃-FAVSGAA were determined by ELISA 0.05 h, 4 h, and 24 h after the first injection, as well as 4 h and 24 after the last injection of the first cycle, showing that similar serum concentrations were reached after the first and last injection (Figure 37B). Additionally, the pharmacokinetics of Db10-Glyco-scTRAIL₃-FAVSGAA were studied in immunocompetent CD-1 mice, revealing a terminal half-life of 3.6 ± 0.1 h and an AUC of 8.8 ± 1.4 ($\mu\text{g/ml}$)*h (Figure 37C).

The safety of the applied doses of Db10-Glyco-scTRAIL-FAVSGAA was monitored by measuring serum activities of alanine aminotransferase (ALT) and α -amylase (Figure 37D, E). Serum samples were taken from all groups 4 hours and 24 hours after the first injection as well as 24 h after the last injection of the first treatment cycle and compared to untreated animals. In both assays, no statistically significant increase of ALT and α -amylase levels were observed for all treatment groups compared to the untreated animals, except for the α -amylase value of the 1 nmol group at day 9 (24 h after last injection, 199 U/L vs. 161 U/L, $p < 0.05$).

Example 21: Fusion proteins of scTRAIL₃-FAVSGAA with Fc

In order to demonstrate the suitability of scTRAIL₃-FAVSGAA (SEQ ID NO: 227) in a dimeric protein format comprising the Fc moiety of human IgG1 for dimerization and elongation of serum half-life, we generated fusion proteins with C-terminal (Fc-scTRAIL₃-FAVSGAA; SEQ ID NO: 246) or N-terminal configuration (scTRAIL₃-FAVSGAA-Fc; SEQ ID NO: 245) of the scTRAIL₃ moiety (see **Table 14** for additional details on the structure of the constructs used in this example). In addition, a tetrameric assembly of scTRAIL₃-FAVSGAA (scTRAIL₃-FAVSGAA-Fc-scTRAIL₃-FAVSGAA; SEQ ID NO: 247) was investigated to prove that higher numbers of scTRAIL units in one molecule correlate directly with higher bioactivity. In this first series of molecules, scTRAIL₃-FAVSGAA and Fc were connected via a 16 aa residues linker with two N-glycosylation sites (GSGNGTSNGTSGSSGG). Linkers of that kind were used in one polypeptide chain in case of the tetrameric protein scTRAIL₃-FAVSGAA-Fc-scTRAIL₃-FAVSGAA (GSGNGTSNGTSGSSRT and GSGNGTSNGTSGSSGG). The resulting proteins Fc-scTRAIL₃-FAVSGAA, scTRAIL₃-FAVSGAA-Fc and scTRAIL₃-FAVSGAA-Fc-scTRAIL₃-FAVSGAA were expressed in transiently or stably transfected HEK293 cells in a soluble form and purified by anti-FLAG affinity chromatography. As shown by SEC, all proteins were present in a correctly dimerized form (Figure 38A). ELISA studies on TRAIL R1-Fc and TRAIL R2-Fc revealed better binding properties for Fc-scTRAIL₃-FAVSGAA, but also scTRAIL₃-FAVSGAA-Fc and scTRAIL₃-FAVSGAA-Fc-scTRAIL₃-FAVSGAA and the monomeric control protein scTRAIL₃-FLVGGGGPQRVA bound concentration-dependent to the TRAIL

receptors (Figure 38B). Higher bioactivities compared with monomeric scTRAIL₃-FLVGGGGPQRVA were observed for Fc-scTRAIL₃-FAVSGAA and scTRAIL₃-FAVSGAA-Fc in *in vitro* bioactivity assays on Colo205 cells, putatively due to dimeric configuration of scTRAIL (Figure 38C, Table 11). The tetrameric assembly scTRAIL₃-FAVSGAA-Fc-scTRAIL₃-FAVSGAA showed the highest bioactivity of all tested scTRAIL₃ Fc fusion proteins. The sensitization with bortezomib resulted in additionally enhanced bioactivities for all tested proteins.

In a subsequent study, we investigated the optimal length and composition of the peptide linker connecting scTRAIL₃-FAVSGAA with Fc. Therefore, scTRAIL₃-FAVSGAA was fused C-terminally with Fc using glycine/serine linkers of 5, 10, 15, 20 and 25 aa residues length, yielding the proteins scTRAIL₃-FAVSGAA-5 G/S-Fc (SEQ ID NO: 249), scTRAIL₃-FAVSGAA-10 G/S-Fc (SEQ ID NO: 250), scTRAIL₃-FAVSGAA-15 G/S-Fc (SEQ ID NO: 251), scTRAIL₃-FAVSGAA-20 G/S-Fc (SEQ ID NO: 252) and scTRAIL₃-FAVSGAA-25 G/S-Fc (SEQ ID NO: 253). In addition, scTRAIL₃-FAVSGAA and Fc were fused without linker (scTRAIL₃-FAVSGAA-0-Fc; SEQ ID NO: 248) or with an alpha-helical linker comprising 33 aa residues (scTRAIL₃-FAVSGAA-“W”-Fc; SEQ ID NO: 254).

All of these scTRAIL₃-FAVSGAA-Fc fusion proteins could be expressed in a soluble form from transiently or stably transfected HEK293 cells, as proven by TRAIL ELISA (Figure 39A). Affinity-purified full length proteins were obtained from six variants and analyzed by SEC for molecular composition (Figure 39B). The variants scTRAIL₃-FAVSGAA-0-Fc, scTRAIL₃-FAVSGAA-5 G/S-Fc, scTRAIL₃-FAVSGAA-10 G/S-Fc, scTRAIL₃-FAVSGAA-15 G/S-Fc, scTRAIL₃-FAVSGAA-20 G/S-Fc, scTRAIL₃-FAVSGAA-25 G/S-Fc and scTRAIL₃-FAVSGAA-“W”-Fc were present in a correct dimeric constitution. Dynamic light scattering analysis revealed a melting point of 60 °C for scTRAIL₃-FAVSGAA-20 G/S-Fc (Figure 39C). The melting points of the other fusion proteins scTRAIL₃-FAVSGAA-25 G/S-Fc, scTRAIL₃-FAVSGAA-“W”-Fc and scTRAIL₃-FAVSGAA-Fc were in the range of 54 - 55 °C, indicating that the higher thermal stability of scTRAIL₃-FAVSGAA (54 °C) contributed to a higher overall protein stability also in the scTRAIL Fc fusion protein format.

Regarding binding to TRAIL R1-Fc and TRAIL R2-Fc in ELISA, the proteins scTRAIL₃-FAVSGAA-20 G/S-Fc, scTRAIL₃-FAVSGAA-25 G/S-Fc and scTRAIL₃-FAVSGAA-“W”-Fc bound, depending of the specific receptor, with EC₅₀ values in the nanomolar or sub-nanomolar range without major differences among each other (Figure 40A). Lowest EC₅₀ values were

detected for molecules with N-terminal fusion of Fc to scTRAIL₃. In contrast, scTRAIL₃-FAVSGAA-Fc showed less effective binding than the other proteins. All proteins bound with comparable properties to Colo205 or HCT116 tumor cells in flow cytometry, whereupon molecules with flexible glycine/serine linkers showed stronger binding signals than the variant scTRAIL₃-FAVSGAA-“W”-Fc comprising a mostly rigid alpha-helical peptide linker (Figure 40B). The bioactivity of scTRAIL₃-FAVSGAA-20 G/S-Fc, scTRAIL₃-FAVSGAA-25 G/S-Fc and scTRAIL₃-FAVSGAA-“W”-Fc was evaluated *in vitro* by comparison with the reference molecules scTRAIL₃-FLVGGGPQRVA, Fc-scTRAIL₃-FAVSGAA, scTRAIL₃-FAVSGAA-Fc on Colo205 and HCT116 cells (Table 13, Figures 41, 42). On Colo205 cells, scTRAIL₃-FAVSGAA-20 G/S-Fc, scTRAIL₃-FAVSGAA-“W”-Fc and scTRAIL₃-FLVGGGPQRVA showed the best bioactivities with almost equal EC₅₀ values of ~ 20 pM (w/o bortezomib) and ~ 5 pM (with bortezomib), respectively. In contrast, on HCT116 cells, the molecule scTRAIL₃-FAVSGAA-20 G/S-Fc showed the best bioactivity among the analyzed scTRAIL₃ fusion proteins with Fc.

Example 22: Db-scTRAIL fusion proteins targeting FAP

Targeting of tumor stroma markers like fibroblast activation protein (FAP) is considered as a promising approach to enhance the specificity and anti-tumor activity of TRAIL based protein therapeutics, e.g. because of the lower genetic variability of activated tumor fibroblasts. We accomplished FAP targeting in the Db-scTRAIL and scFv-Fc-scTRAIL format by use of humanized antigen binding domains derived from scFv36 (Baum et al., 2007).

In the molecule Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA (SEQ ID NO: 255), the FAP-specific V_H and V_L domains were genetically connected with a peptide linker according to SEQ ID NO: 260 (GGGGS), facilitating diabody formation and hence a dimeric configuration of scTRAIL₃-FAVSGAA, which is fused via a 16 aa residues peptide linker comprising two N-glycosylation sites (AAAGNGTSGTSEFGG, SEQ ID NO: 194). Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA was expressed as soluble protein from stably transfected HEK293 cells and showed mostly monomolecular, dimerized constitution in SEC upon anti-FLAG affinity purification (Figure 43A). A melting point of 55 °C was determined for the protein by dynamic light scattering, which resembles data obtained from EGFR-specific Db-scTRAIL or IgG-scTRAIL molecules comprising stability-enhanced scTRAIL₃-FAVSGAA (Figure 43B). Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA bound concentration dependent with an EC₅₀ of 14 ± 5 nM to HT1080 fibrosarcoma cells overexpressing the FAP antigen in flow cytometry, whereas no binding was detected on HT1080 wild-type cells (Figure 43C). Most importantly, functional FAP targeting was revealed in an *in vitro* bioactivity assay on HT1080 FAP⁺ cells, using competition with a

molar excess of an anti-FAP IgG (Figure 43D). In this setting, an EC₅₀ value of 40 ± 1 pM was determined for Db anti-FAP-Glyco-scTRAIL₃-FAVSGAA under competing conditions, whereas incubation without αFAP IgG resulted in higher cytotoxicity (EC₅₀ = 29 pM). Simultaneous sensitization with bortezomib resulted in generally higher bioactivities, reflected by EC₅₀ values of 4.1 pM and 2.0 pM for competing and non-competing conditions, respectively.

Example 23: scFv-Fc-scTRAIL₃ fusion protein targeting FAP

A single-chain fragment variable (scFv) targeting fibroblast activation protein (FAP) was fused to the N-terminus (polypeptide linker: AAAGGSGG) and the scTRAIL₃-FLVGGGPQRVA to the C-terminus (polypeptide linker: GSGGGSSGG) of the Fc-part and produced in stably transfected HEK293 cells. Stably transfected HT1080 FAP⁺ cells (20,000/well) were cultivated for 24 h at 37 °C, 5% CO₂. After preincubation with bortezomib (13 nM final concentration) or medium for 30 min, cells were treated with titration of the Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 210) or the FAP-targeting fusion protein scFv36-Fc-scTRAIL₃-FLVGGGPQRVA for 16 hours. Viability was measured by staining cells with crystal violet. For the dimeric FAP-targeting fusion protein scFv36-Fc-scTRAIL₃-FLVGGGPQRVA, EC₅₀ values of 5.2 pM and 1.4 pM were determined in the absence or presence of bortezomib, respectively (Figure 44). In contrast, Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 210) showed reduced bioactivity on HT1080 FAP⁺ cells with EC₅₀ values of 15.2 pM (without bortezomib) and 2.6 pM (in combination with bortezomib).

Example 24: Binding of scTRAIL₃ fusion proteins to human TRAIL receptors

The binding properties of the scTRAIL₃-fusion proteins to all five human TRAIL receptors were analyzed via ELISA. The extracellular domain (ECD) of the human death receptors (DR) 4 (TRAIL-R1) and 5 (TRAIL-R2), or of the human decoy receptors (DcR) 1 (TRAIL-R3) and 2 (TRAIL-R4) as well as human osteoprotegerin (OPG) was fused to the N-terminus of the Fc-part and was expressed in transiently transfected HEK293 cells. ELISA-plates were coated with ECD-Fc or OPG-Fc fusion proteins (3 µg/ml). After blocking with MPBS (2% milk in PBS), titrations of scFvhu225-Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 209), Fc-scTRAIL₃-FLVGGGPQRVA (SEQ ID NO: 210), and Db10hu225-Glyco-scTRAIL₃-FAVSGAA were incubated for 1 h at room temperature. Bound scTRAIL₃-fusion proteins were detected via HRP-conjugated anti-Flag antibody. ELISA studies revealed concentration-dependent binding and EC₅₀ values in the nanomolar or sub-nanomolar range (Figure 45).

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Tables

Table 1

Name	Subunit (aa)	Subunit C-Term.*	Peptide	Subunit N-Terminus**	Mutations
	114-281	..FLVG	—	VRERGPQRVA ~~AAH~~ ..	-
scTRAIL-95L8	95-281	..FLVG	GGSGGGGS	TSEETISTVQEK QQNISPLVRERG PQRVA ~~AAH~~ .	-
scTRAIL ₃ - FLVGGGGSGGGSV RERGPQRVA	114-281	..F ~~FLVG~~	GGSGGGGS	VRERGPQRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGGGSVRERG PQRVA	114-281	..F ~~FLVG~~	GGGS	VRERGPQRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGGPQRVA	118-281	..F ~~FLVG~~	GG	GPQRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGGPQRVA	118-281	..F ~~FLVG~~	G	GPQRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGPQRVA	118-281	..F ~~FLVG~~	—	GPQRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGGGSGGGSQ RVA	120-281	..F ~~FLVG~~	GGSGGGGS	QRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGGGSQRVA	120-281	..F ~~FLVG~~	GGGS	QRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGQRVA	120-281	..F ~~FLVG~~	G	QRVA ~~AAH~~ ..	-
scTRAIL ₃ - FLVGGRVA	121-281	..F ~~FLVG~~	G	RVA ~~AAH~~ ..	-
scTRAIL ₃ -FLVGGVA	122-281	..F ~~FLVG~~	G	V ~~AAH~~ ..	-
scTRAIL ₃ -FLVGGAA	122-281	..F ~~FLVG~~	G	A ~~AAH~~ ..	V122A
scTRAIL ₃ -FLVGGIA	122-281	..F ~~FLVG~~	G	I ~~AAH~~ ..	V122I
scTRAIL ₃ -FLVGGGA	122-281	..F ~~FLVG~~	G	G ~~AAH~~ ..	V122G
scTRAIL ₃ -FLVGGLA	122-281	..F ~~FLVG~~	G	L ~~AAH~~ ..	V122L
scTRAIL ₃ -FLVGGMA	122-281	..F ~~FLVG~~	G	M ~~AAH~~ ..	V122M
scTRAIL ₃ -FLGGGGA	122-281	..F ~~FLGG~~	G	G ~~AAH~~ ..	V280G / V122G
scTRAIL ₃ -FLGGGA	122-281	..F ~~FLGG~~	—	G ~~AAH~~ ..	V280G / V122G
scTRAIL ₃ -FGVGGGA	122-281	..F ~~FGVG~~	G	G ~~AAH~~ ..	L279G / V122G
scTRAIL ₃ -FAVGGAA	122-281	..F ~~FAVG~~	G	A ~~AAH~~ ..	L279A / V122A
scTRAIL ₃ -FAVGGIA	122-281	..F ~~FAVG~~	G	I ~~AAH~~ ..	L279A / V122I
scTRAIL ₃ -FIVGGIA	122-281	..F ~~FIVG~~	G	I ~~AAH~~ ..	L279I / V122I
scTRAIL ₃ -FAVSGAA	122-281	..F ~~FAVS~~	G	A ~~AAH~~ ..	L279A / G281S / V122A
scTRAIL ₃ -FLVSGIA	122-281	..F ~~FLVS~~	G	I ~~AAH~~ ..	G281S / V122I
scTRAIL ₃ -FIVSGIA	122-281	..F ~~FIVS~~	G	I ~~AAH~~ ..	L279I / G281S / V122I

scTRAIL ₃ -FAVSGIA	122-281	. . F AVS	G	I A ^{AH} . .	L279A / G281S / V122I

- * The C-terminal amino acid of scTRAIL, preferably human scTRAIL that is included according to above outlined nomenclature of the constructs is highlighted in bold. It corresponds to -X₁ of the C-terminal consensus sequence according to SEQ ID NO: 1).
- ** The N-terminal amino acids of scTRAIL, preferably human scTRAIL that are included according to above outlined nomenclature of the constructs are highlighted in bold. They correspond to X₂-V/A/F- of the N-terminal consensus sequence according to SEQ ID NO: 2.

Table 2: Solubility of scTRAIL molecules

Name	Solubility
sTRAIL	yes
scTRAIL-95L8	yes
scTRAIL ₃ -FLVGGGGSGGGSVRERGPQRVA	yes
scTRAIL ₃ -FLVGGGGSVRERGPQRVA	yes
scTRAIL ₃ -FLVGGGGPQRVA	yes
scTRAIL ₃ -FLVGGGPQRVA	yes
scTRAIL ₃ -FLVGGPQRVA	yes
scTRAIL ₃ -FLVGGGGSGGGSQRVA	yes
scTRAIL ₃ -FLVGGGGSQRVA	yes
scTRAIL ₃ -FLVGGQRVA	yes
scTRAIL ₃ -FLVGGRVA	yes
scTRAIL ₃ -FLVGGVA	yes
scTRAIL ₃ -FLVGGAA	yes
scTRAIL ₃ -FLVGGIA	yes
scTRAIL ₃ -FLVGGGA	no
scTRAIL ₃ -FLVGGLA	no
scTRAIL ₃ -FLVGGMA	no
scTRAIL ₃ -FLGGGGA	no
scTRAIL ₃ -FLGGGA	no
scTRAIL ₃ -FGVGGGA	no
scTRAIL ₃ -FAVGGAA	yes
scTRAIL ₃ -FAVGGIA	yes
scTRAIL ₃ -FIVGGIA	yes
scTRAIL ₃ -FAVSGAA	yes
scTRAIL ₃ -FLVSGIA	yes
scTRAIL ₃ -FIVSGIA	yes
scTRAIL ₃ -FAVSGIA	yes

Table 3: Thermal stability of scTRAIL molecules

Name	Tm (°C)
sTRAIL	46
scTRAIL-95L8	47
scTRAIL ₃ -FLVGGGGSGGGSVRERGPQRVA	46
scTRAIL ₃ -FLVGGGGSVRERGPQRVA	47
scTRAIL ₃ -FLVGGGGPQRVA	52
scTRAIL ₃ -FLVGGGPQRVA	52
scTRAIL ₃ -FLVGGPQRVA	53
scTRAIL ₃ -FLVGGGGSGGGSQRVA	49
scTRAIL ₃ -FLVGGGGSQRVA	50
scTRAIL ₃ -FLVGGQRVA	nd
scTRAIL ₃ -FLVGGRVA	54
scTRAIL ₃ -FLVGGVA	57
scTRAIL ₃ -FLVGGAA	53.5
scTRAIL ₃ -FLVGGIA	54
scTRAIL ₃ -FLVGGGA	nd
scTRAIL ₃ -FLVGGIA	nd
scTRAIL ₃ -FLVGGMA	nd
scTRAIL ₃ -FLGGGGA	nd
scTRAIL ₃ -FLGGGA	nd
scTRAIL ₃ -FGVGGGA	nd
scTRAIL ₃ -FAVGGAA	53
scTRAIL ₃ -FAVGGIA	53
scTRAIL ₃ -FIVGGIA	52
scTRAIL ₃ -FAVSGAA	54
scTRAIL ₃ -FLVSGIA	51.5
scTRAIL ₃ -FIVSGIA	51
scTRAIL ₃ -FAVSGIA	50.5

Table 4: EC₅₀ values of cell death induction of various Db-scTRAIL molecules on tumor cells in the presence of Bortezomib+/- antibody Cetuximab (Cet) competing for EGFR binding.

EC ₅₀ , Mean ± SE (pM)	Colo205	HT1080	HCT116
Db-scTRAIL-95L8	24 ± 4	3 ± 0.7	127 ± 2
Db-scTRAIL-95L8 + Cet.	48 ± 5	15 ± 4	381 ± 16
Db-Glyco-scTRAIL ₃ -FAVSGAA	33 ± 6	1 ± 0.6	66 ± 10
Db-Glyco-scTRAIL ₃ -FAVSGAA + Cet.	74 ± 15	10 ± 4	450 ± 2
Db-Glyco-scTRAIL ₃ -FAVSGIA	19 ± 3	0.6 ± 0.2	43 ± 2
Db-Glyco-scTRAIL ₃ -FAVSGIA + Cet.	32 ± 5	5 ± 1	161 ± 3
Db8-Glyco-scTRAIL ₃ -FAVSGAA	26 ± 2	0.9 ± 0.2	51 ± 8
Db8-Glyco-scTRAIL ₃ -FAVSGAA + Cet.	61 ± 7	7 ± 2	300 ± 10
Db10-Glyco-scTRAIL ₃ -FAVSGAA	13 ± 2	0.7 ± 0.2	39 ± 4
Db10-Glyco-scTRAIL ₃ -FAVSGAA + Cet.	66 ± 17	8 ± 2	298 ± 3

Table 5: EC₅₀ values [nM] of binding to target antigens (EGFR, HER2, HER3*), TRAIL-R2 and to Colo205 cells measured by ELISA and flow cytometry, respectively.

Construct	Antigen binding ELISA	TRAIL-R2 binding ELISA	FACS Colo205
Dbhu225-scTRAIL ₃ -FLVGGGPQRVA	0.77	0.79	0.87
dsDbhu225-scTRAIL ₃ -FLVGGGPQRVA	0.98	1.29	0.86
scFvhu225-EHD2-scTRAIL ₃ -FLVGGGPQRVA	1.25	1.82	0.58
scFvhu225-Fc-scTRAIL ₃ -FLVGGGPQRVA	0.86	1.52	1.33
Db4D5-scTRAIL ₃ -FLVGGGPQRVA	8.33	1.55	18.08
dsDb4D5-scTRAIL ₃ -FLVGGGPQRVA	4.53	1.48	23.21
scFv4D5-EHD2-scTRAIL ₃ -FLVGGGPQRVA	4.98	2.21	14.34
scFv4D5-Fc-scTRAIL ₃ -FLVGGGPQRVA	4.13	0.73	27.96
Db3M6-scTRAIL ₃ -FLVGGGPQRVA	5.88	2.81	10.44
dsDb3M6-scTRAIL ₃ -FLVGGGPQRVA	8.54	2.83	32.49
scFv3M6-EHD2-scTRAIL ₃ -FLVGGGPQRVA	11.63	2.65	19.93
scFv3M6-Fc-scTRAIL ₃ -FLVGGGPQRVA	-	-	13.11

scTRAIL ₃ -FLVGGGPQRVA	-	1.74	5.67
EHD2-scTRAIL ₃ -FLVGGGPQRVA	-	1.94	>100
Fc-scTRAIL ₃ -FLVGGGPQRVA	-	2.42	>100

*target antigens and DR5 for ELISA are Fc fusion proteins

Table 6 EC₅₀ values [pM] of cell death induction on Colo205 and HCT-116 cells in the absence and presence of Bortezomib(250 ng/ml, 650 nM) in the absence and presence of Cetuximab (200-fold molar excess).

Construct	Colo205 without / with bortezomib	Colo205 in presence of Cetuximab without / with bortezomib	HCT-116 without / with bortezomib
Dbhu225-scTRAIL ₃ -FLVGGGPQRVA	41.0 / 3.8	101.6 / 13.8	35.2 / 1.2
dsDbhu225-scTRAIL ₃ -FLVGGGPQRVA	21.2 / 2.5	42.3 / 7.2	25.2 / 0.8
scFvhu225-EHD2-scTRAIL ₃ -FLVGGGPQRVA	11.9 / 1.8	69.3 / 6.7	11.1 / 0.6
scFvhu225-Fc-scTRAIL ₃ -FLVGGGPQRVA	12.5 / 2.3	66.2 / 5.7	11.9 / 0.5

Table 7: Initial and terminal half-lives [h] and areas under the curve [%h] of EGFR-targeting scTRAIL₃-FLVGGGPQRVA fusion proteins. Data are represented as mean ± S.D. (n=3).

Construct	t _{1/2α} [h]	t _{1/2β} [h]	AUC [%h]
Dbhu225-scTRAIL ₃ -FLVGGGPQRVA	1.09 ± 0.22	2.42 ± 0.09	215.98 ± 52.04
dsDbhu225-scTRAIL ₃ -FLVGGGPQRVA	1.28 ± 0.15	2.32 ± 0.12	291.96 ± 20.87
scFvhu225-EHD2-scTRAIL ₃ -FLVGGGPQRVA	1.49 ± 0.11	6.55 ± 0.20	401.94 ± 51.37
scFvhu225-Fc-scTRAIL ₃ -FLVGGGPQRVA	2.05 ± 0.23	17.37 ± 3.14	543.20 ± 39.90

Table 8: EC₅₀ values of binding of cetuximab and derived humanized anti-EGFR IgG to EGFR⁺ cell lines.

Molecule	Colo205	HCT116
	EC ₅₀ of binding (pM)	EC ₅₀ of binding (pM)
cetuximab	19 ± 8	9 ± 3
anti-EGFR IgG	28 ± 6	8 ± 3

Table 9: EC₅₀ values of binding of anti-EGFR IgG-scTRAIL₃-FAVSGAA fusion proteins to EGFR⁺ cell lines (flow cytometry) or EGFR-Fc (ELISA).

Molecule	Colo205	HCT116	EGFR-Fc
	EC ₅₀ of binding (pM)	EC ₅₀ of binding (pM)	EC ₅₀ of binding (pM)
anti-EGFR IgG	60 ± 41	70 ± 21	156 ± 19
anti-EGFR IgG LC- scTRAIL ₃ -FAVSGAA	148 ± 83	181 ± 79	460 ± 27
anti-EGFR IgG HC- scTRAIL ₃ -FAVSGAA	81 ± 59	118 ± 33	343 ± 72
anti-EGFR IgG LC/HC- scTRAIL ₃ -FAVSGAA	146 ± 28	91 ± 72	315 ± 87

Table 10: EC₅₀ values of bioactivity of anti-EGFR IgG-scTRAIL₃-FAVSGAA fusion proteins on EGFR⁺ cell lines in presence or without bortezomib (BZB). An excess of cetuximab was used for competition, where indicated.

Molecule	HT1080 (pM)		Colo205 (pM)		HCT116 (pM)	
	- BZB	+ BZB	- BZB	+ BZB	- BZB	+ BZB
Db10-Glyco-scTRAIL ₃ - FAVSGAA	1.8 ± 0.3	0.23 ± 0.17	35 ± 9	8.1 ± 0.8	7.7 ± 1.7	2.1 ± 1.4
anti-EGFR IgG LC- scTRAIL ₃ -FAVSGAA	1.4 ± 0.2	0.4 ± 0.1	41 ± 14	10 ± 4	8.5 ± 1.8	5.2 ± 0.8
anti-EGFR IgG LC- scTRAIL ₃ -FAVSGAA + cetuximab	52 ± 8	4.2 ± 1.9	304 ± 168	25 ± 11	75 ± 33	19 ± 4
anti-EGFR IgG HC- scTRAIL ₃ -FAVSGAA	1.3 ± 0.1	0.2 ± 0.17	32 ± 17	5.9 ± 2.9	4.8 ± 0.5	1.3 ± 0.6
anti-EGFR IgG HC- scTRAIL ₃ -FAVSGAA + cetuximab	34 ± 8	6.3 ± 0.5	74 ± 38	17 ± 1	47 ± 25	3.9 ± 0.6
anti-EGFR IgG LC/HC- scTRAIL ₃ -FAVSGAA	0.3 ± 0.01	0.036	6.9 ± 2.5	1.1 ± 0.2	0.6 ± 0.2	0.3 ± 0.1

anti-EGFR IgG LC/HC-scTRAIL ₃ -FAVSGAA + cetuximab	5.9 ± 0.4	0.13	10 ± 5	1.8 ± 0.7	6.7 ± 0.6	1.5 ± 0.04
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Table 11: EC₅₀ values of bioactivity of scTRAIL₃-FAVSGAA Fc fusion proteins comprising glycosylated peptide linkers between scTRAIL₃-FAVSGAA and Fc on Colo205 in presence or without bortezomib (BZB). Monomeric scTRAIL₃-FLVGGGGPQRVA served as a reference (mean ± S.D., n = 3).

Molecule	Colo205 (EC ₅₀ in pM)	
	- BZB	+ BZB
scTRAIL ₃ -FLVGGGGPQRVA	-	670 ± 96
Fc-scTRAIL ₃ -FAVSGAA	220 ± 50	35 ± 7
scTRAIL ₃ -FAVSGAA-Fc	218 ± 9	31 ± 9
scTRAIL ₃ -FAVSGAA-Fc-scTRAIL ₃ -FAVSGAA	62 ± 13	21 ± 7

Table 12: EC₅₀ values of binding (ELISA) of scTRAIL₃-FAVSGAA Fc fusion proteins comprising glycine/serine or glycosylated peptide linkers to TRAIL R1-Fc and TRAIL R2-Fc. Fc-scTRAIL₃-FLVGGGGPQRVA served as a reference (mean ± S.D., n = 3).

Molecule	TRAIL R1-Fc (EC ₅₀ in nM)	TRAIL R2-Fc (EC ₅₀ in nM)
scTRAIL ₃ -FAVSGAA-20 G/S-Fc	4.45 ± 0.93	0.43 ± 0.26
scTRAIL ₃ -FAVSGAA-25 G/S-Fc	3.24 ± 0.58	0.42 ± 0.23
scTRAIL ₃ -FAVSGAA-“W”-Fc	3.91 ± 0.84	0.54 ± 0.34
scTRAIL ₃ -FAVSGAA-Fc	6.36 ± 1.29	0.69 ± 0.37
Fc-scTRAIL ₃ -FAVSGAA	2.15 ± 1.01	0.34 ± 0.15
Fc-scTRAIL ₃ -FLVGGGGPQRVA	1.63 ± 1.07	0.34 ± 0.23

Table 13: EC₅₀ values of bioactivities of scTRAIL₃-FAVSGAA or scTRAIL₃-FLVGGGPQRVA fusion proteins with Fc on Colo205 and HCT116 tumor cell lines in presence or without bortezomib (BZB) (mean ± S.D., n = 3).

Molecule	Colo205 (EC ₅₀ in pM)		HCT116 (EC ₅₀ in pM)	
	- BZB	+ BZB	- BZB	+ BZB
Fc-scTRAIL ₃ -FLVGGGPQRVA	17.2 ± 8.0	3.8 ± 0.8	92.1 ± 38.0	38.7 ± 17.6
Fc-scTRAIL ₃ -FAVSGAA	45.6 ± 6.6	15.7 ± 8.9	35.6 ± 18.2	27.4 ± 10.2
scTRAIL ₃ -FAVSGAA-Fc	288 ± 97	24.9 ± 17.8	261 ± 84	145 ± 25
scTRAIL ₃ -FAVSGAA-20 G/S-Fc	20.7 ± 1.0	5.3 ± 1.3	23.8 ± 7.7	16.9 ± 3.4
scTRAIL ₃ -FAVSGAA-25 G/S-Fc	56.7 ± 24.8	12.5 ± 3.3	61.3 ± 16.1	42.9 ± 8.9
scTRAIL ₃ -FAVSGAA-“W”-Fc	20.1 ± 4.0	4.7 ± 2.1	35.4 ± 7.5	26.4 ± 5.4

Tab

le 14: Structure of constructs tested in Examples 17 to 24.

Molecule	Sequence (connecting linker)	Length (aa)
scTRAIL ₃ -FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---GSGNGTSNGTSGSSGG---Fc	16
Fc-scTRAIL ₃ -FAVSGAA	Fc---GSGNGTSNGTSGSSGG---scTRAIL ₃ -FAVSGAA	16
scTRAIL ₃ -FAVSGAA-Fc-scTRAIL ₃ -FAVSGAA	scTRAIL ₃ -FAVSGAA---GSGNGTSNGTSGSSRT---Fc---GSGNGTSNGTSGSSGG---scTRAIL ₃ -FAVSGAA	2×16
scTRAIL ₃ -0-FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---Fc	-
scTRAIL ₃ -5 G/S-FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---GGSGG---Fc	5
scTRAIL ₃ -10 G/S-FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---GGSGGGSGG---Fc	10
scTRAIL ₃ -15 G/S-FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---GGSGGGSGGGSGG---Fc	15
scTRAIL ₃ -20 G/S-FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---GGSGGGSGGGSGGGSGG---Fc	20
scTRAIL ₃ -25 G/S-FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---GGSGGGSGGGSGGGSGGGSGG---Fc	25
scTRAIL ₃ -“W”-FAVSGAA-Fc	scTRAIL ₃ -FAVSGAA---GGSGEAAAKEAAAKEAAAKEAAAKEAAKSGG---Fc	33
DbαFAP-Glyco-scTRAIL ₃ -FAVSGAA	DbαFAP---AAAGNGTSNGTSEFGG---scTRAIL ₃ -FAVSGAA	16

References

1. Arnau J, Lauritzen C, Petersen GE, Pedersen J. Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein Expr Purif.* 2006 Jul;48(1):1-13.
5
2. Baum, P., Müller, D., Rüger, R., and Kontermann, R.E. (2007). Single-chain Fv immunoliposomes for the targeting of fibroblast activation protein-expressing tumor stromal cells. *J. Drug Target.* 15, 399–406.
10
3. Bodmer JL, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. *Trends Biochem Sci.* 2002 Jan;27(1):19-26.
4. Chen W, Qiu L, Hou J, Zhang X, Ke X, Wang Z *et al.* (2012a). Phase Ib study of recombinant circularly permuted TRAIL (CPT) in relapsed or refractory multiple myeloma patients. 54th ASH annual meeting abstr 1857.
15
5. Chen W, Hou J, Zhao Y, Qiu L, Ke X, Wang Z *et al.* (2012b). Circularly permuted TRAIL (CPT) combined with thalidomide for the treatment of relapsed or refractory multiple myeloma: an open-label, multicenter phase II clinical trial. 54th ASH annual meeting abstr 2958.
20
6. de Bruyn M, Bremer E, Helfrich W. Antibody-based fusion proteins to target death receptors in cancer. *Cancer Lett.* 2013 May 28;332(2):175-83.
25
7. Gao D, Narasimhan DL, Macdonald J, Brim R, Ko MC, Landry DW, Woods JH, Sunahara RK, Zhan CG. Thermostable variants of cocaine esterase for long-time protection against cocaine toxicity. *Mol Pharmacol.* 2009 Feb;75(2):318-23.
8. Herbst RS, Kurzrock R, Hong DS, Valdivieso M, Hsu CP, Goyal L, Juan G, Hwang YC, Wong S, Hill JS, Friberg G, LoRusso PM. A first-in-human study of conatumumab in adult patients with advanced solid tumors. *Clin Cancer Res.* 2010a Dec 1;16(23):5883-91.
30

9. Herbst RS, Eckhardt SG, Kurzrock R, Ebbinghaus S, O'Dwyer PJ, Gordon MS, Novotny W, Goldwasser MA, Tohny TM, Lum BL, Ashkenazi A, Jubb AM, Mendelson DS. Phase I dose-escalation study of recombinant human Apo2L/TRAIL, a dual proapoptotic receptor agonist, in patients with advanced cancer. *J Clin Oncol*. 2010b Jun 10;28(17):2839-46.
10. Holland PM, Death receptor agonist therapies for cancer, which is the right TRAIL?, *Cytokine and Growth Factor Reviews*, 2014, 25: 185-193-
11. Hymowitz SG, Christinger HW, Fuh G, Ultsch M, O'Connell M, Kelley RF, Ashkenazi A, de Vos AM. Triggering cell death: the crystal structure of Apo2L/TRAIL in a complex with death receptor 5. *Mol Cell*. 1999 Oct;4(4):563-71.
12. Hymowitz SG, O'Connell MP, Ultsch MH, Hurst A, Totpal K, Ashkenazi A, de Vos AM, Kelley RF. A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. *Biochemistry*. 2000 Feb 1;39(4):633-40.
13. Micheau O, Shirley S, Dufour F. Death receptors as targets in cancer. *Br J Pharmacol*. 2013 Aug;169(8):1723-44.
14. Plummer R, Attard G, Pacey S, Li L, Razak A, Perrett R, Barrett M, Judson I, Kaye S, Fox NL, Halpern W, Corey A, Calvert H, de Bono J. Phase 1 and pharmacokinetic study of lexatumumab in patients with advanced cancers. *Clin Cancer Res*. 2007 Oct 15;13(20):6187-94.
15. Schneider B, Munkel S, Krippner-Heidenreich A, Grunwald I, Wels WS, Wajant H, Pfizenmaier K, Gerspach J. Potent antitumoral activity of TRAIL through generation of tumor-targeted single-chain fusion proteins. *Cell Death Dis*. 2010 Aug 26;1:e68.
16. Seifert O, Plappert A, Fellermeier S, Siegemund M, Pfizenmaier K, Kontermann RE. Tetravalent antibody-scTRAIL fusion proteins with improved properties. *Mol Cancer Ther*. 2014 Jan;13(1):101-11.

17. Sergeeva A, Kolonin MG, Molldrem JJ, Pasqualini R, Arap W. Display technologies: application for the discovery of drug and gene delivery agents. *Adv Drug Deliv Rev.* 2006 Dec 30;58(15):1622-54.
- 5
18. Siegemund M, Pollak N, Seifert O, Wahl K, Hanak K, Vogel A, Nussler AK, Göttisch D, Münkkel S, Bantel H, Kontermann RE, Pfizenmaier K. Superior antitumoral activity of dimerized targeted single-chain TRAIL fusion proteins under retention of tumor selectivity. *Cell Death Dis.* 2012 Apr 12;3:e295.
- 10
19. Tolcher AW, Mita M, Meropol NJ, von Mehren M, Patnaik A, Padavic K, Hill M, Mays T, McCoy T, Fox NL, Halpern W, Corey A, Cohen RB. Phase I pharmacokinetic and biologic correlative study of mapatumumab, a fully human monoclonal antibody with agonist activity to tumor necrosis factor-related apoptosis-inducing ligand receptor-1. *J Clin Oncol.* 2007 Apr 10;25(11):1390-5. Erratum in: *J Clin Oncol.* 2007 Oct 10;25(29):4701.
- 15
20. Trarbach T, Moehler M, Heinemann V, Köhne CH, Przyborek M, Schulz C, Sneller V, Gallant G, Kanzler S. Phase II trial of mapatumumab, a fully human agonistic monoclonal antibody that targets and activates the tumour necrosis factor apoptosis-inducing ligand receptor-1 (TRAIL-R1), in patients with refractory colorectal cancer. *Br J Cancer.* 2010 Feb 2;102(3):506-12.
- 20

Claims

1. A polypeptide comprising:

(i) component A comprising:

at least three TNF homology domains of TNF-ligand family member proteins (THD), wherein the C-terminus of the first and second THD, respectively, which is in each case defined by the C-terminal consensus sequence

-S/T/V-F/Y/S-F-G-A/L/V/I-X₁ (SEQ ID NO: 1),

is linked to the N-terminus of the second and third THD, respectively, which is in each case defined by the N-terminal consensus sequence

X₂-V/A/F-A-H-V/L/I/Y (SEQ ID NO: 2)

or

X₃-V/W/F/C-A/L-E/Y/Q/H-L (SEQ ID NO: 3)

through a peptide X_a, which is in each case independently selected and has a length of 3 to 8 amino acids,

wherein X₁ is a non-polar/hydrophob or polar/neutral amino acid, preferably selected from the group consisting of F, V, Q, A, I, L, and Y;

wherein X₂ is selected from the group consisting of P, K, V, I, and A;

wherein X₃ is selected from the group consisting of D, S, M, and I;

(ii) component B comprising

a dimerization domain consisting of an antibody V_L and V_H region linked directly to each other with a peptide that has a length of between 7 and 11 amino acids.

2. The polypeptide according to claim 1, wherein the V_L and V_H region of an antibody specifically binding to a target molecule on the cell surface, preferably the target molecule is selected from the group consisting of tyrosine-kinase-receptors (EGFR, HER2, HER3, HER4), VEGFRs, heteromeric integrin α - or β -receptor family, tumor stroma markers, preferably fibroblast activation protein (FAP), galectin, EpCAM, CEA, CD44 and tumor specific variants thereof and other tumor selective cell surface markers, CD2, CD5, CD7, CD19, CD20, CD21, CD22, CD24, CD25, CD30, CD33, CD38, CD40, CD52, CD56, CD71, CD72, CD73, CD105, CD117, CD123, CD133, c-Met, PDGFR, IGF1-R, HMW-

MAA, TAG-72, GD2, GD3, GM2, folate receptor, Lgr5, Ley, Muc-1, Muc-2, PSMA, PSCA and uPAR.

3. The polypeptide according to any of claims 1 to 2, wherein component A is linked to
 5 component B by a peptide X_b, preferably of a length of 5 to 20 amino acids, wherein X_b optionally comprises at least one glycosylation motif, preferably at least one motif is glycosylated; and wherein X_b is preferably selected from the group consisting of SEQ ID NO: 159, SEQ ID NO: 165, SEQ ID NO: 166, SEQ ID NO: 167, SEQ ID NO: 168, SEQ ID NO: 169, SEQ ID NO: 170, SEQ ID NO: 171, SEQ ID NO: 172, SEQ ID NO: 173,
 10 SEQ ID NO: 174 and SEQ ID NO: 175

4. A polypeptide comprising:
 component C comprising:
 at least three TNF homology domains of TNF-ligand family member proteins (THD),
 15 wherein the C-terminus of the first and second THD, respectively, which is in each case defined by the C-terminal consensus sequence

$$-S/T/V-F/Y/S-F-G-A/L/V/I-X_1 \text{ (SEQ ID NO: 1),}$$
 is linked to the N-terminus of the second and third THD, respectively, which is in each case defined by the N-terminal consensus sequence

$$X_2-V/A/F-A-H-V/L/I/Y \text{ (SEQ ID NO: 2)}$$
 20 or

$$X_3-V/W/F/C-A/L-E/Y/Q/H-L \text{ (SEQ ID NO: 3),}$$
 through a peptide X_c, which is in each case independently selected and has a length of 3 to 5 amino acids,
 25 wherein X₁ is a non-polar/hydrophob or polar/neutral amino acid, preferably selected from the group consisting of F, V, Q, A, I, L, and Y;
 wherein X₂ is selected from the group consisting of P, K, V, I, and A; and
 wherein X₃ is selected from the group consisting of D, S, M, and I.

5. The polypeptide according to claim 4 further comprising a component D which is selected from the group consisting of a dimerization or multimerization domain, a half-life extension domain, a target specific binding domain or combinations thereof.

6. The polypeptide according to claim 5, wherein the target specific binding domain is an antibody or fragment thereof; a diabody, preferably the diabody consists of a V_L and V_H domain linked directly to each other with a peptide of between 5 and 15 amino; a single chain antibody or an antibody mimetic, preferably the antibody mimetic is selected from the group consisting of LACI-D1 (lipoprotein-associated coagulation inhibitor); affilins, e.g. human- γ B crystalline or human ubiquitin; cystatin; Sac7D from *Sulfolobus acidocaldarius*; lipocalin and anticalins derived from lipocalins; DARPin (designed ankyrin repeat domains); a domain of a membrane receptor; SH3 domain of Fyn; Kunitz domain of protease inhibitors ; monobodies, e.g. the 10th type III domain of fibronectin; adnectins: knottins (cysteine knot miniproteins); fynomers; atrimers; evibodies, e.g. CTLA4-based binders, affibodies, e.g. three-helix bundle from Z-domain of protein A from *Staphylococcus aureus*; Trans-bodies, e.g. human transferrin; tetranectins, e.g. monomeric or trimeric human C-type lectin domain; microbodies, e.g. trypsin-inhibitor-II; affilins; armadillo repeat proteins.
7. The polypeptide according to any of claims 4 to 6, wherein the at least three THDs are linked to component D by a peptide X_d , preferably of a length of 5 to 15 amino acids wherein X_d optionally comprises at least one glycosylation motif, preferably at least one motif is glycosylated; and wherein X_d is preferably selected from the group consisting of SEQ ID NO: 192, SEQ ID NO: 193 and SEQ ID NO: 194.
8. The polypeptide according to any of claims 1 to 7 wherein the peptide X_a or X_c consists of
- $$X_e-X_f-X_g,$$
- wherein
- X_e is selected from the group consisting of L, L-V, L-V-, L-V-G-G (SEQ ID NO: 159), L-V-S, L-V-S-G (SEQ ID NO: 161), A, A-V, A-V-S, A-V-G, A-V-G-G (SEQ ID NO: 162), A-V-S-G (SEQ ID NO: 163), I-V, I-V-S, I-V-G, I-V-G-G (SEQ ID NO: 164), I-V-S-G (SEQ ID NO: 165), K, K-L, M, M-V, A, A-L, M-V-G, M-V-Q, G, G-V, G-V-H, W, W-V, W-V-R, W-V-R-P (SEQ ID NO: 167), K-L-L, I, A-V, Q, Q-V, and Q-V-H;
- X_f is absent or selected from the group consisting of G, S, G-G, S-G, G-S, S-S, G-G-G, G-G-S, G-S-G, S-G-G, G-S-S, S-G-S and S-S-S; and

X_g is absent or selected from the group consisting of R, Q-R, P-Q-R, G-P-Q-R, L-R, N, V-N, K, L-K, L, G-L, K, D-K, Q, P-Q, A, R-A, W, G-W, Q, and T-Q;

preferably

- (i) X_e is selected from L, L-V, L-V-G, L-V-G-G (SEQ ID NO: 159), L-V, L-V-S-G (SEQ ID NO: 161), A, A-V, A-V-S, A-V-G, A-V-G-G (SEQ ID NO: 162), A-V-S-G (SEQ ID NO: 163), I-V, I-V-S, I-V-G, I-V-G-G (SEQ ID NO: 164), and I-V-S-G (SEQ ID NO: 165); X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from R, Q-R, P-Q-R, and G-P-Q-R (SEQ ID NO: 166);
- (ii) X_e is selected from K, and K-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from R, and L-R,
- (iii) X_e is selected from M, M-V; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from N and V-N;
- (iv) X_e is selected from A, and A-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from K and L-K;
- (v) X_e is selected from M, M-V, and M-V-G; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from L, and G-L;
- (vi) X_e is selected from A, and A-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from K, and D-K;
- (vii) X_e is selected from K, and K-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from Q, and P-Q;
- (viii) X_e is selected from Q, Q-V, and Q-V-H; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from A, and R-A;
- (ix) X_e is selected from W, W-V, W-V-R, and W-V-R-P (SEQ ID NO: 167); X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from W, and G-W; or
- (x) X_e is selected from K, K-L, and K-L-L; X_f is absent or selected from G, S, G-G, S-G, G-S, and S-S; and X_g is absent or selected from Q, and T-Q;

preferably the peptide X_a or X_c is selected from the group consisting of I-V-S-G (SEQ ID NO: 165), A-V-S-G (SEQ ID NO: 163), I-V-G-G (SEQ ID NO: 164), A-V-G-G (SEQ ID NO: 162), L-V-S-G (SEQ ID NO: 161), L-V-G-G (SEQ ID NO: 159), L-V-G-G-R (SEQ ID NO: 168), L-V-G-G-G (SEQ ID NO: 169), L-V-G-G-P (SEQ ID NO: 170) or X_a is selected from the group consisting of L-V-G-G-P-Q-R (SEQ ID

NO: 173), L-V-G-G-G-P-Q-R (SEQ ID NO: 174), and L-V-G-G-G-G-P-Q-R (SEQ ID NO: 75).

- 5 9. A polypeptide according to any of claim 1 to 8, wherein the at least three THDs are identical.
- 10 10. The polypeptide according to any of claims 1 to 9, wherein the sequence of the TNF-ligand family member is selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, preferably the polypeptide comprises or consists of the amino acid sequence selected from the group according to SEQ ID NO: 5, ,SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15.
- 15 11. A nucleic acid encoding a polypeptide according to any of claims 1 to 10.
12. A vector comprising a nucleic acid according to claim 11.
- 20 13. A polypeptide according to claims 1 to 10, a nucleic acid according to claim 11 or a vector according to claim 12 for use as a medicament.
14. A pharmaceutical composition comprising as an active agent a polypeptide according to claims 1 to 10 or a nucleic acid according to claim 11 or a vector according to claim 12.
- 25 15. The pharmaceutical composition according to claim 14 further comprising one or more proteasome inhibitor.
- 30 16. The pharmaceutical composition according to claim 15, wherein the proteasome inhibitor is selected from the group comprising bortezomib, lactacystin, disulfiram, epigallocatechin-3-gallate, salinosporamide A, carfilzomib, epoxomicin, ixazomib, ONX 0912, CEP-18770, and MLN9708.

17. A polypeptide according to claims 1 to 10 or a nucleic acid according to claim 11 or a vector according to claim 12 or the pharmaceutical composition according to claims 14 to 16 for use in the diagnosis, prophylaxis or treatment of hyperproliferative disorders or inflammatory disorders, preferably cancer or malignancies of the hematologic system, autoimmune disorders and neurodegenerative diseases.

Fig. 1

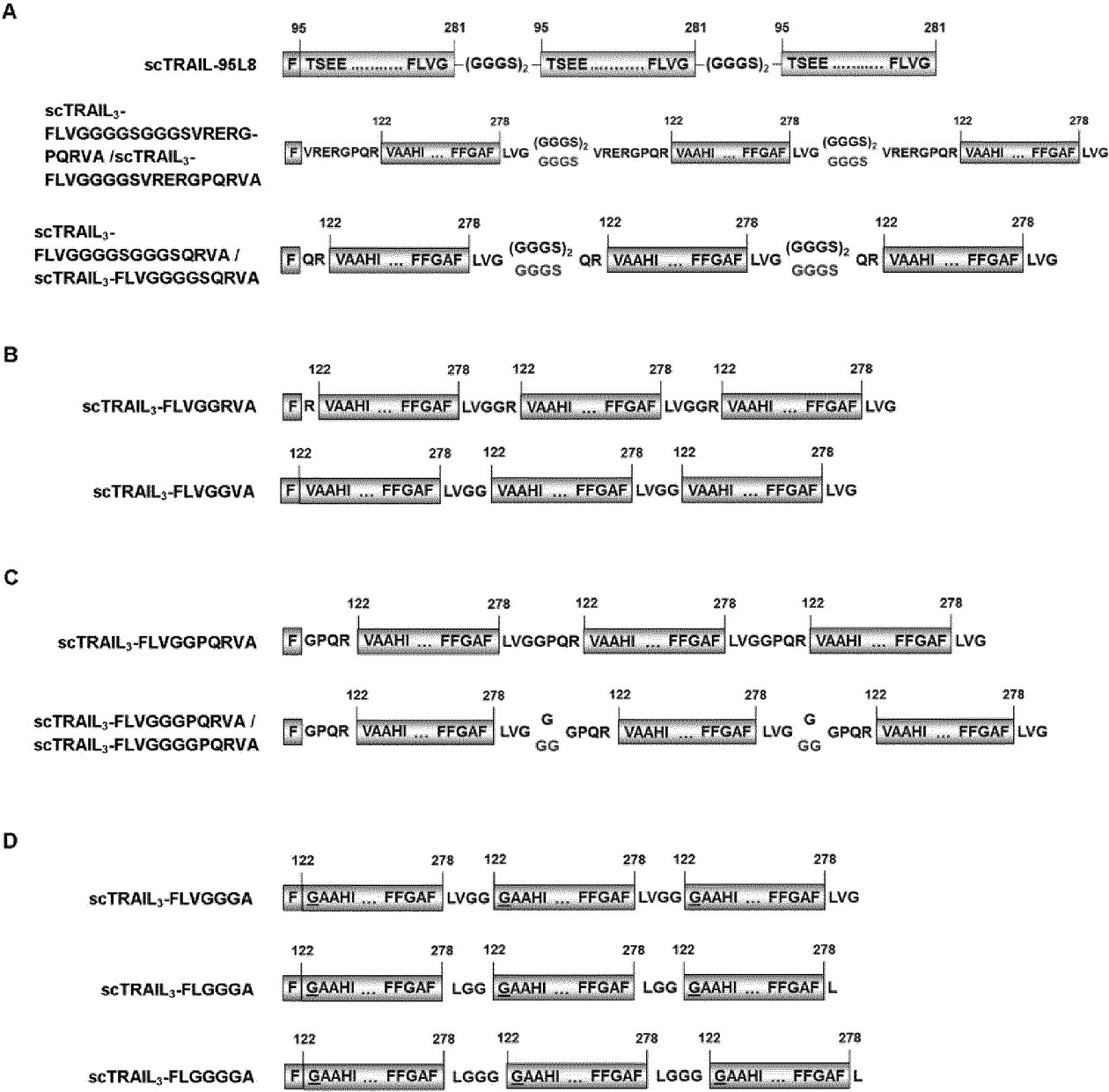
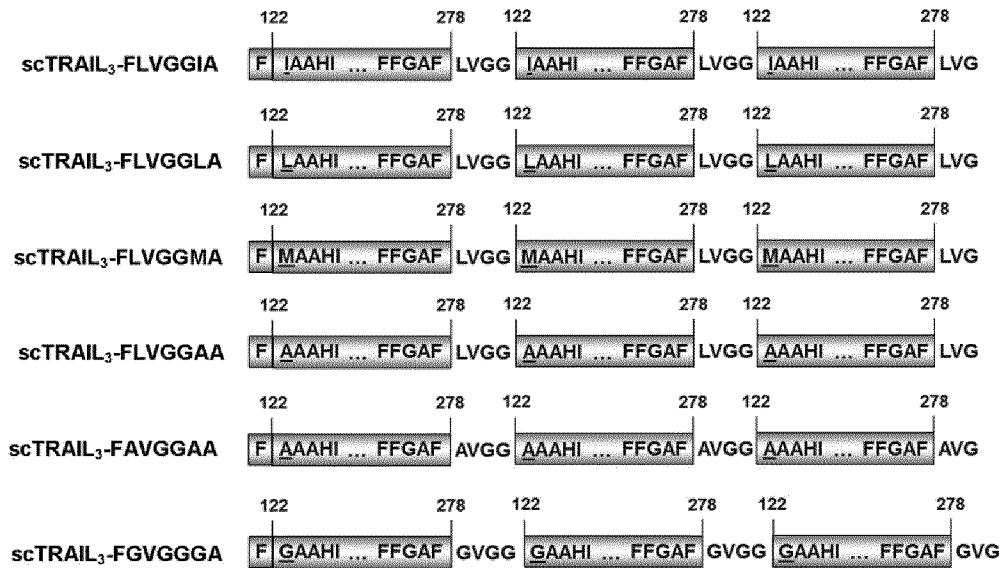


Fig. 1

E



F



Fig. 2

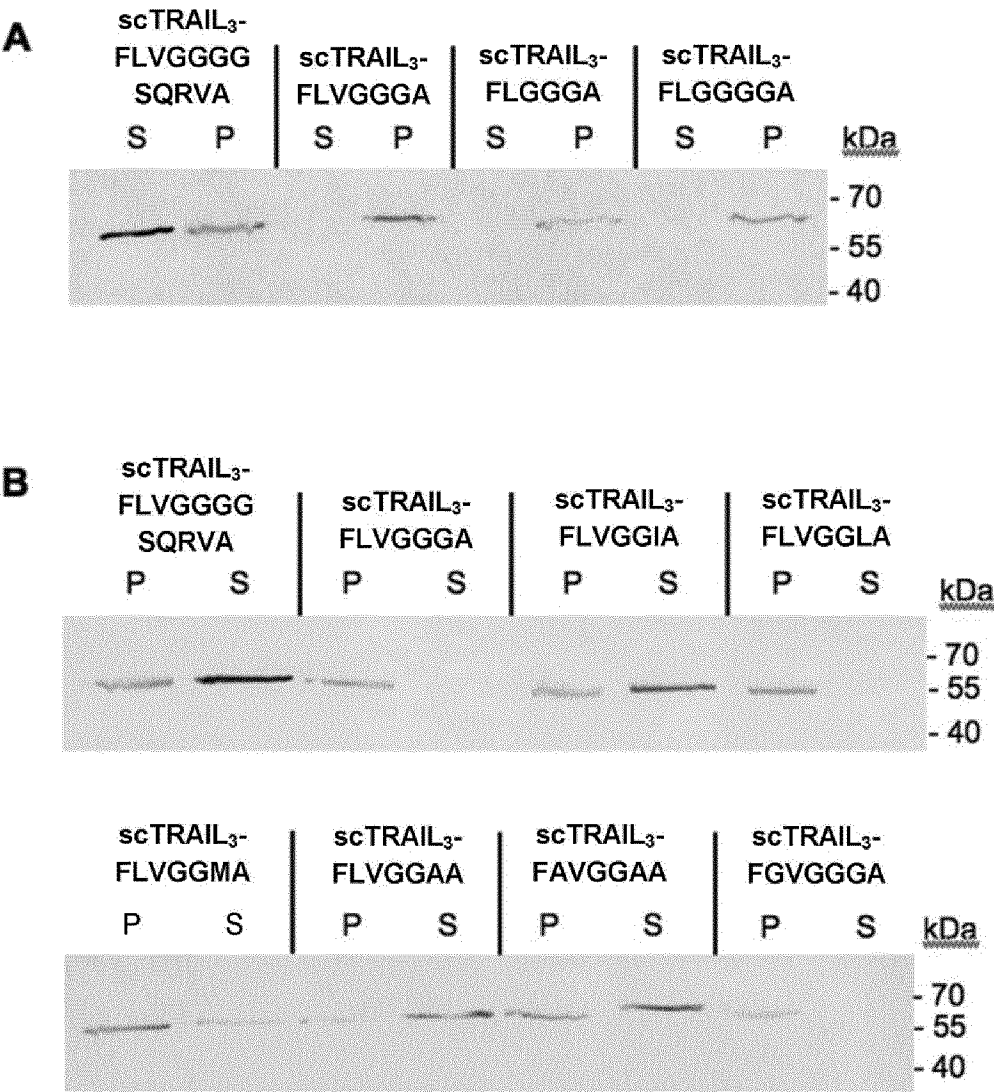
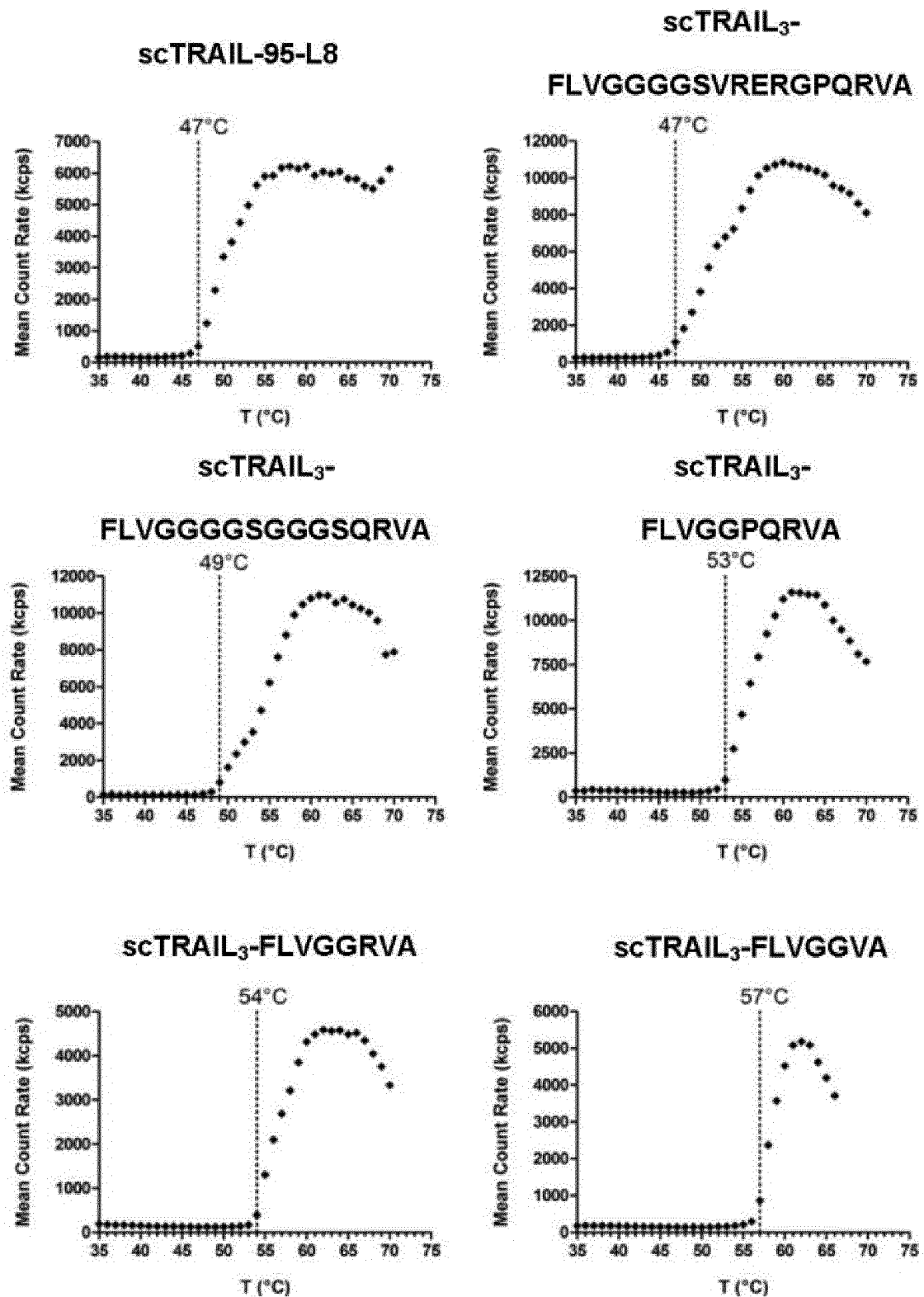
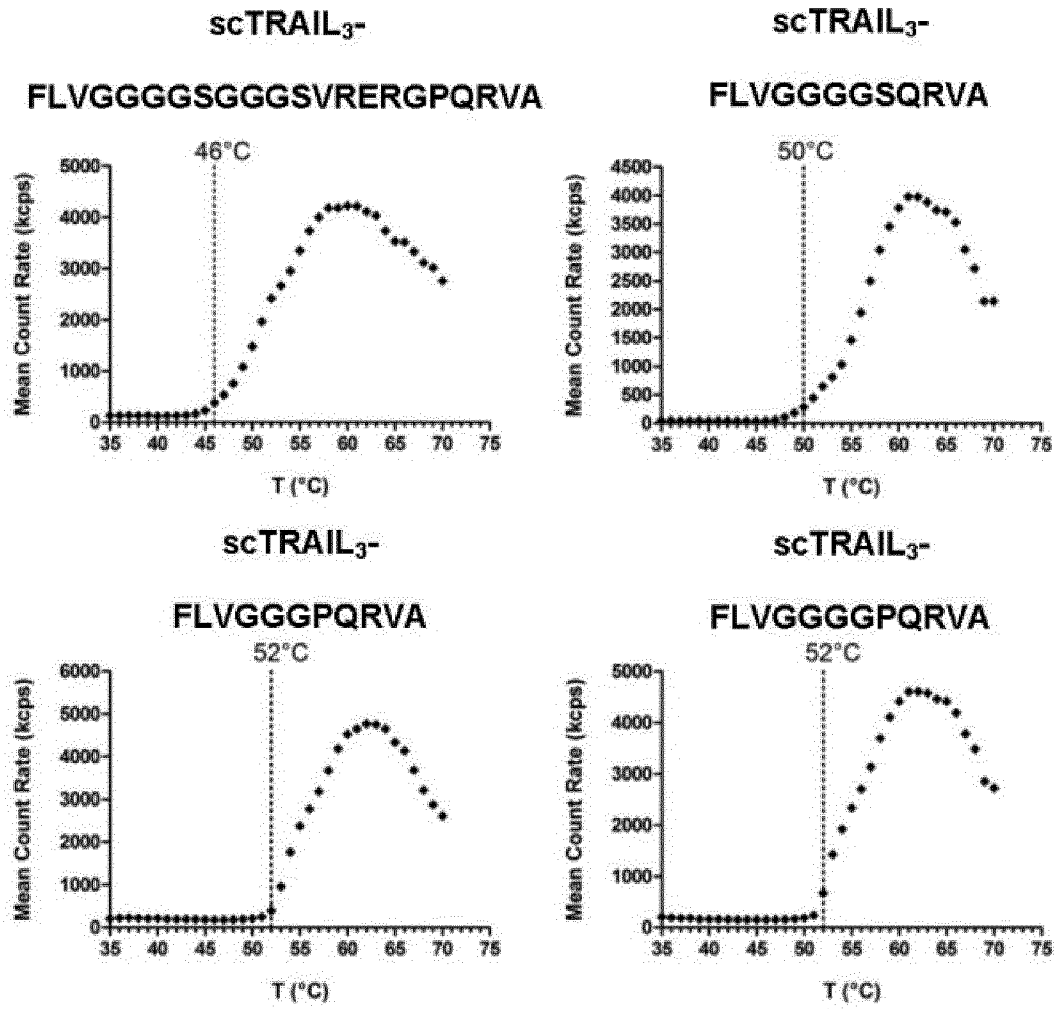


Fig. 3



cont.

Fig. 3



cont.

Fig. 3

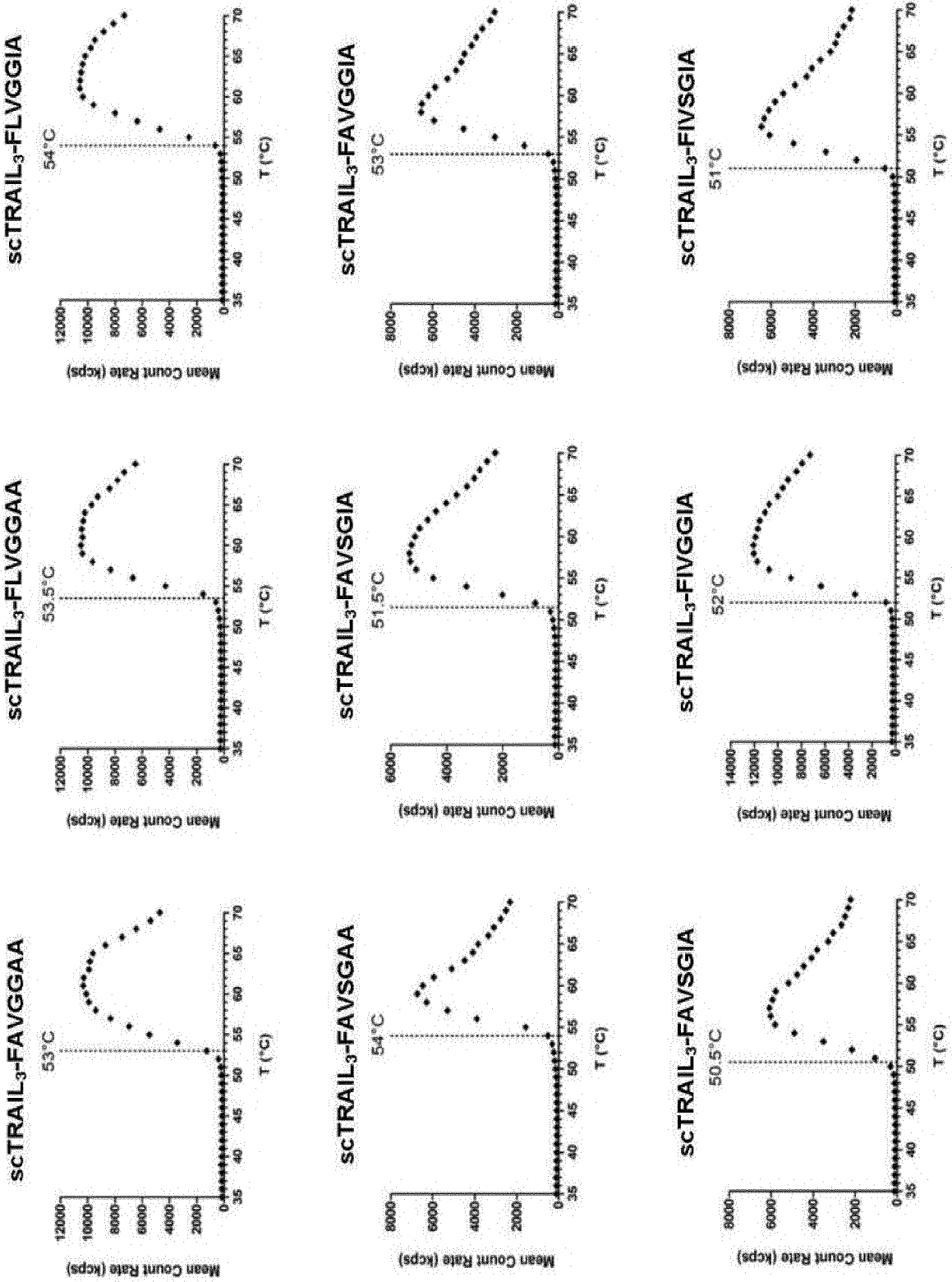
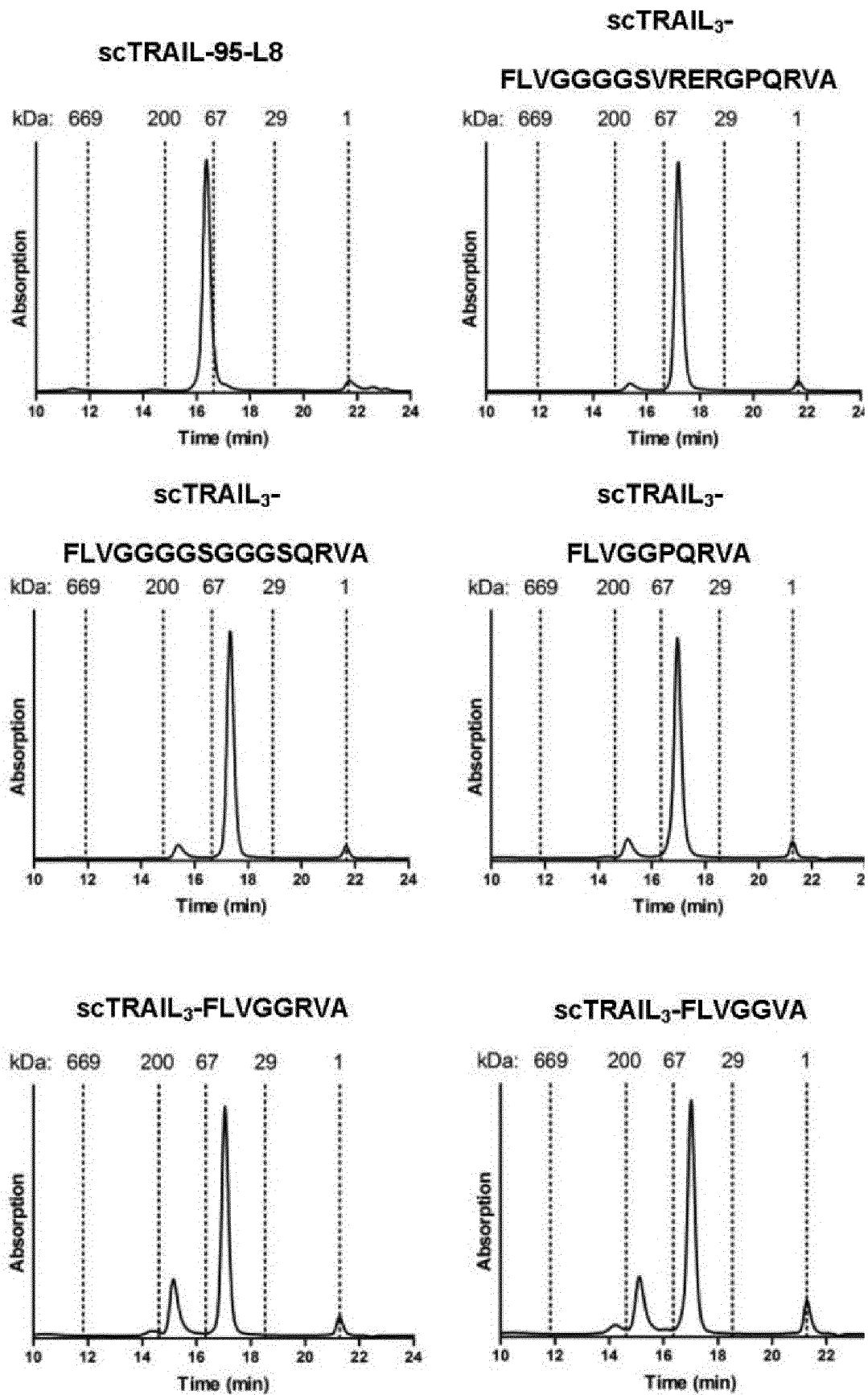
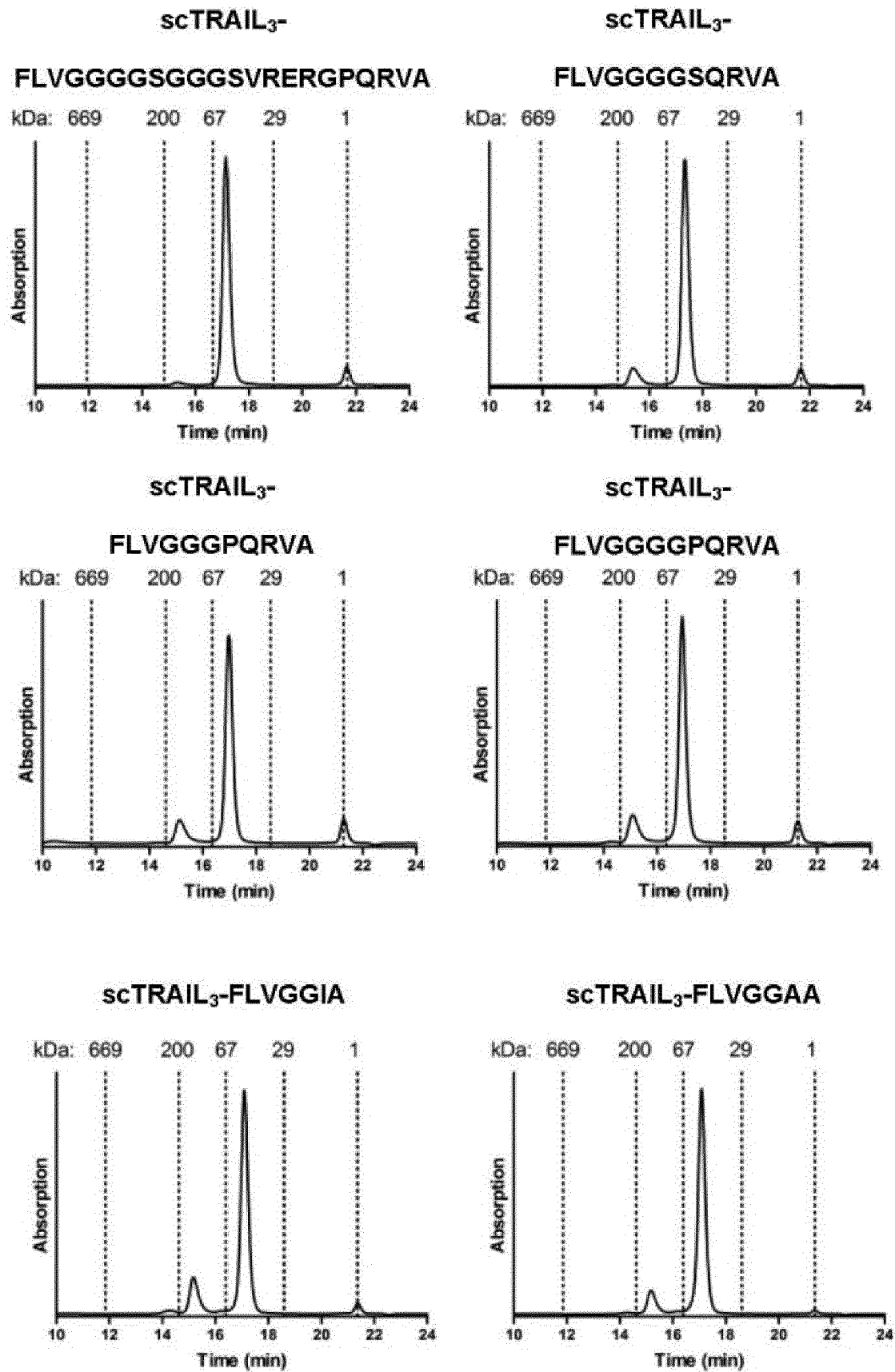


Fig. 4



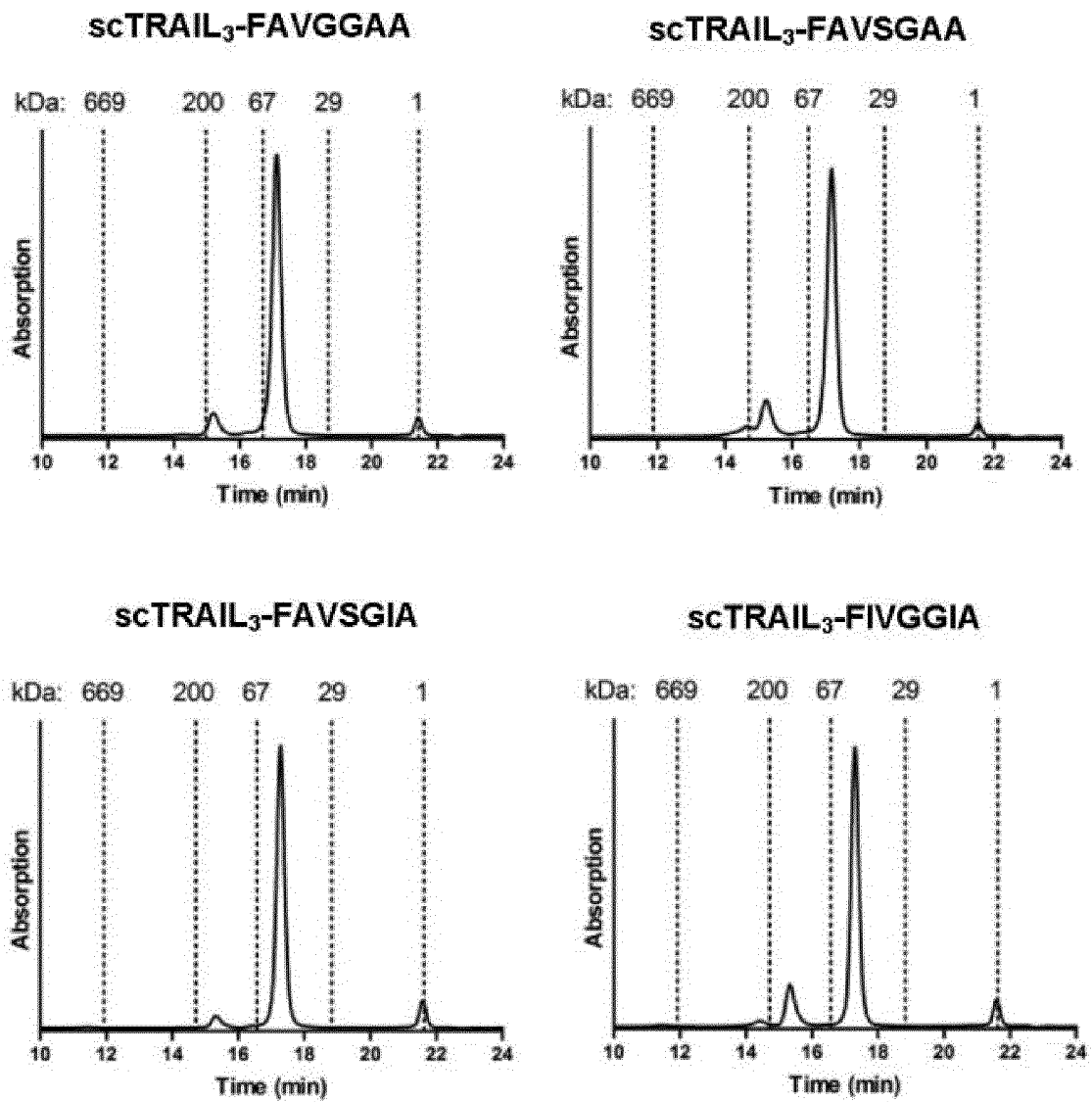
cont.

Fig. 4



cont.

Fig. 4



cont.

Fig. 4

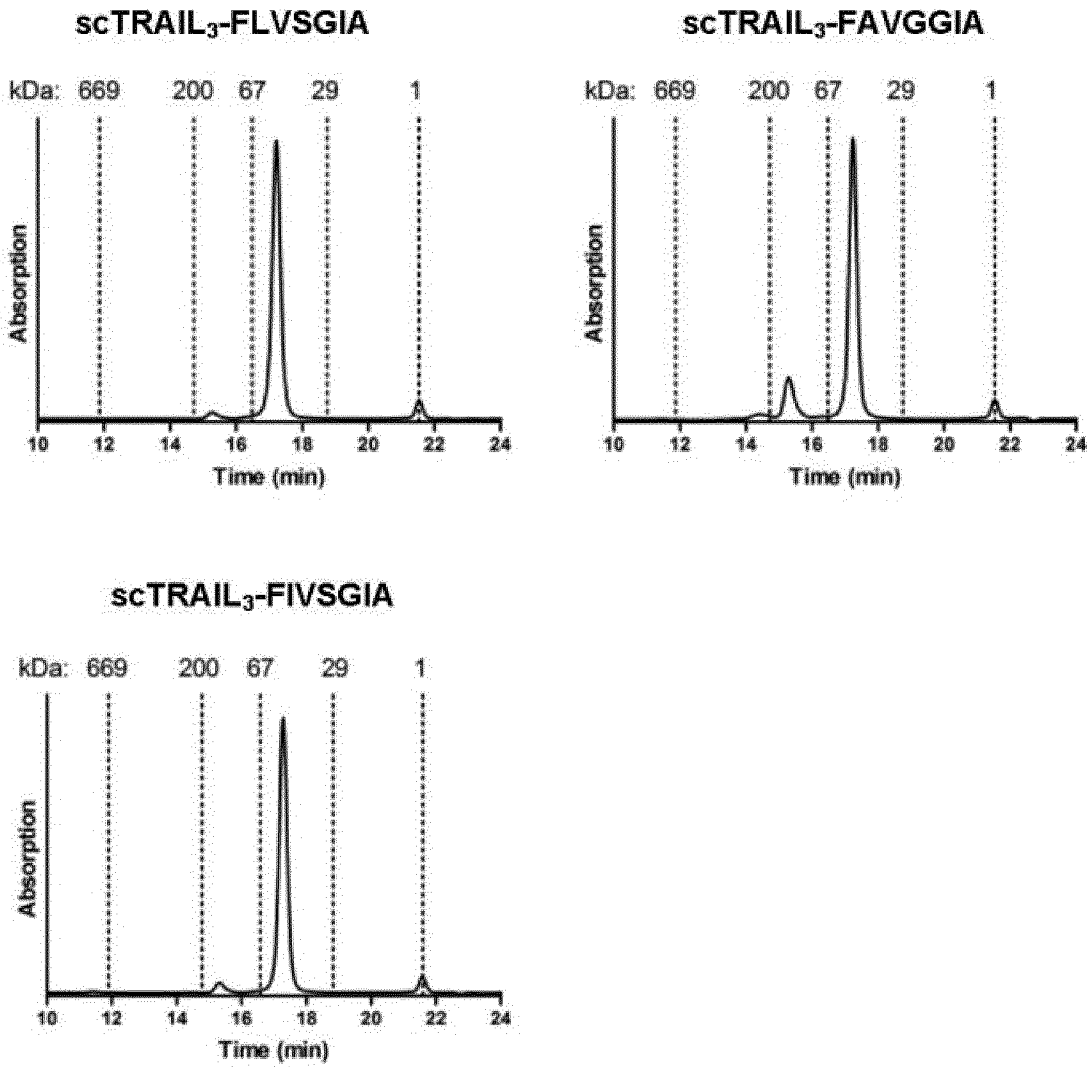


Fig. 5

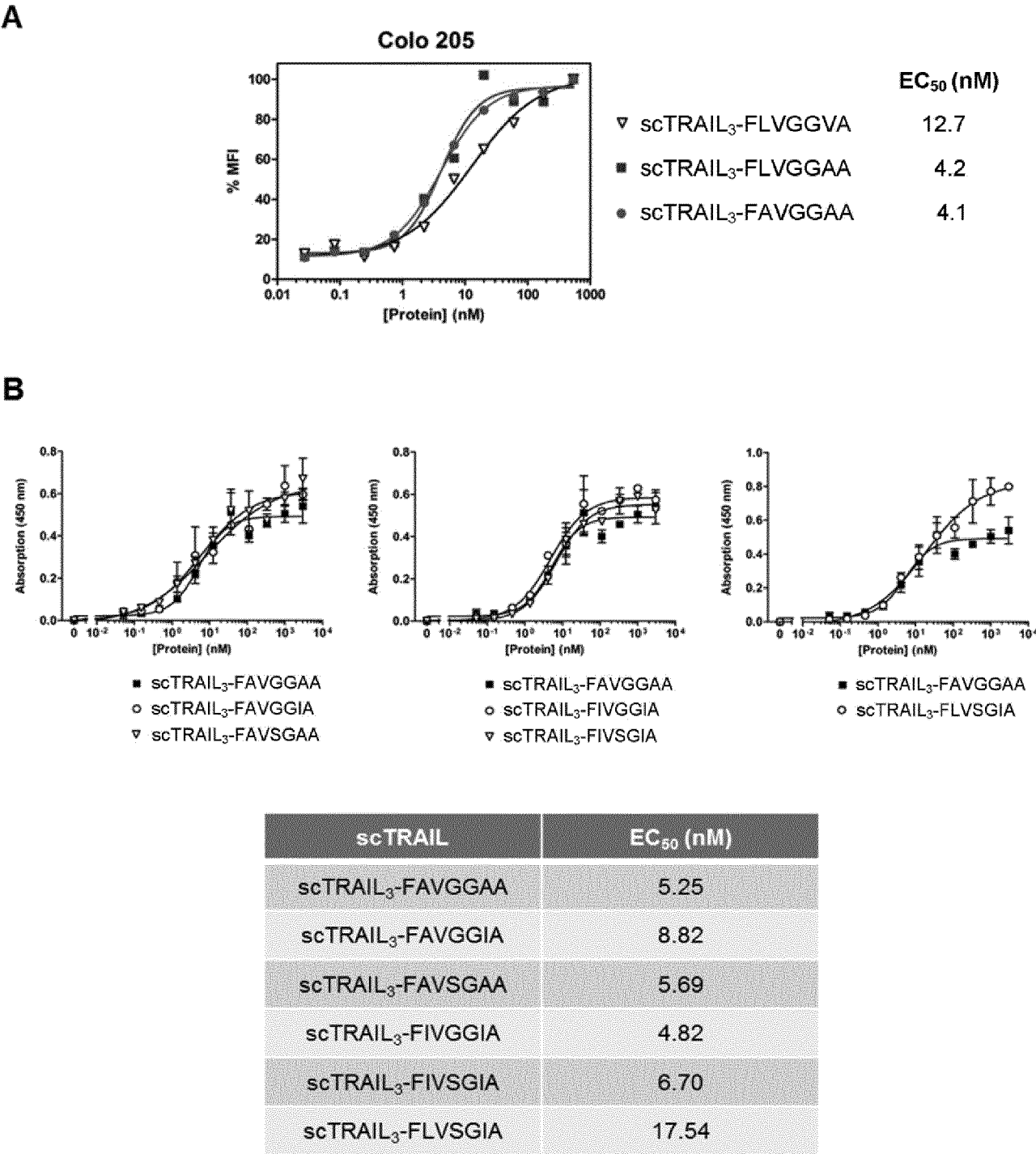
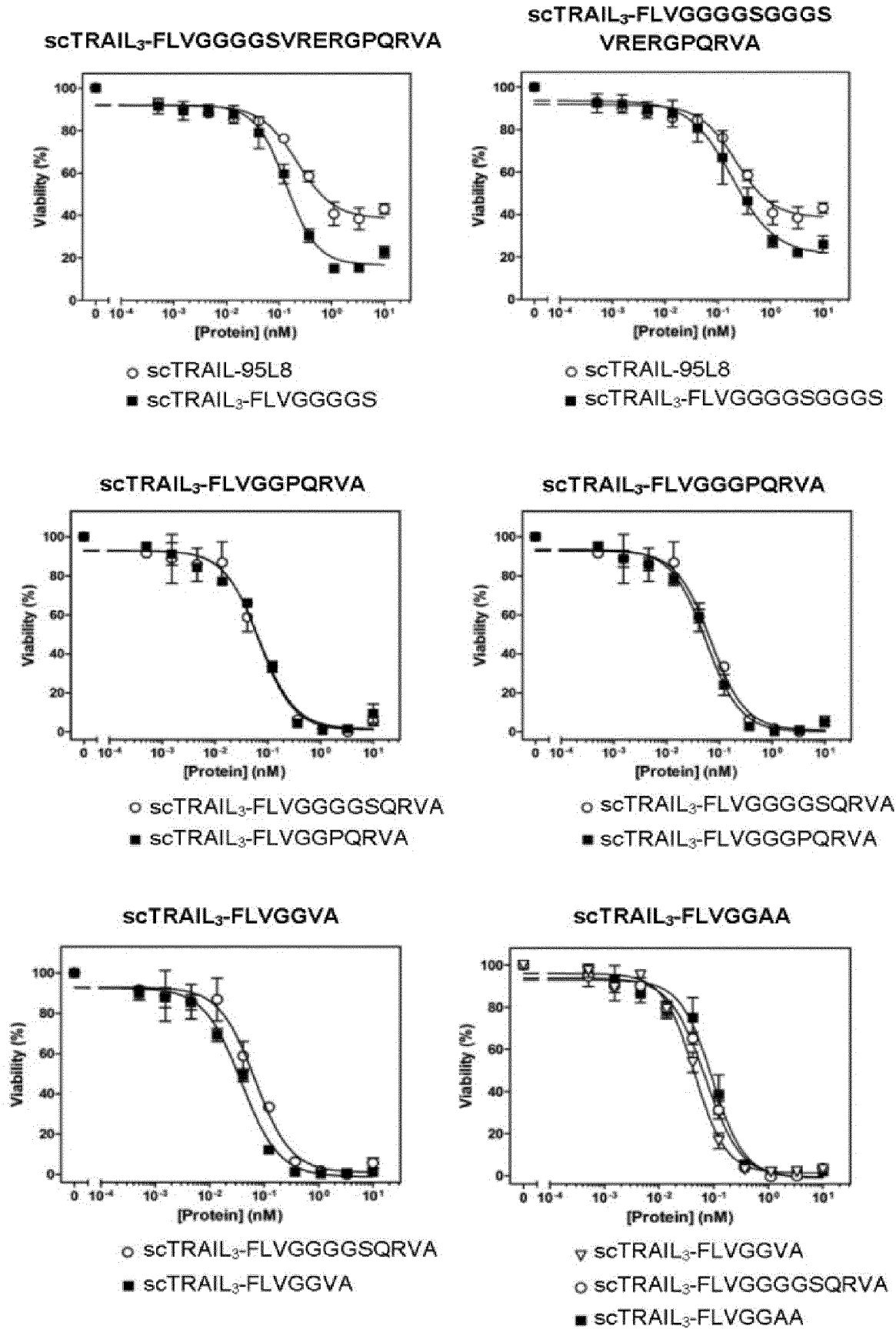
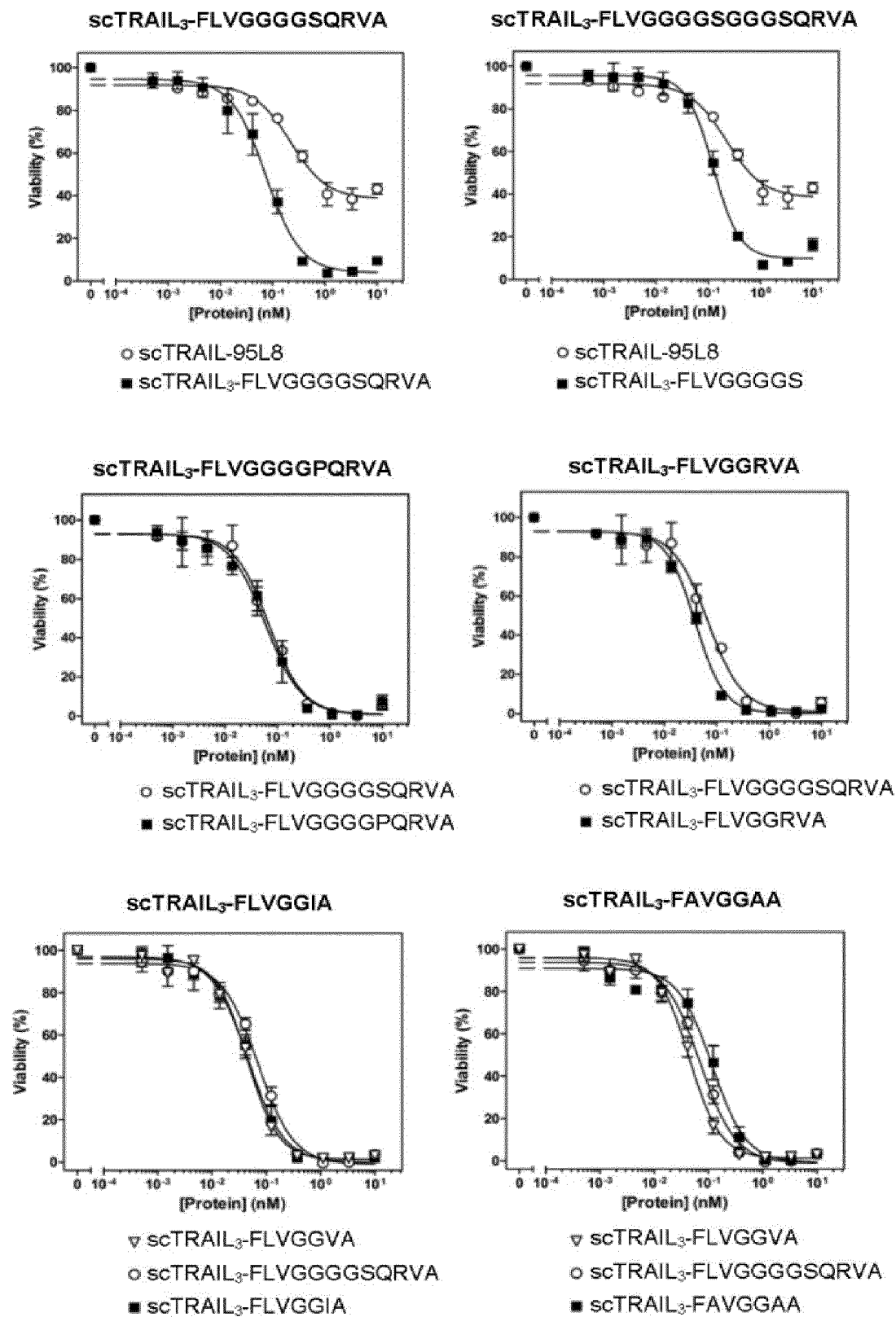


Fig. 6



cont.

Fig. 6



cont.

Fig. 6

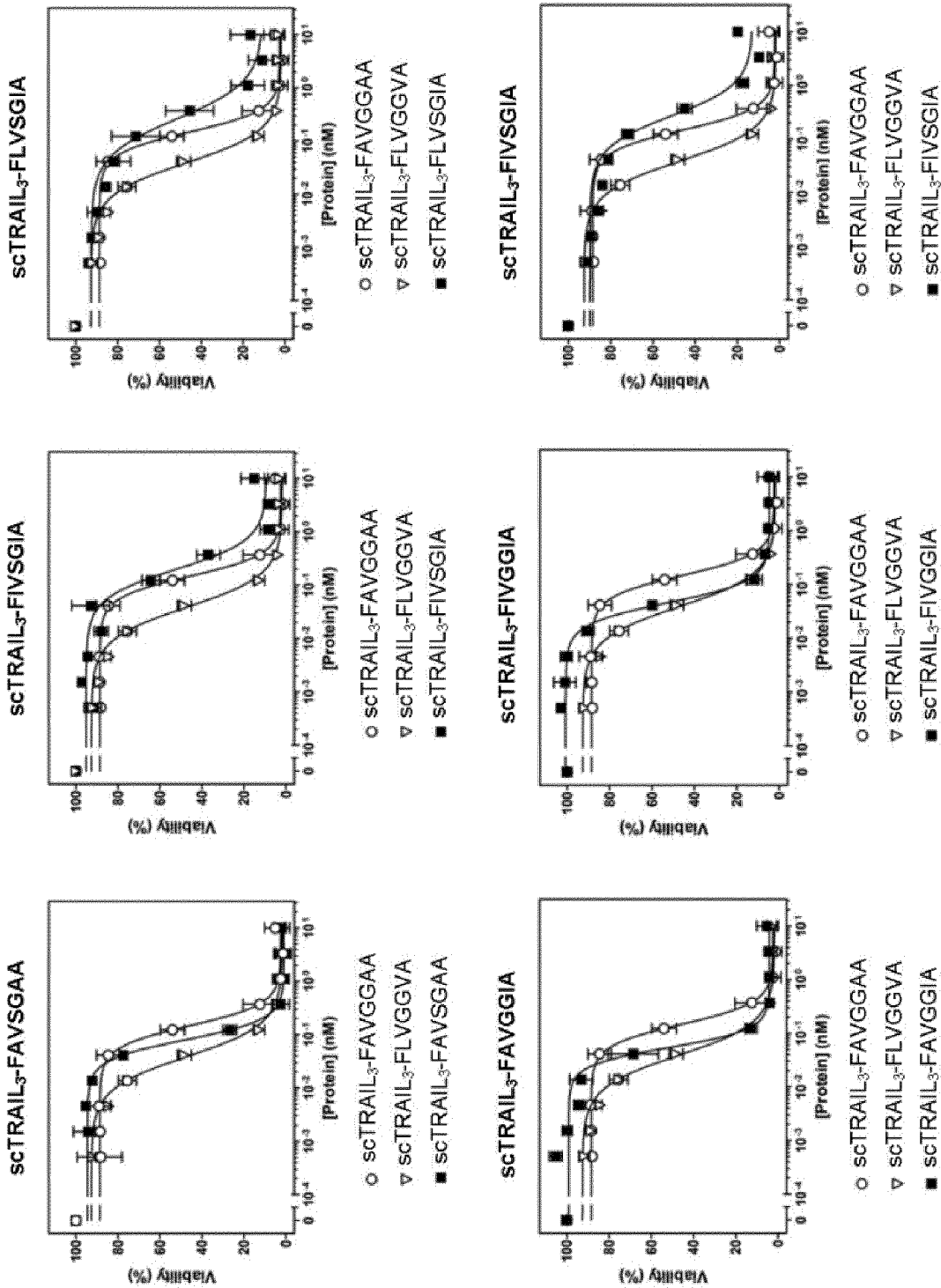


Fig. 7

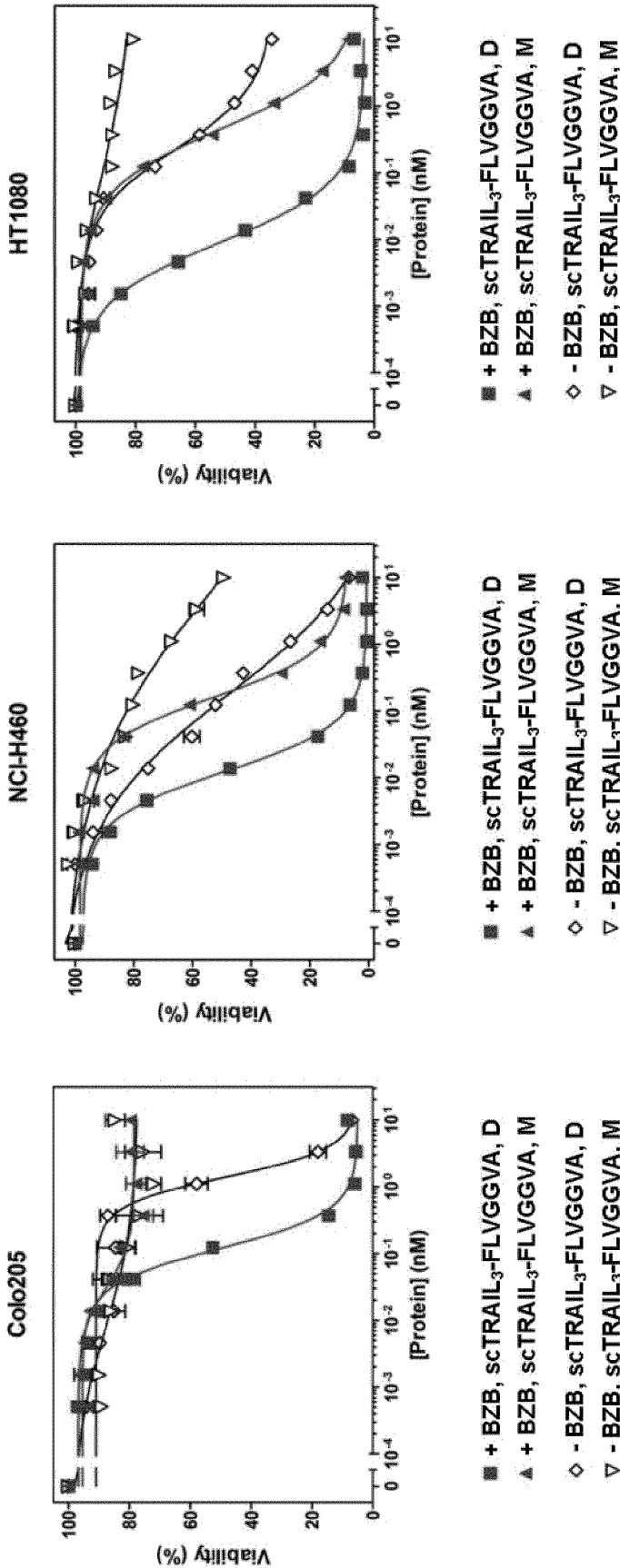


Fig. 8

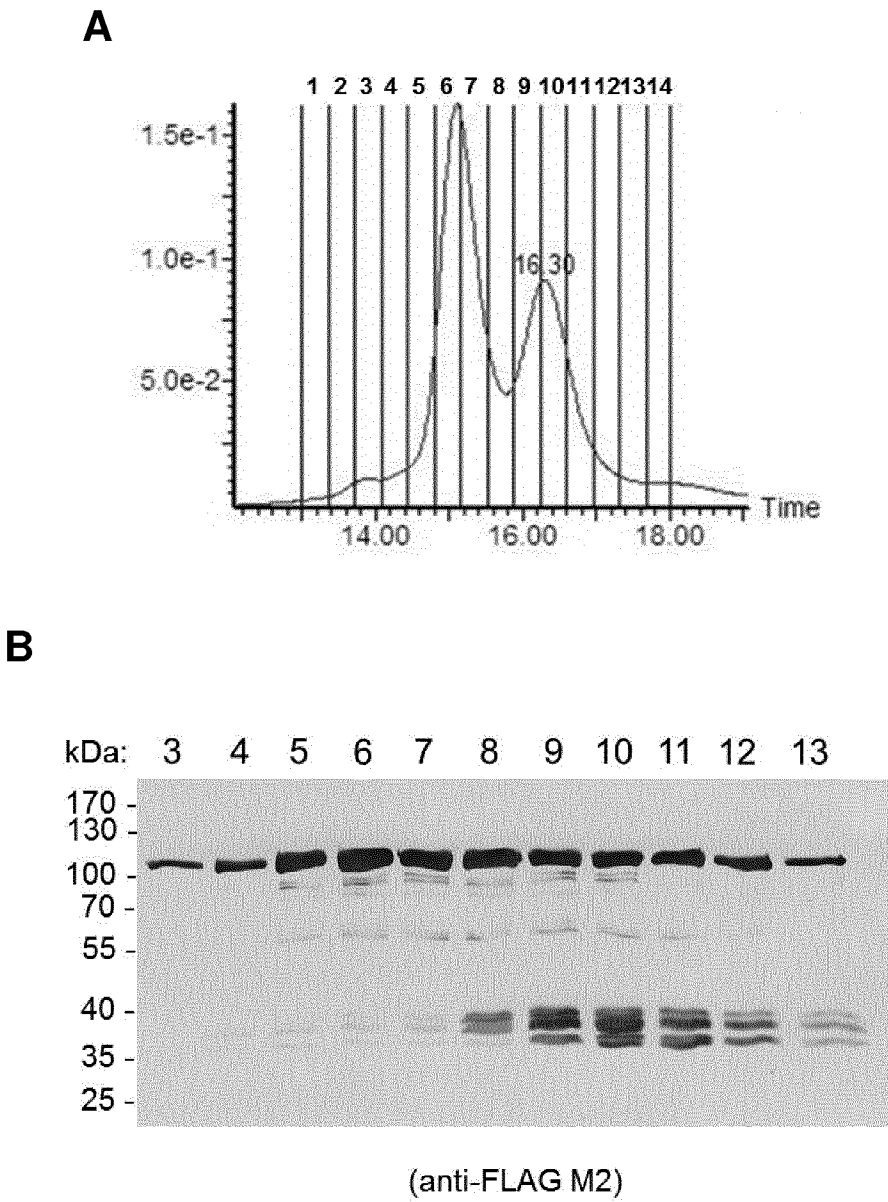
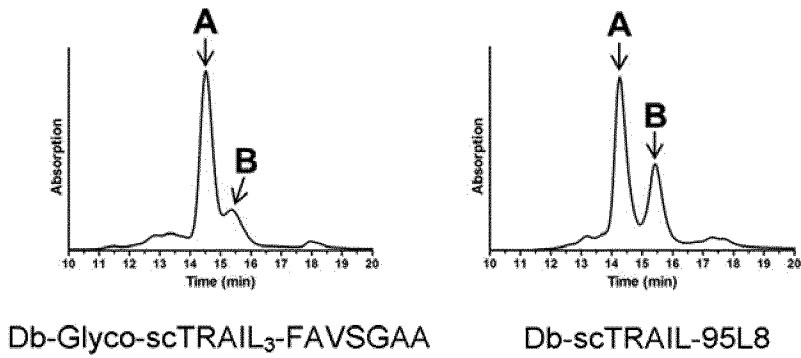


Fig. 9

A



B

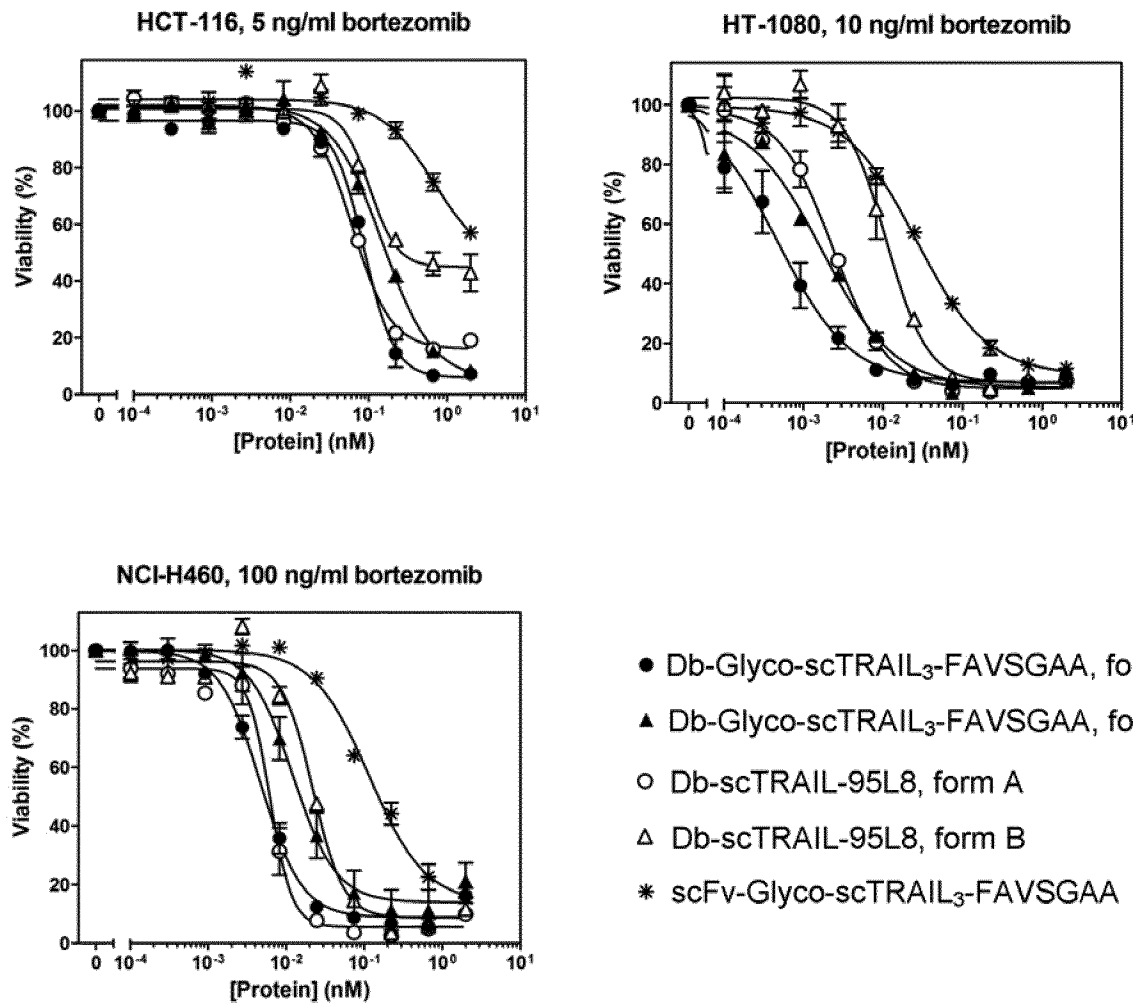


Fig. 10

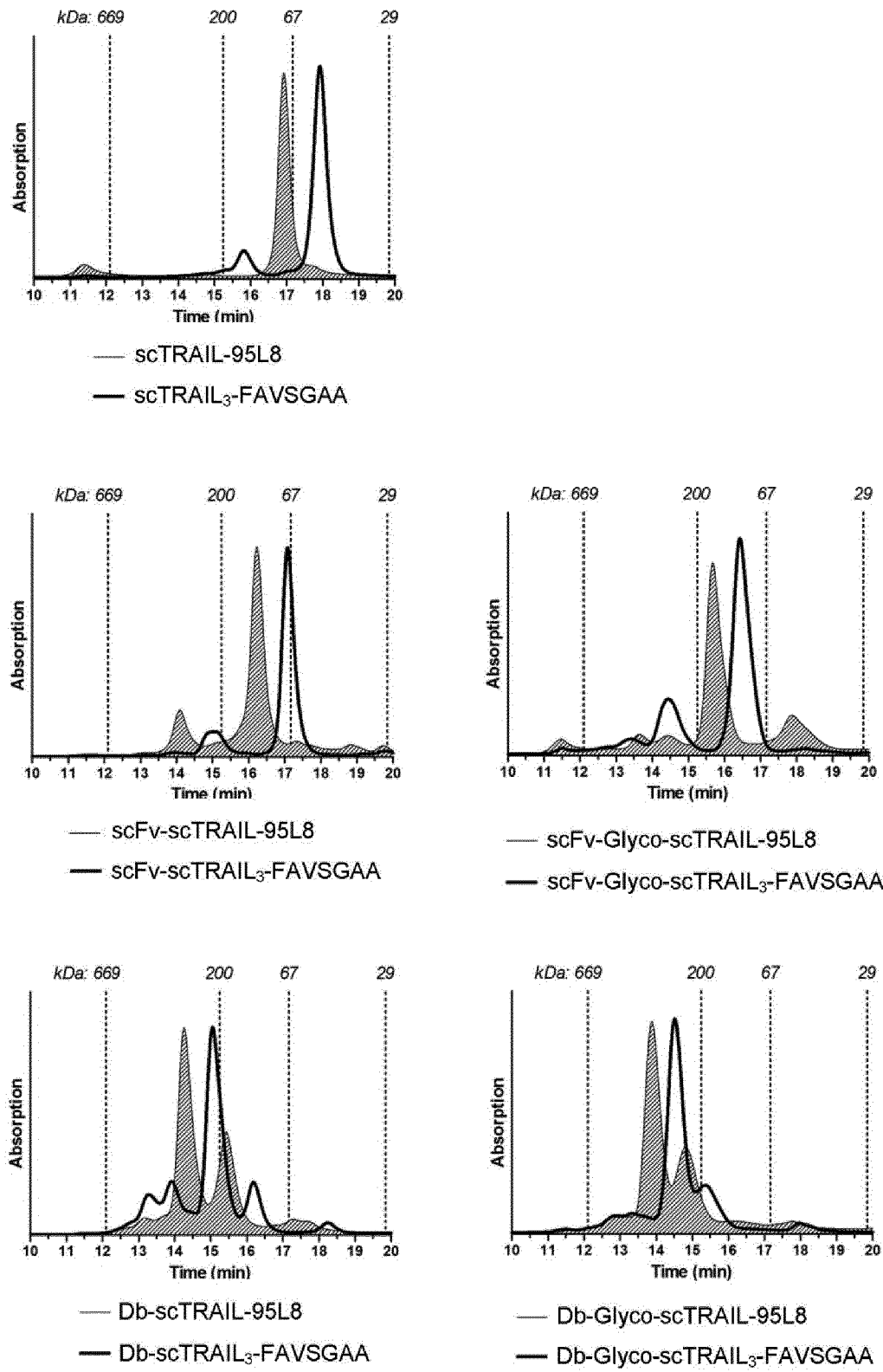


Fig. 11

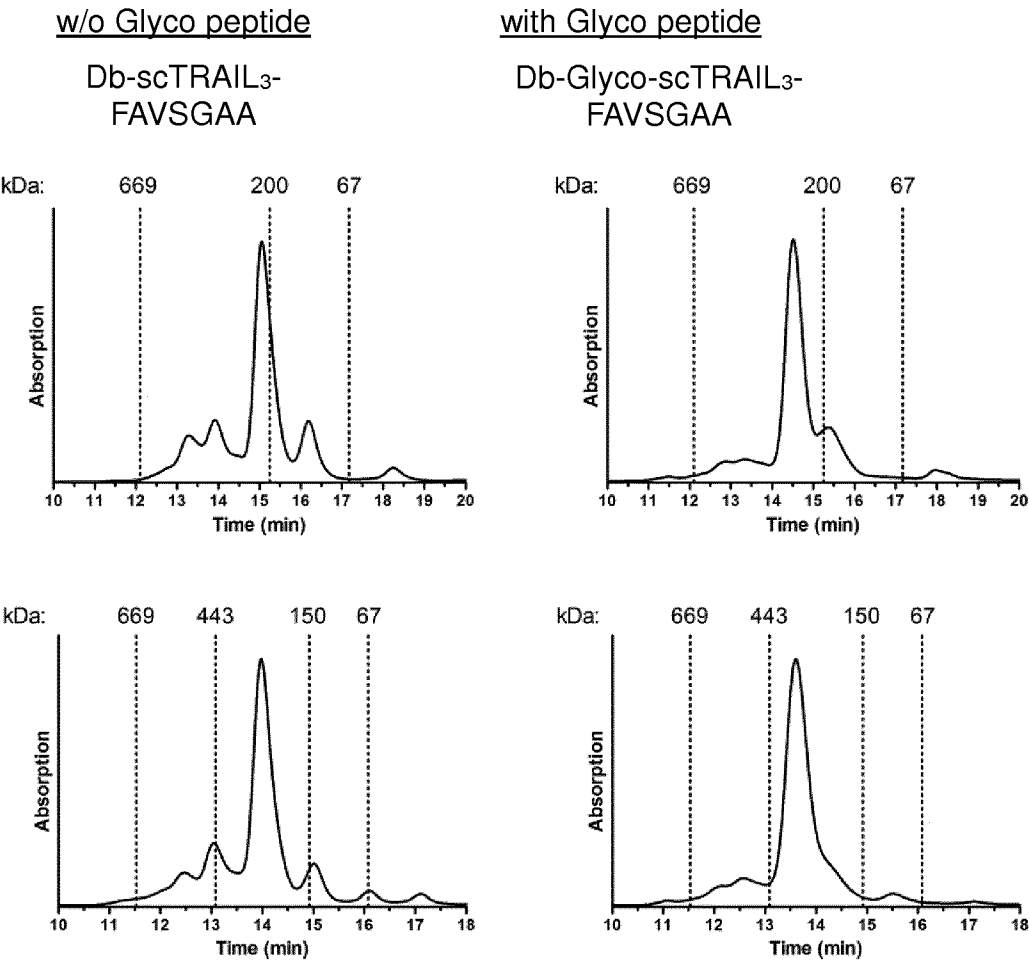
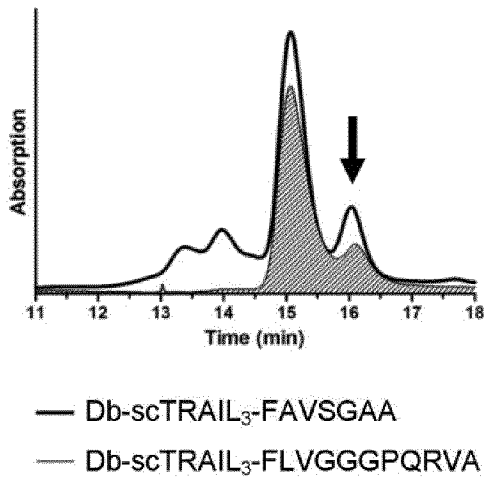
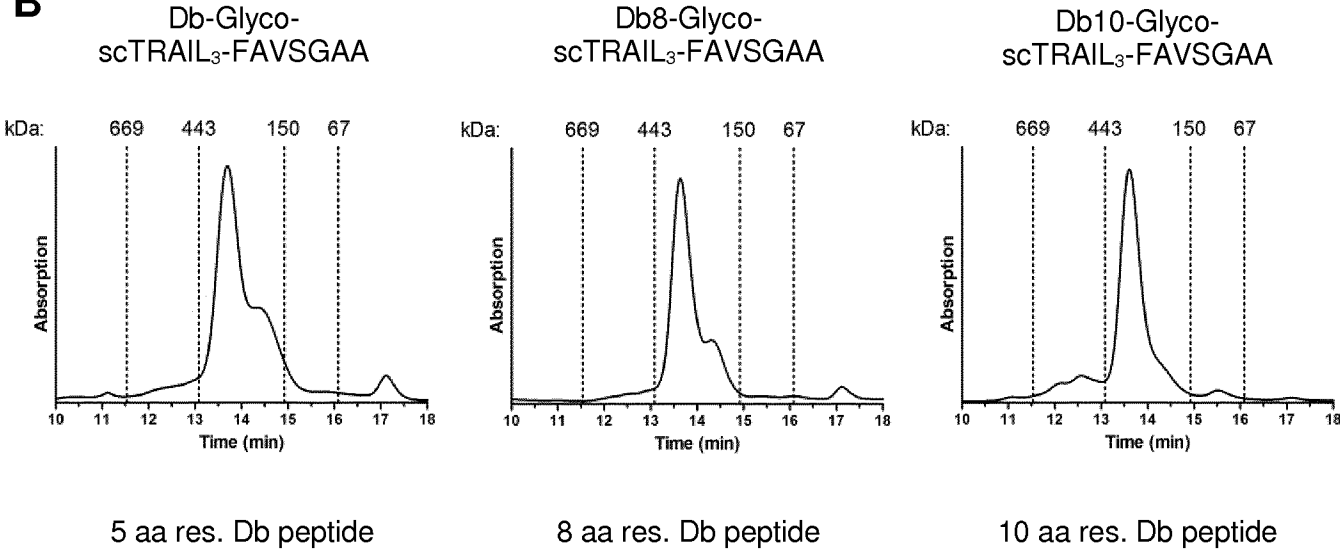


Fig. 12

A



B



C

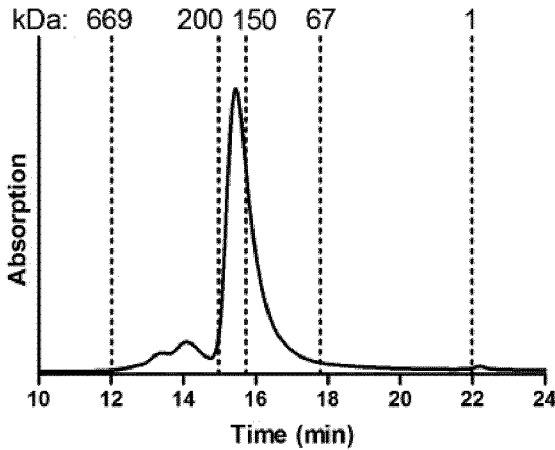
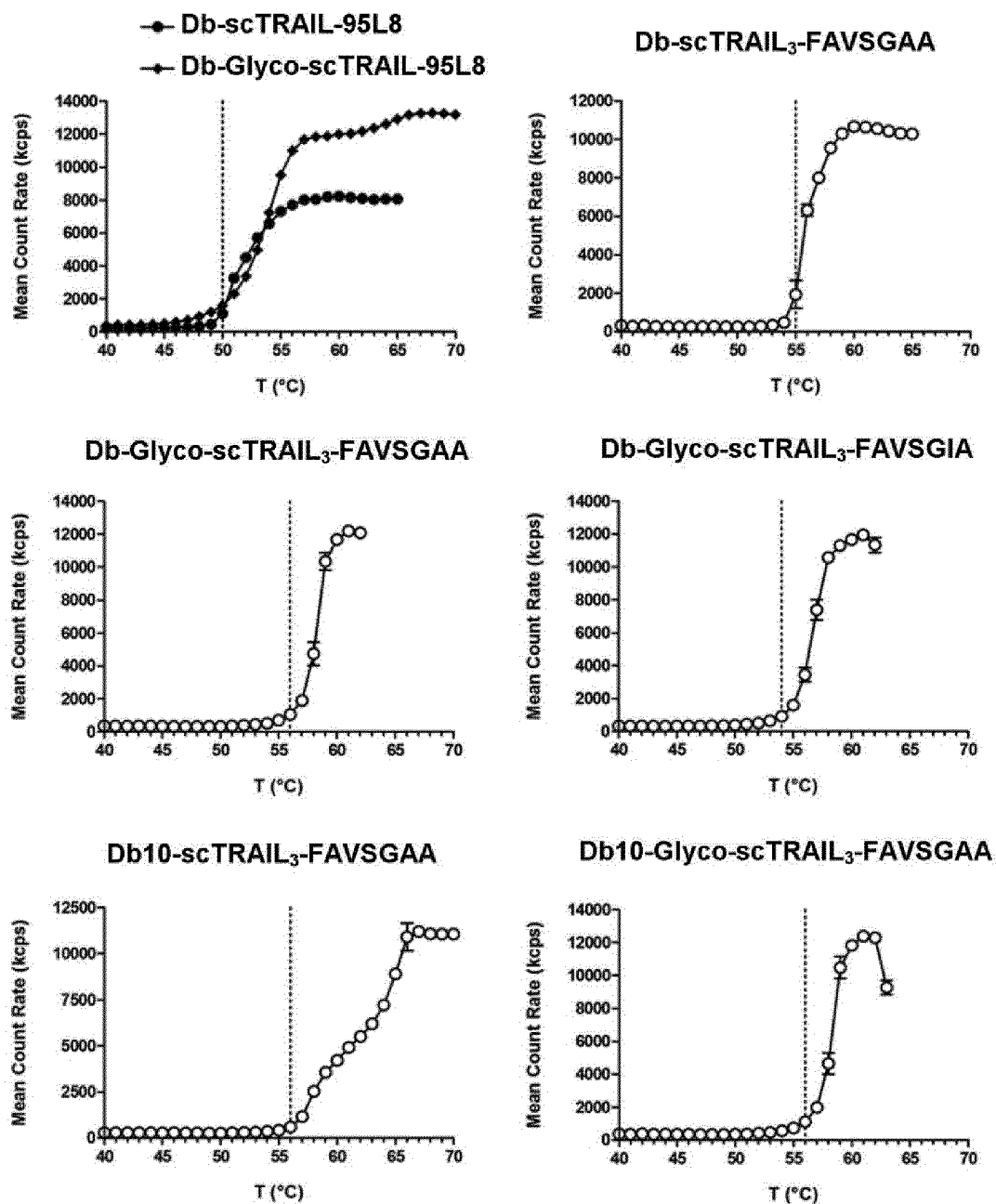
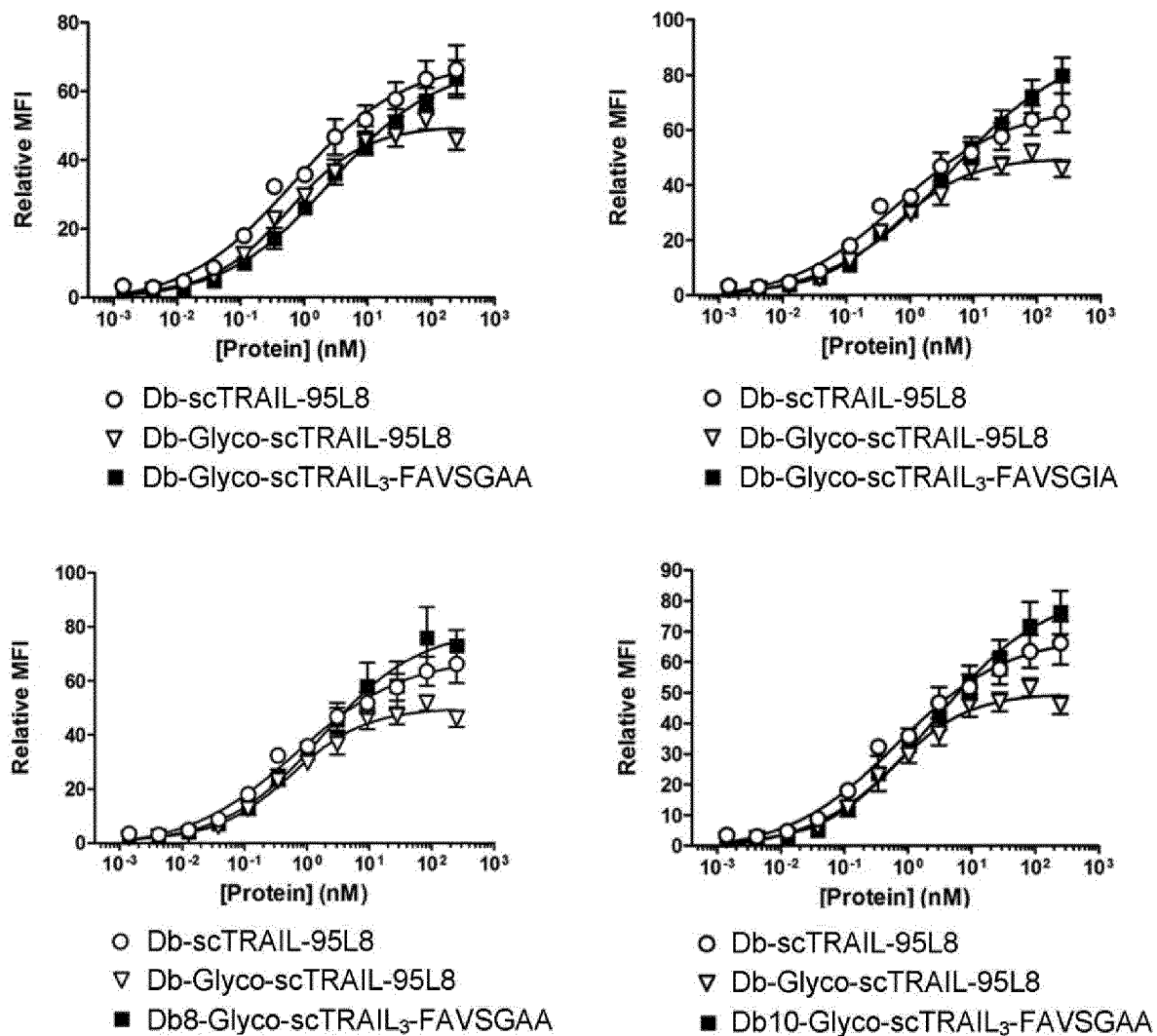


Fig. 13



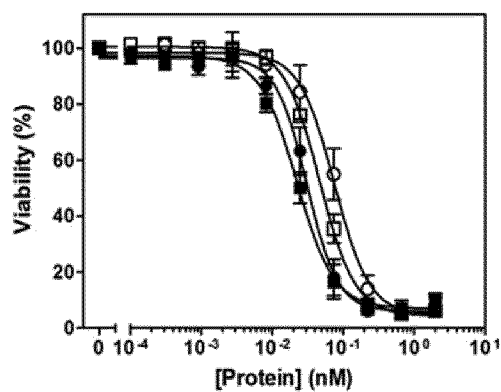
	T _m (°C)
Db-scTRAIL-95L8	50
Db-Glyco-scTRAIL-95L8	50
Db-scTRAIL ₃ -FAVSGAA	55
Db-Glyco-scTRAIL ₃ -FAVSGAA	56
Db-Glyco-scTRAIL ₃ -FAVSGIA	54
Db10-scTRAIL ₃ -FAVSGAA	56
Db10-Glyco-scTRAIL ₃ -FAVSGAA	56

Fig. 14

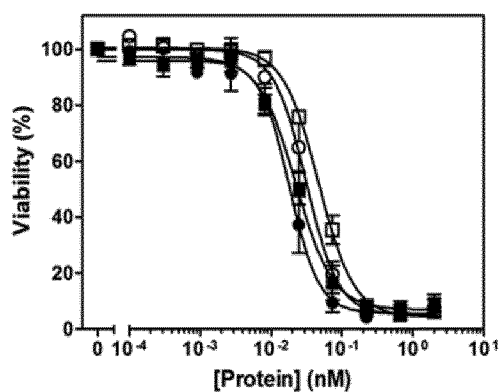


HT1080	EC ₅₀ (nM)
Db-scTRAIL-95L8	0.7 ± 0.3
Db-Glyco-scTRAIL-95L8	0.6 ± 0.1
Db-Glyco-scTRAIL ₃ -FAVSGAA	2.6 ± 0.9
Db-Glyco-scTRAIL ₃ -FAVSGIA	4.3 ± 1.4
Db8-Glyco-scTRAIL ₃ -FAVSGAA	1.9 ± 0.9
Db10-Glyco-scTRAIL ₃ -FAVSGAA	3.0 ± 1.3

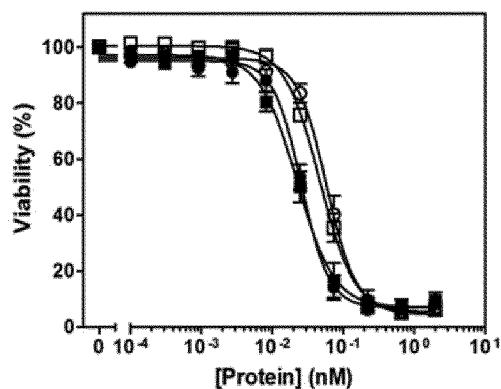
Fig. 15



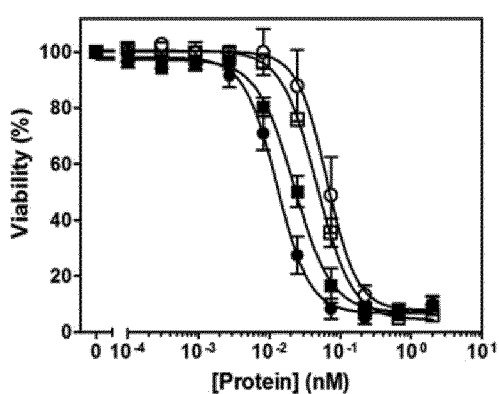
- Db-Glyco-scTRAIL₃-FAVSGAA
- Db-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab



- Db-Glyco-scTRAIL₃-FAVSGIA
- Db-Glyco-scTRAIL₃-FAVSGIA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab

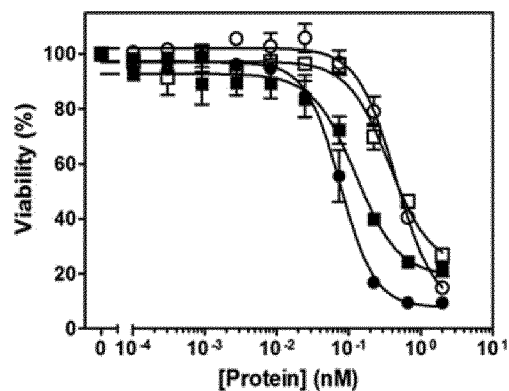


- Db8-Glyco-scTRAIL₃-FAVSGAA
- Db8-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab

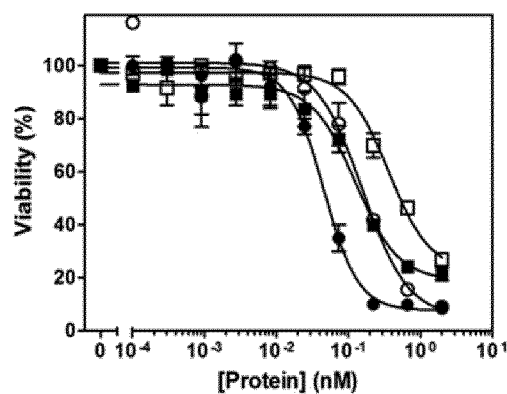


- Db10-Glyco-scTRAIL₃-FAVSGAA
- Db10-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab

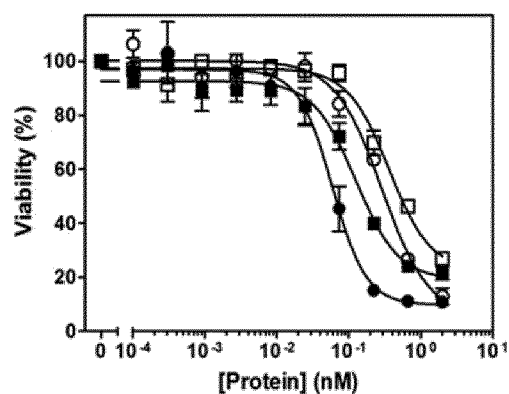
Fig. 16



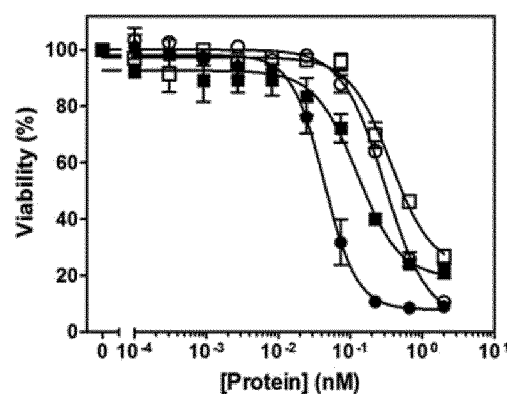
- Db-Glyco-scTRAIL₃-FAVSGAA
- Db-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab



- Db-Glyco-scTRAIL₃-FAVSGIA
- Db-Glyco-scTRAIL₃-FAVSGIA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab

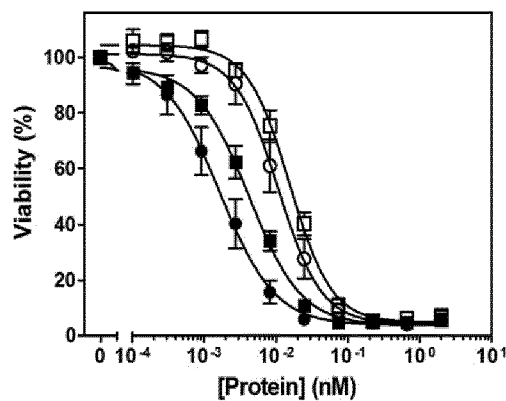


- Db8-Glyco-scTRAIL₃-FAVSGAA
- Db8-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab

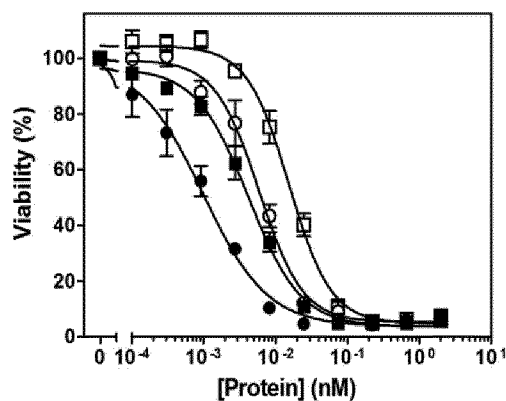


- Db10-Glyco-scTRAIL₃-FAVSGAA
- Db10-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab

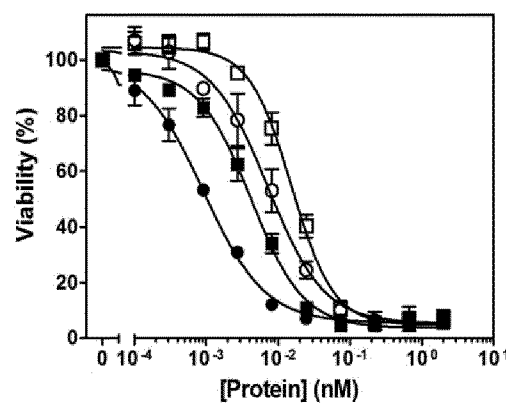
Fig. 17



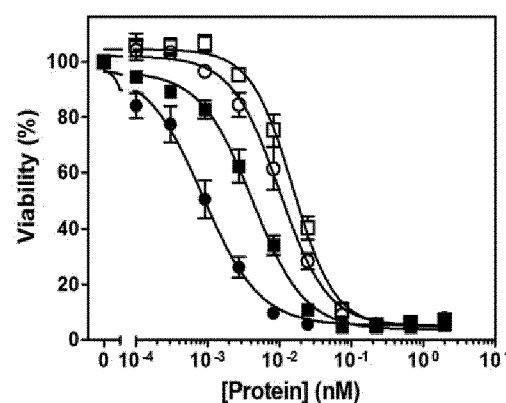
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- Db-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab



- Db-Glyco-scTRAIL₃-FAVSGIA
- Db-Glyco-scTRAIL₃-FAVSGIA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab



- Db8-Glyco-scTRAIL₃-FAVSGAA
- Db8-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab



- Db10-Glyco-scTRAIL₃-FAVSGAA
- Db10-Glyco-scTRAIL₃-FAVSGAA + Cet.
- Db-scTRAIL-95L8
- Db-scTRAIL-95L8 + Cetuximab

Fig. 18

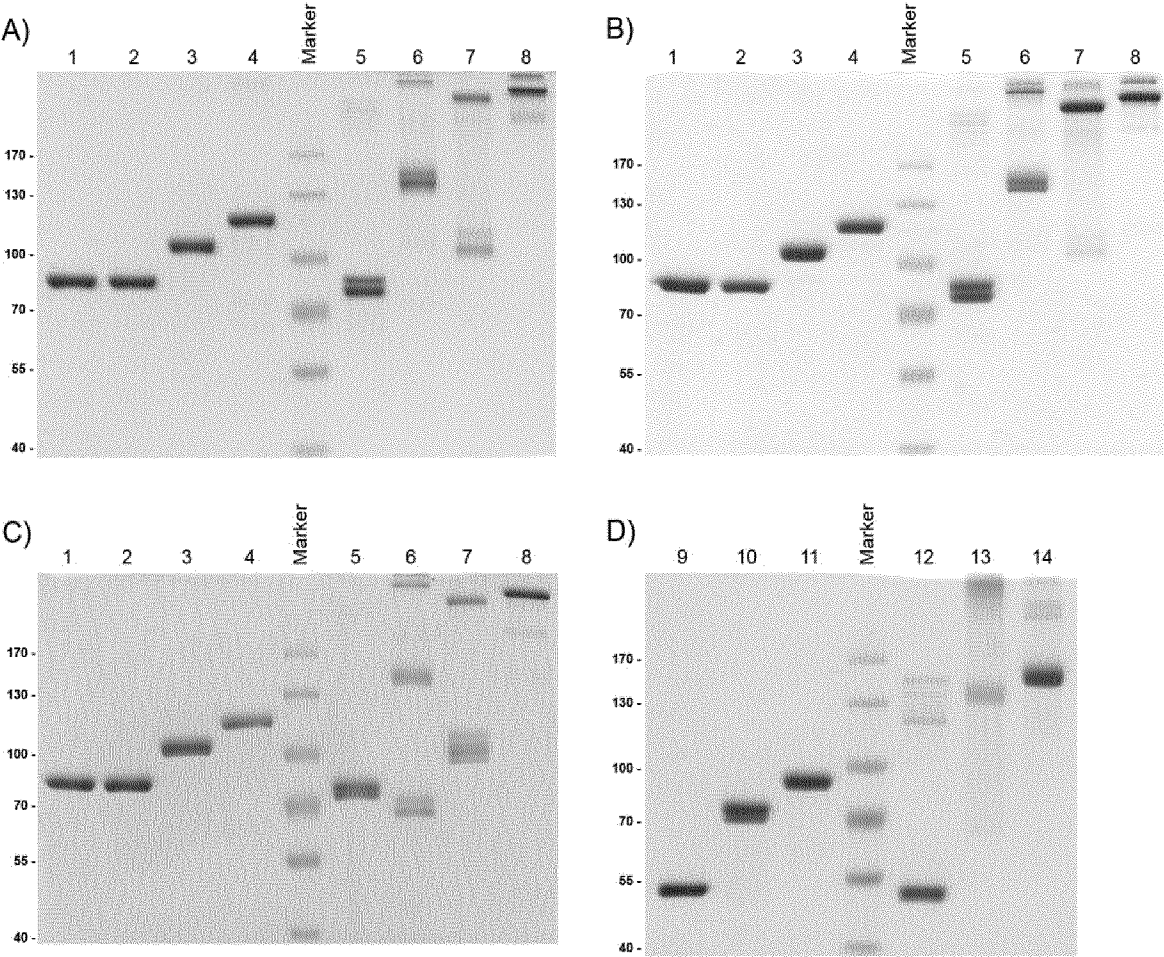


Fig. 19

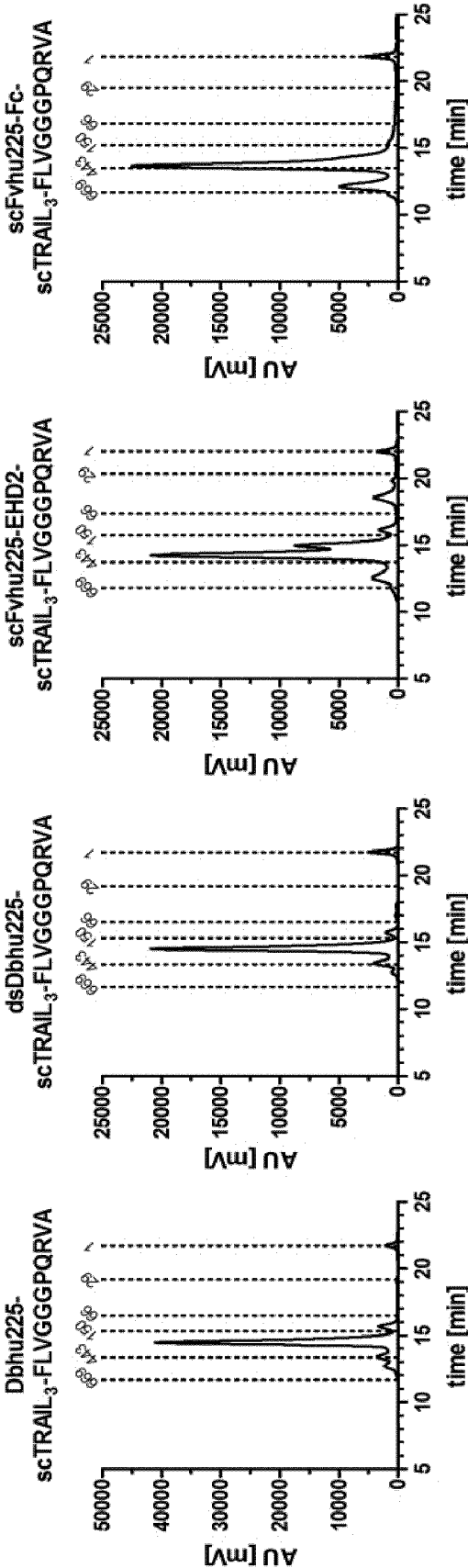


Fig. 20

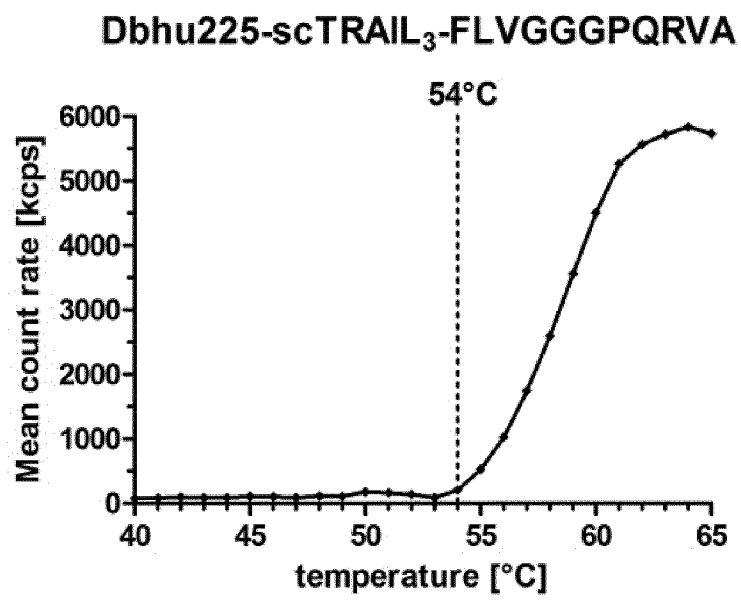


Fig. 21

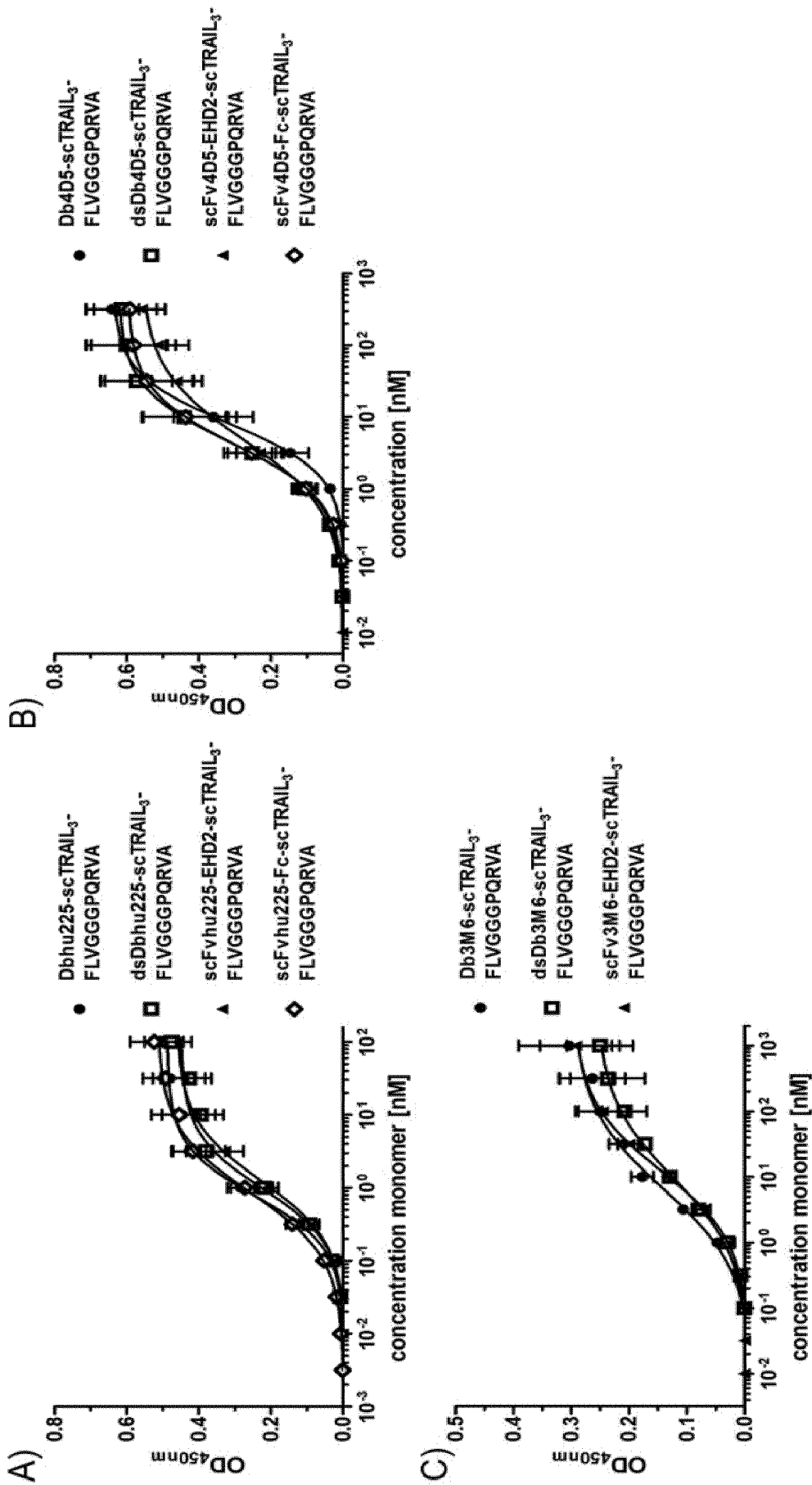


Fig. 22

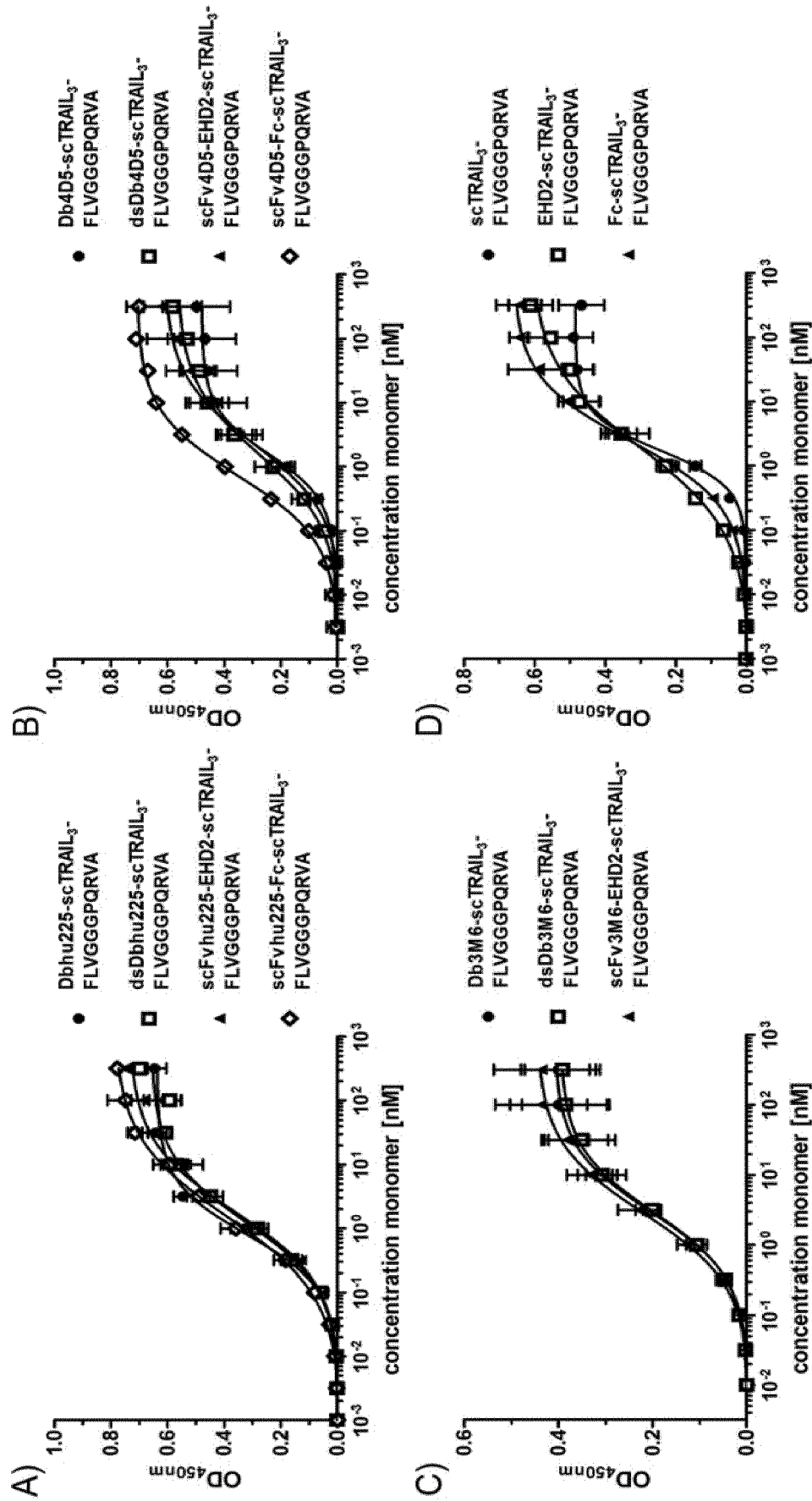


Fig. 23

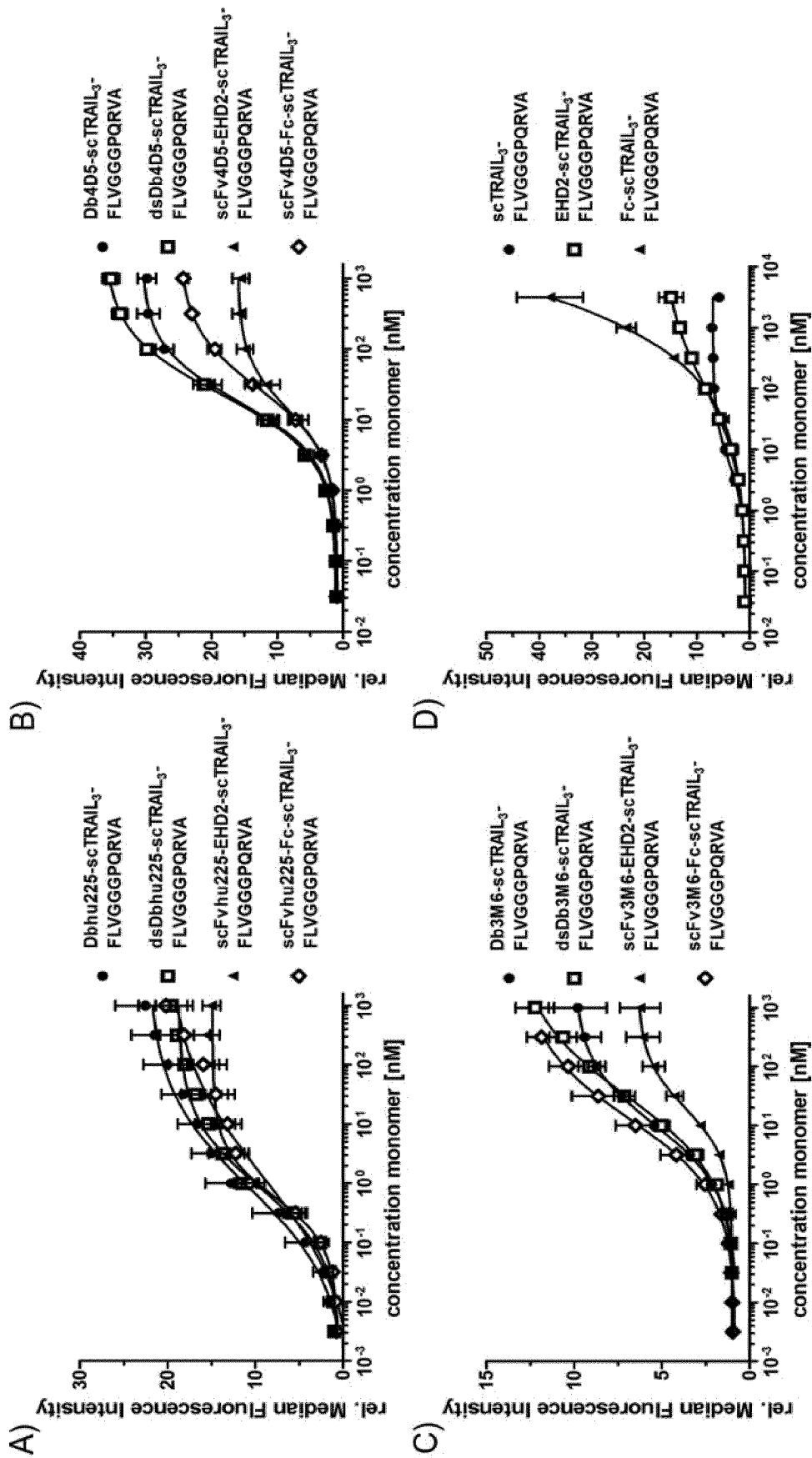
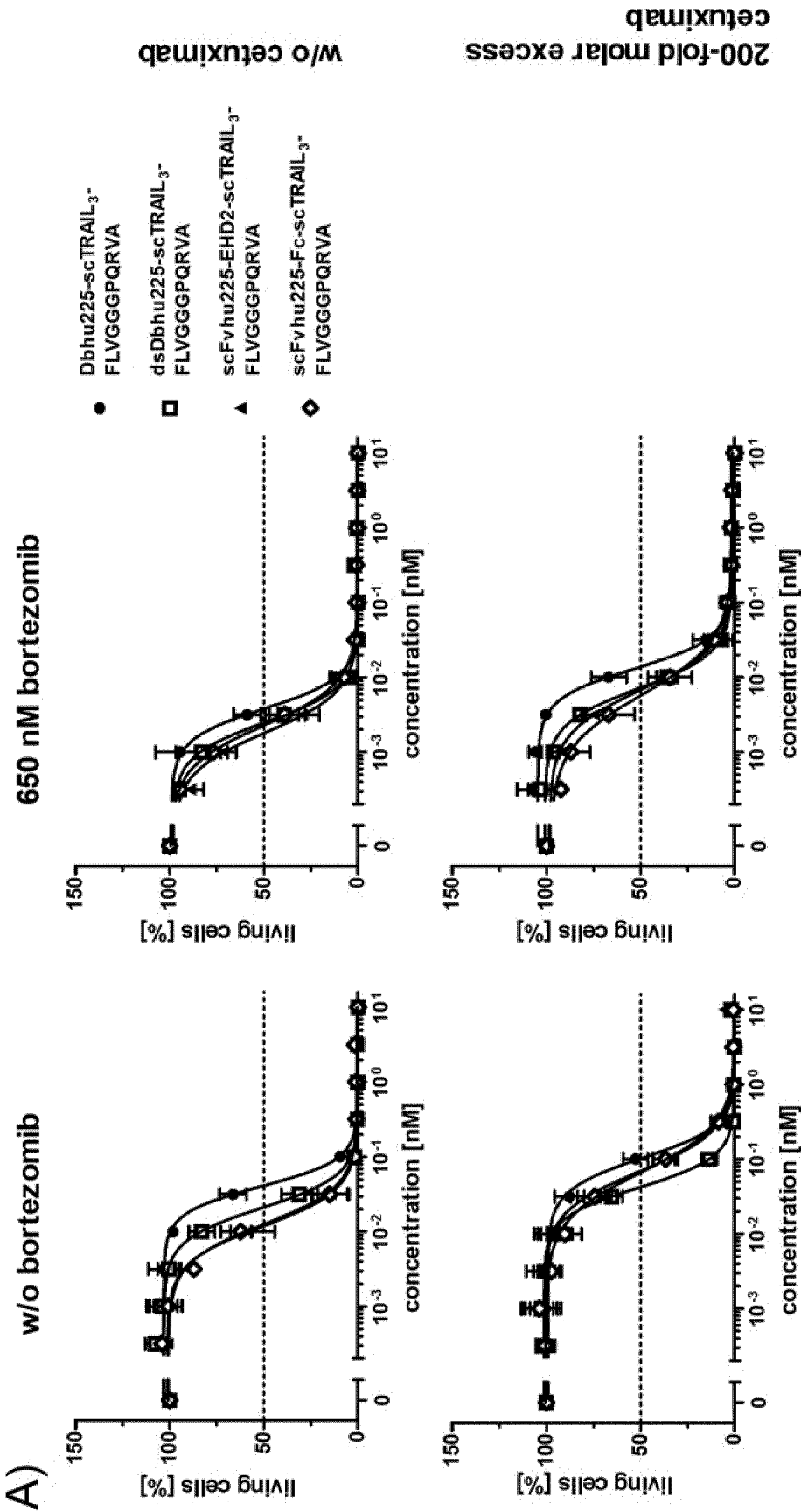


Fig. 24



cont.

Fig. 24

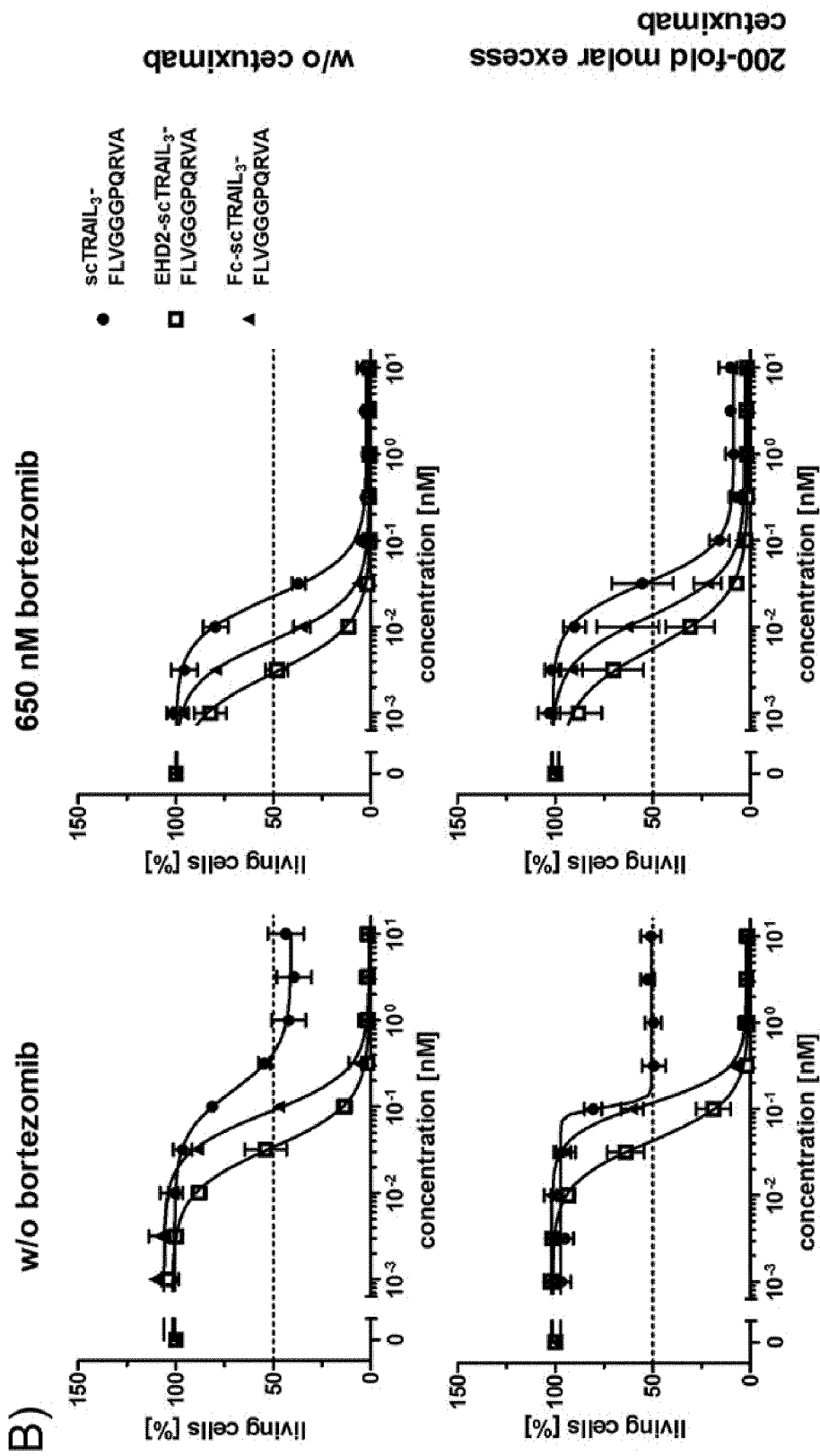


Fig. 25

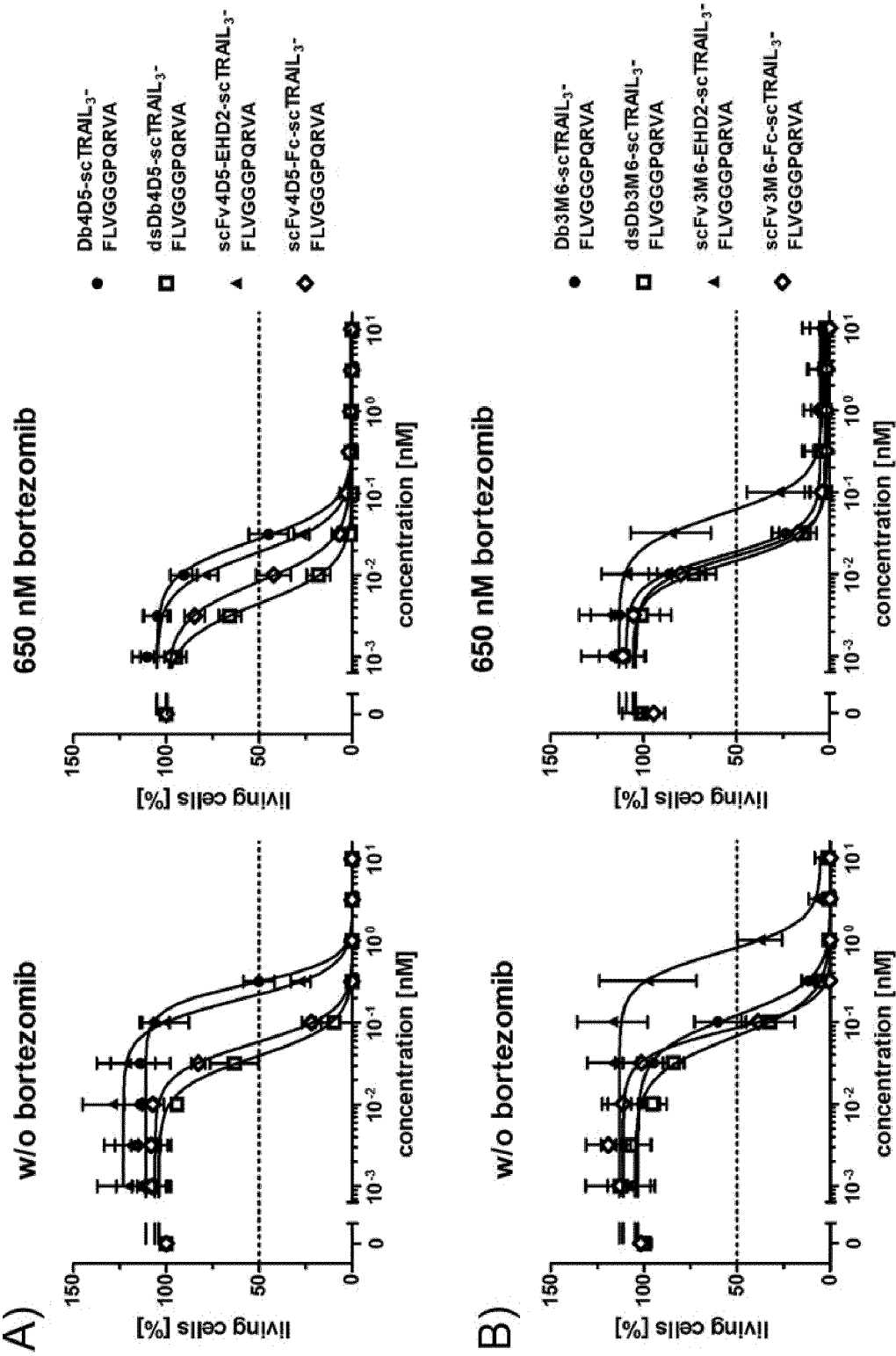
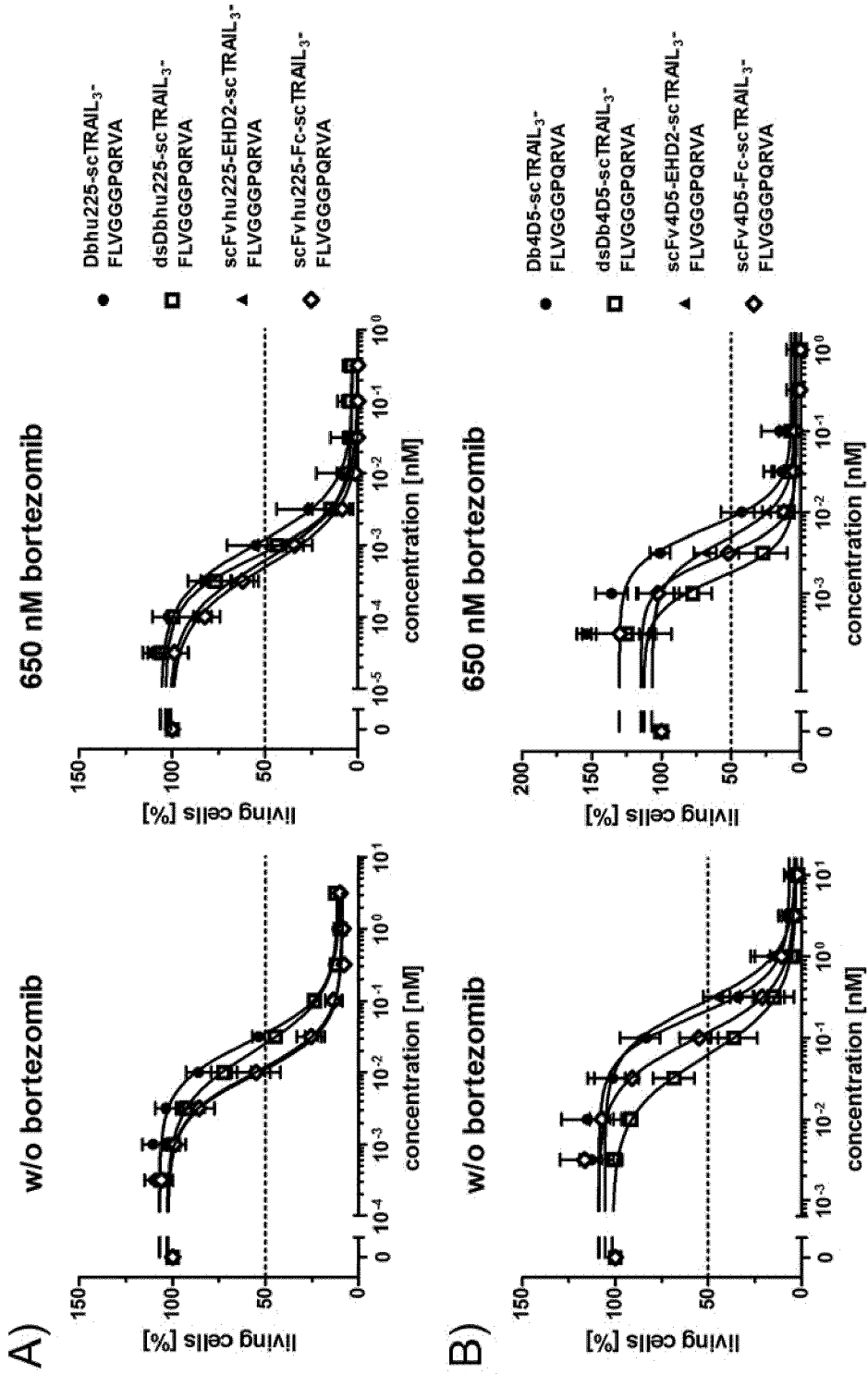


Fig. 26



cont.

Fig. 26

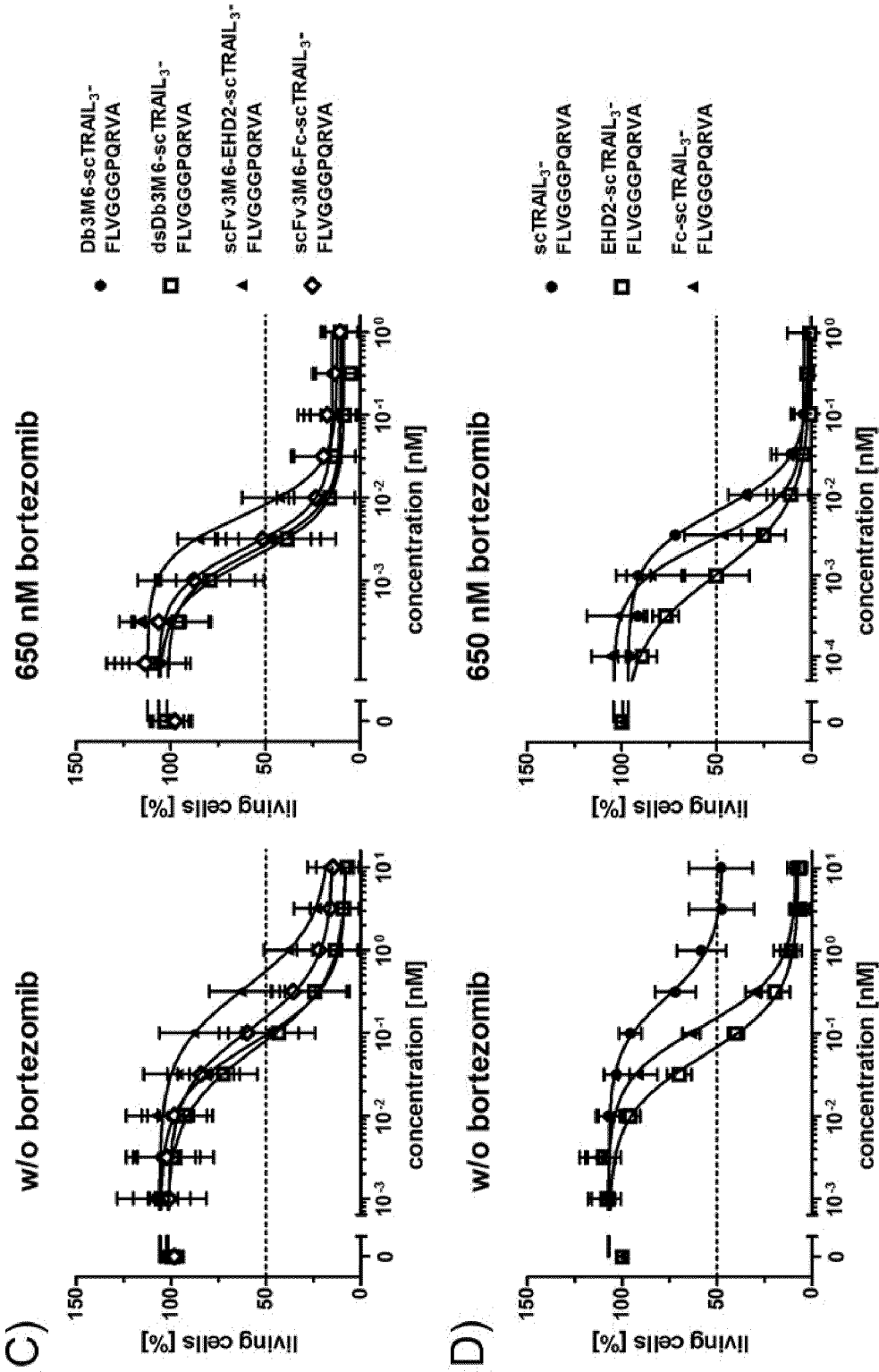


Fig. 27

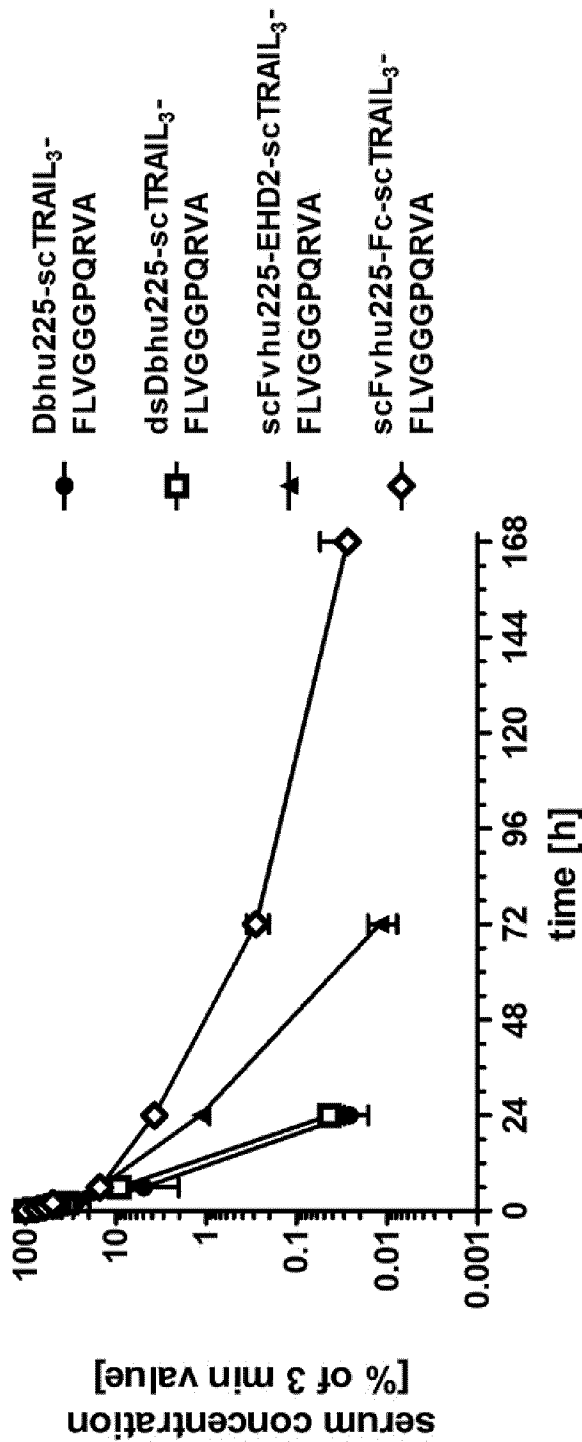


Fig. 28

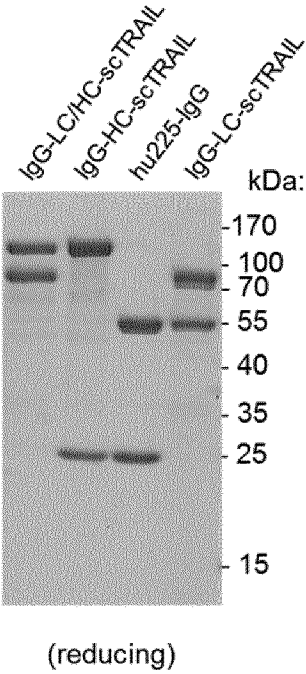


Fig. 29

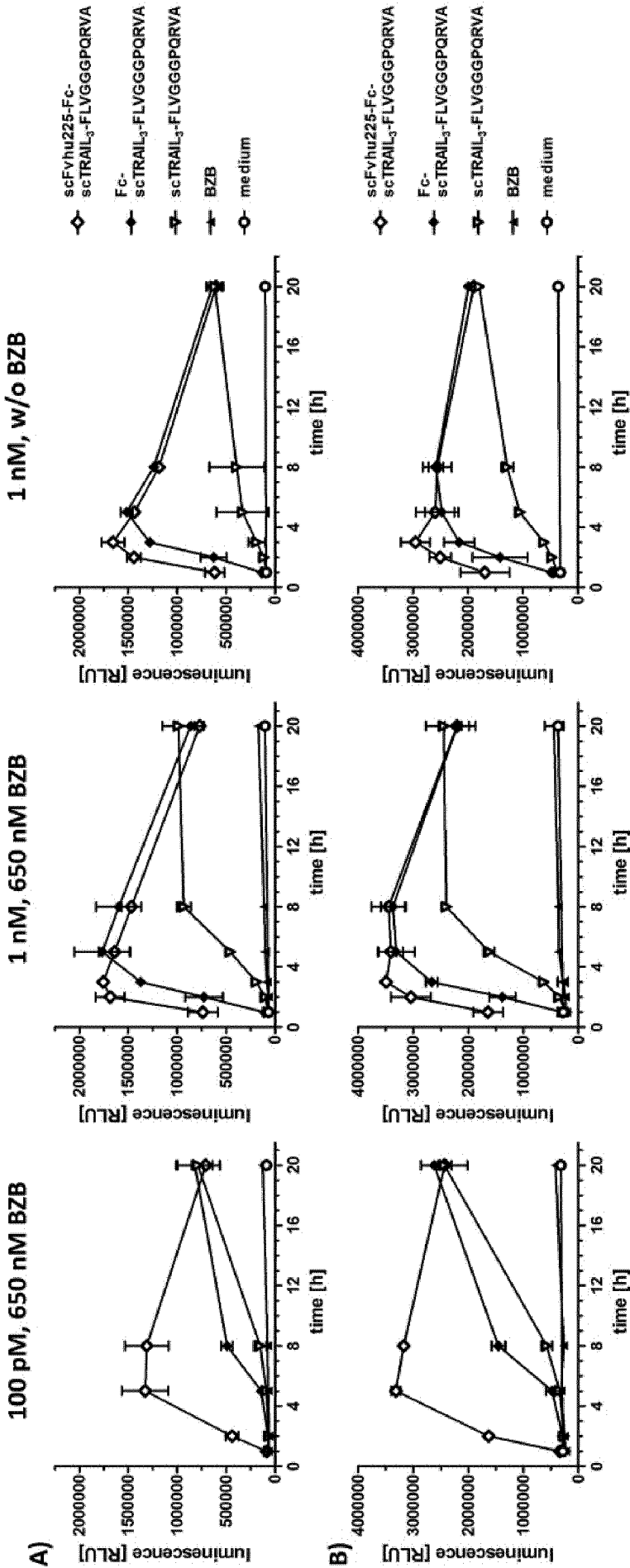


Fig. 30

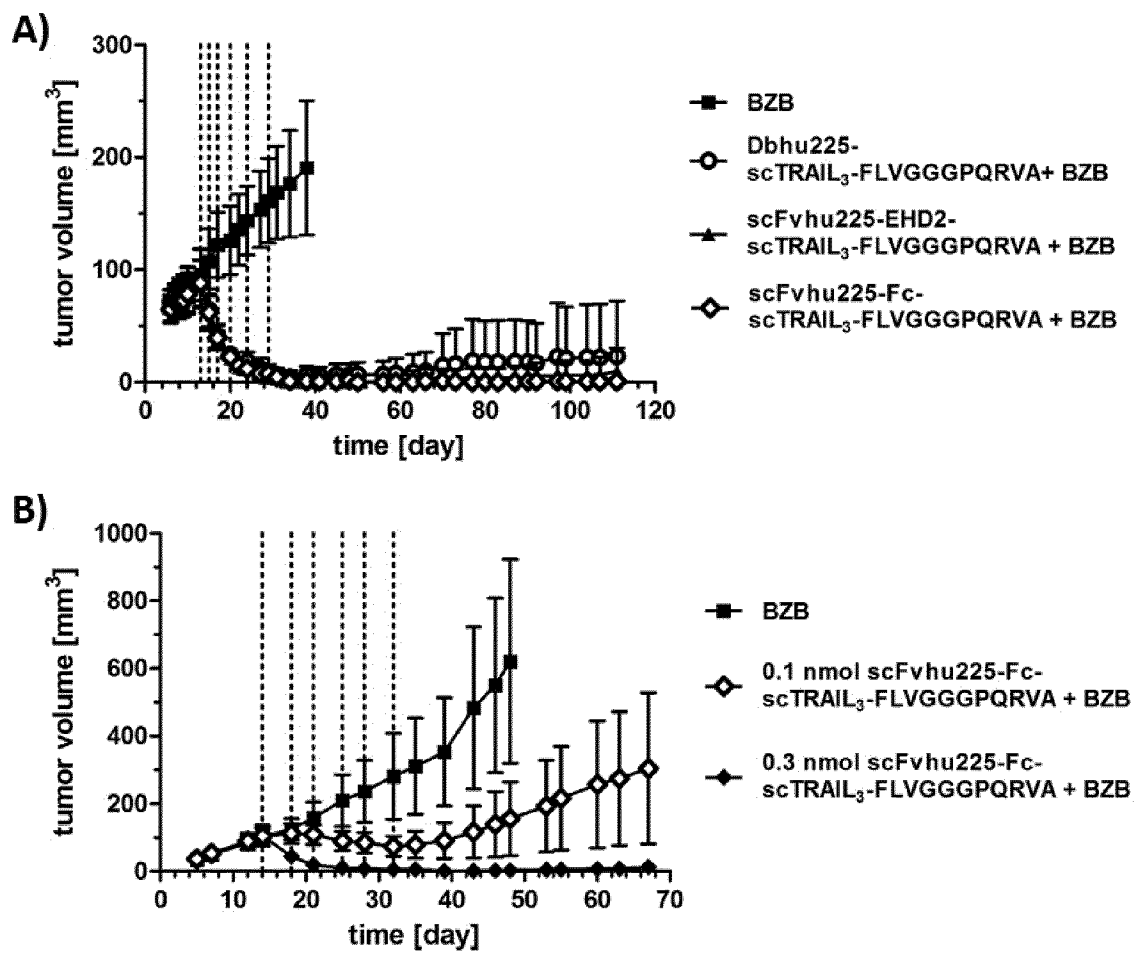
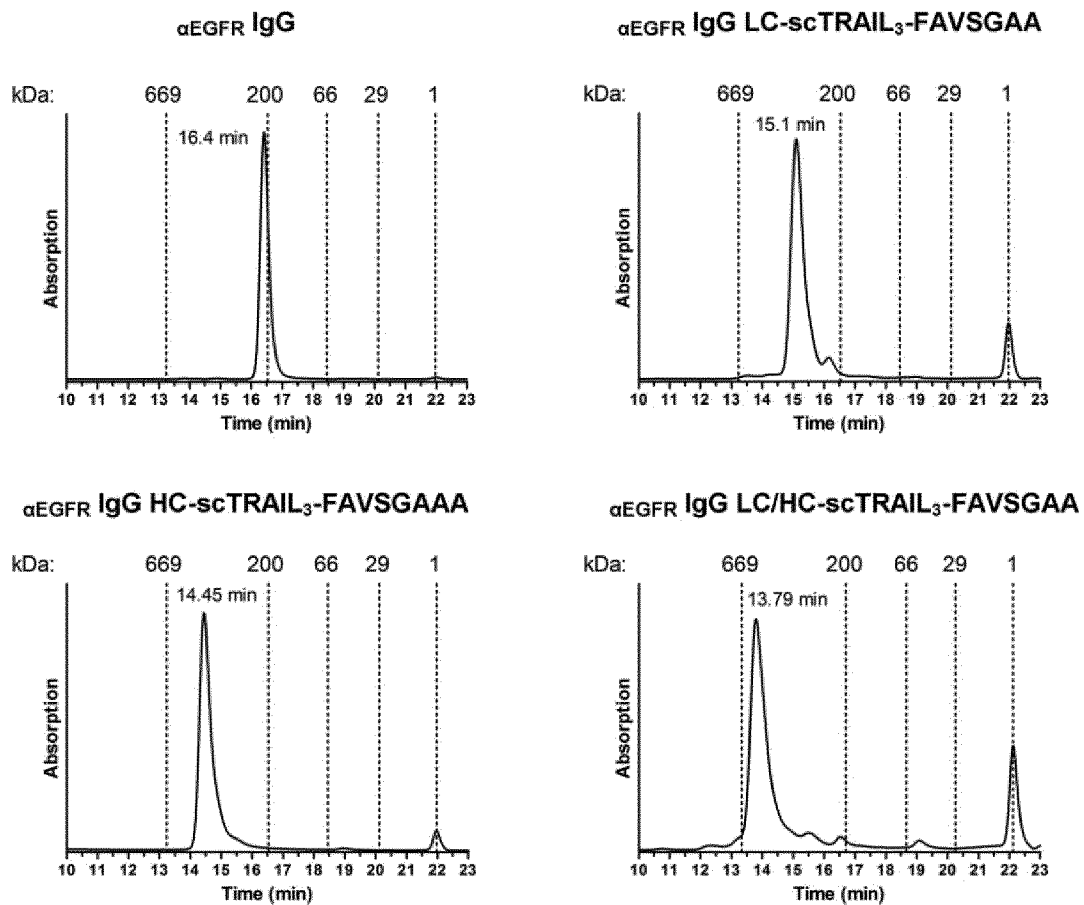


Fig. 31

A



B

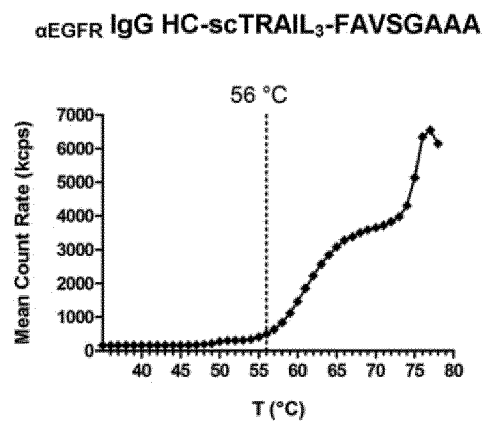


Fig. 32

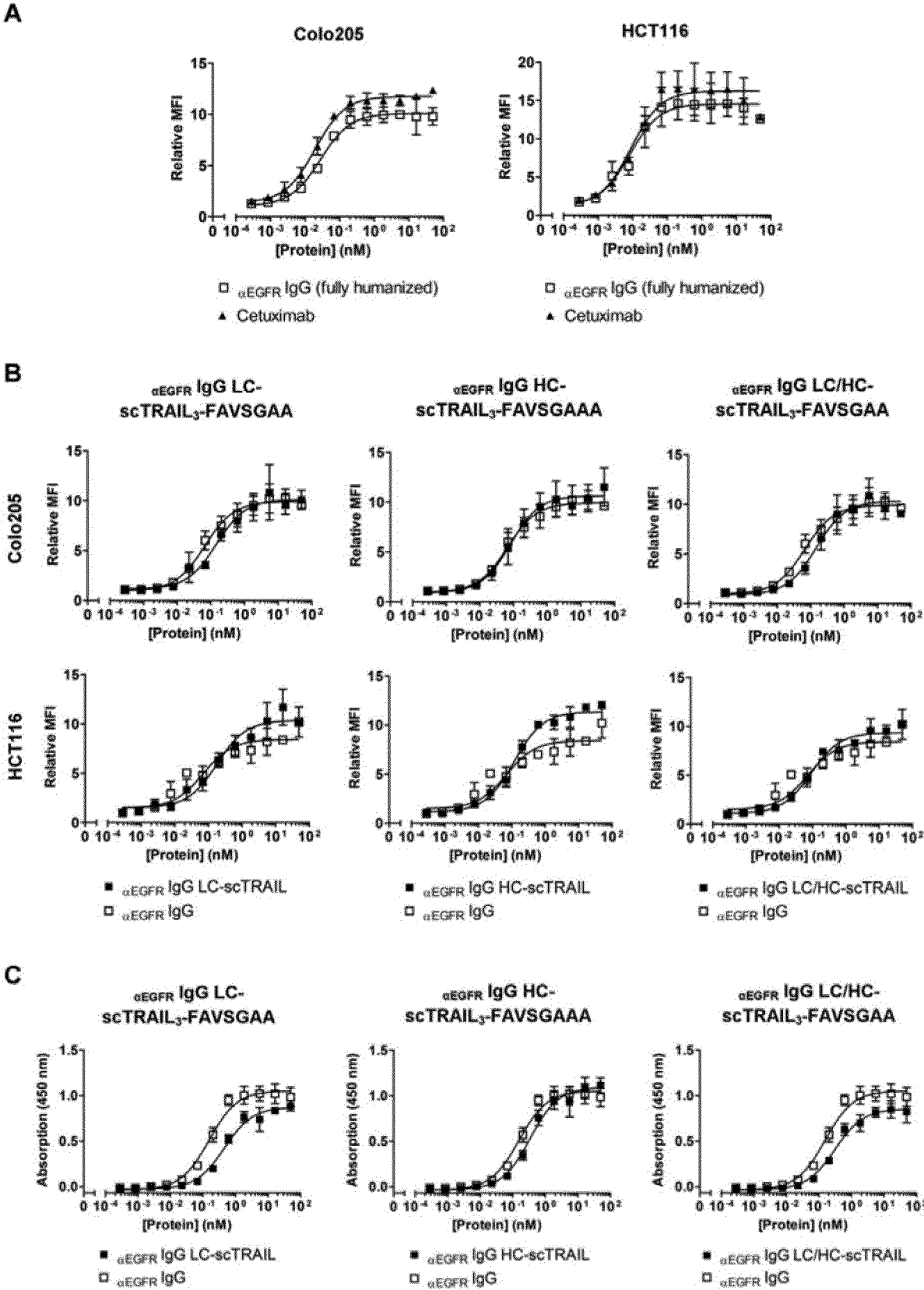


Fig. 33

HCT116

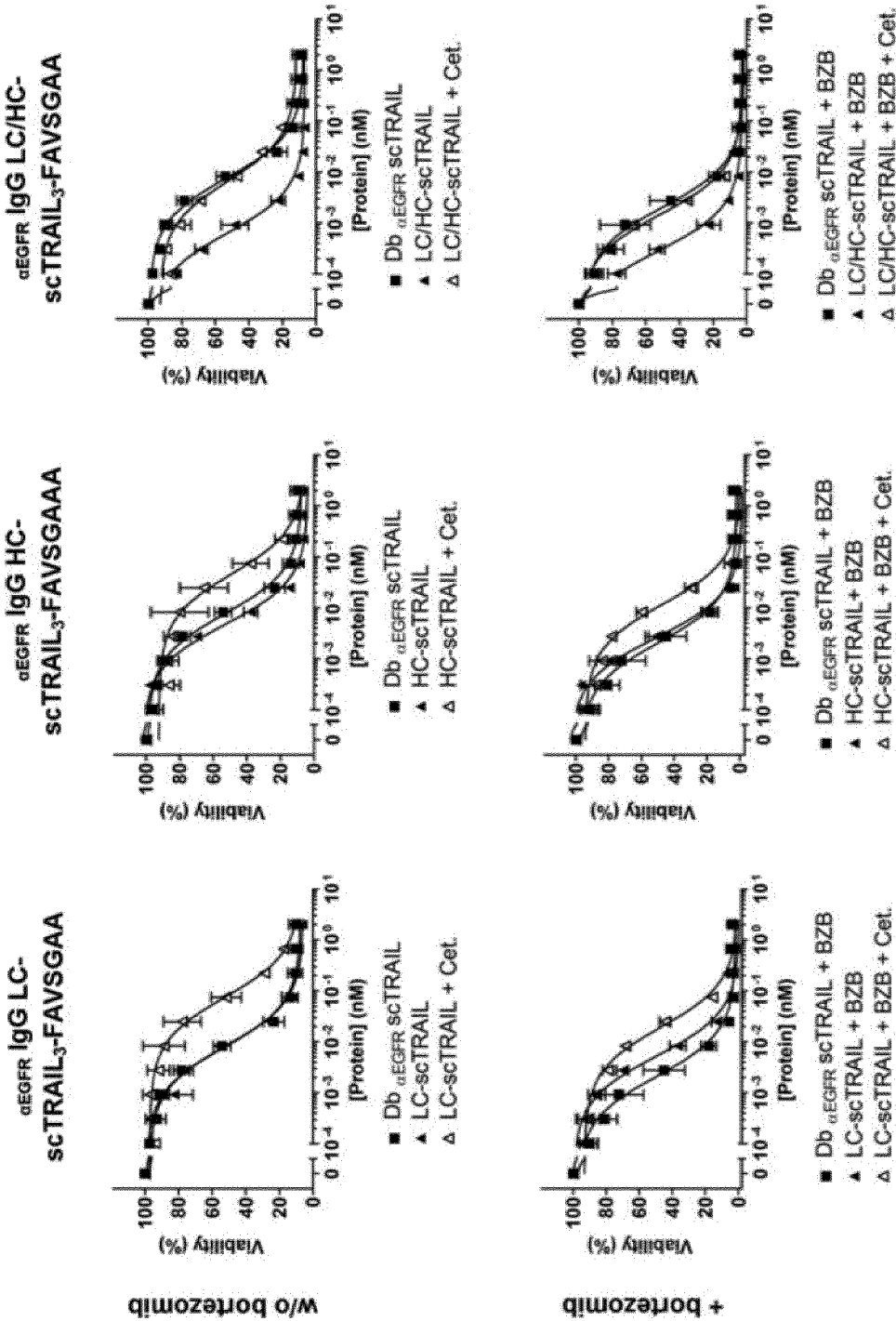


Fig. 34

Colo205

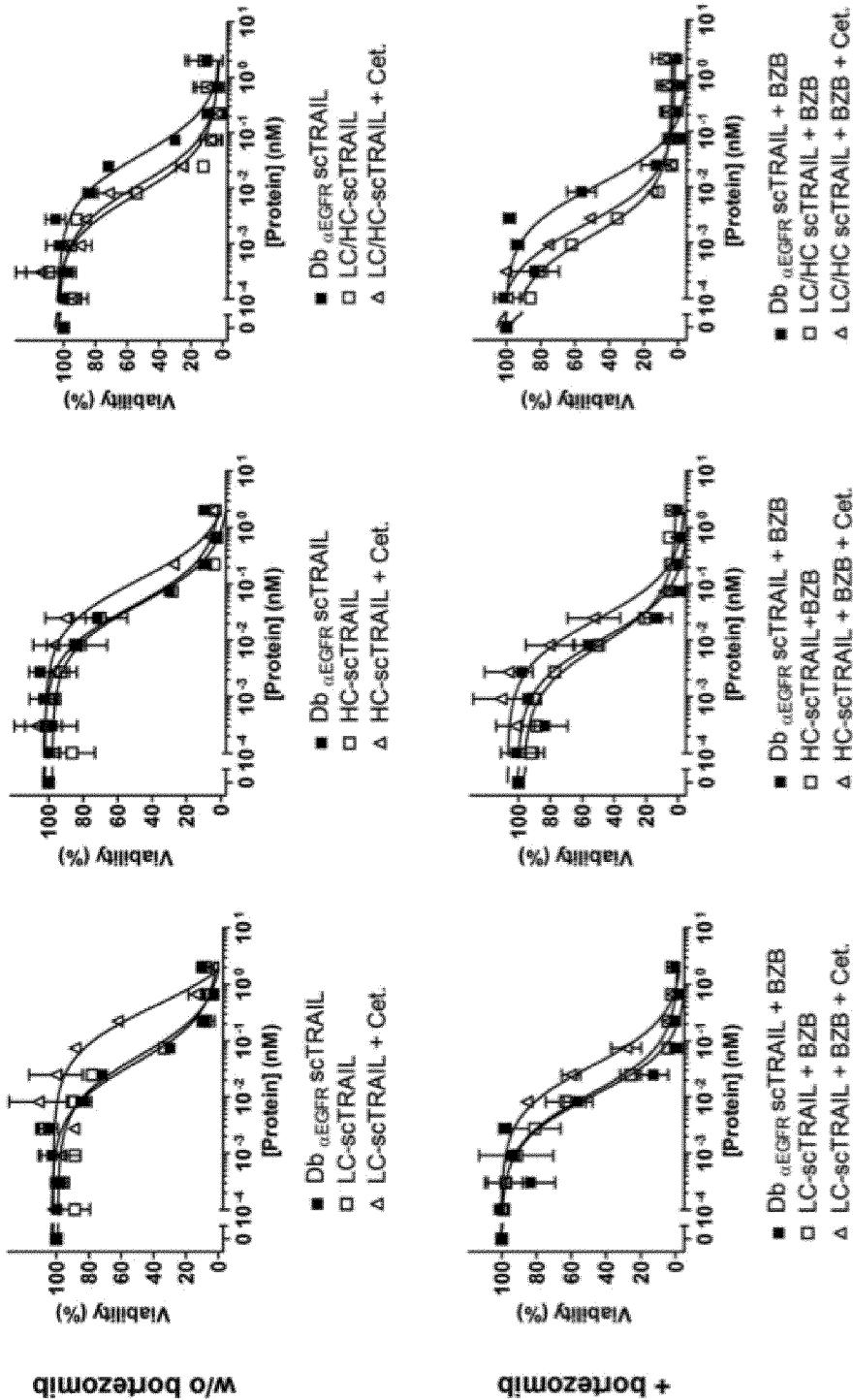


Fig. 35

HT1080

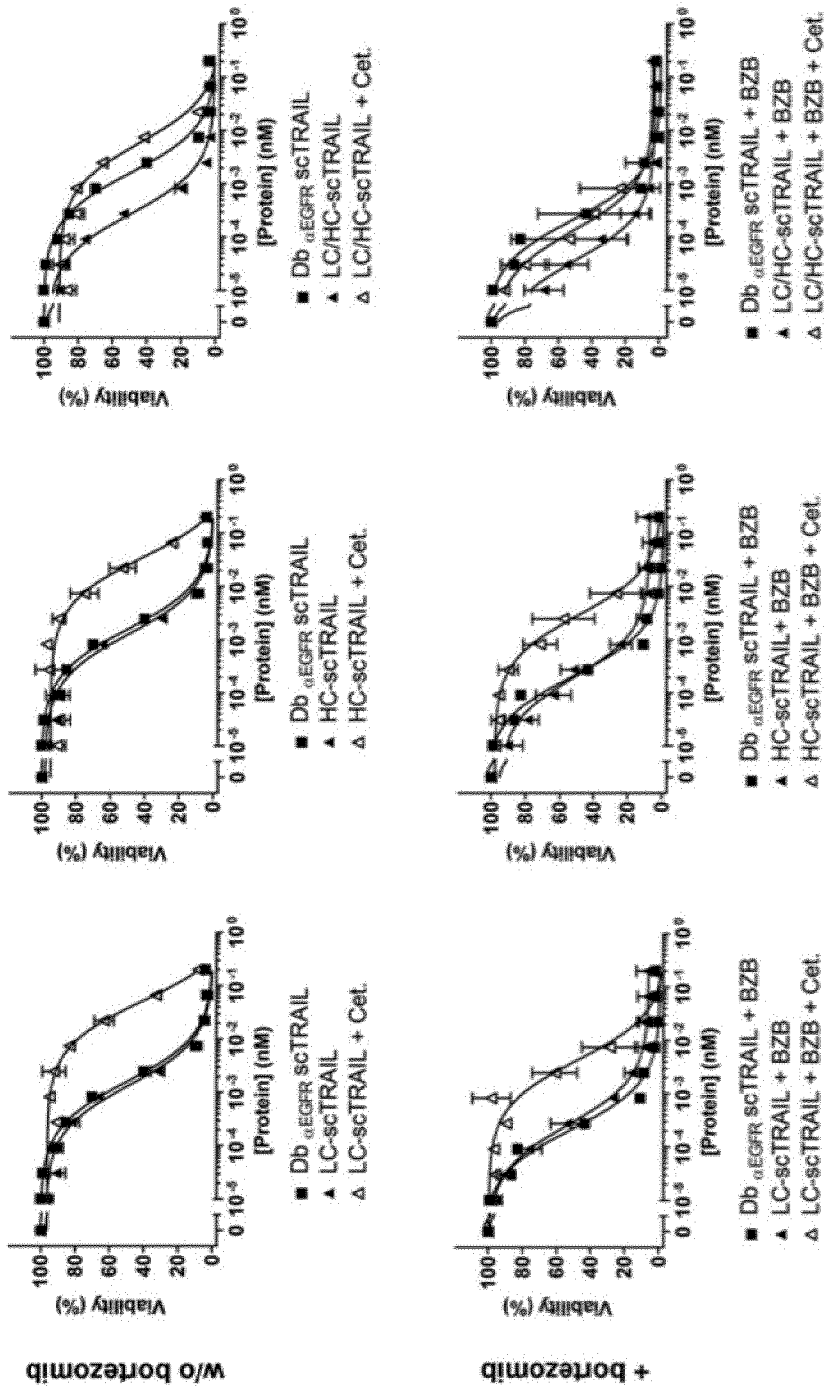


Fig. 36

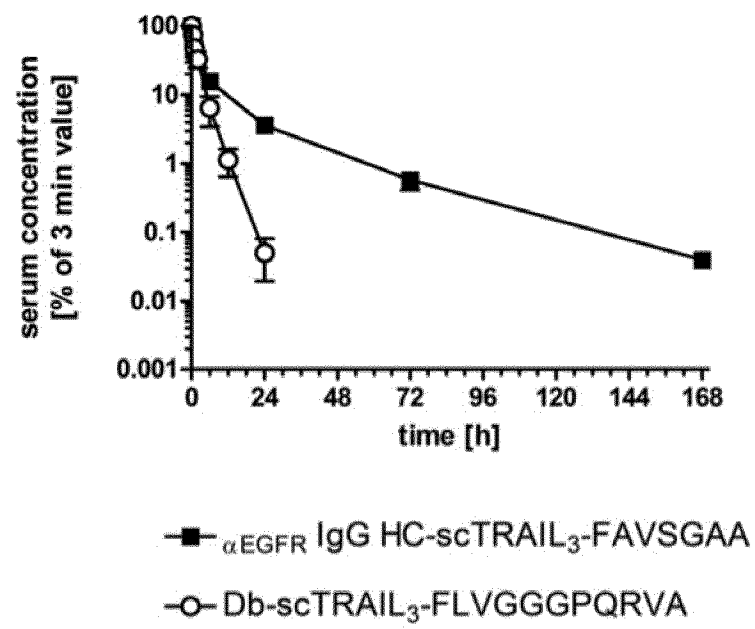


Fig. 37

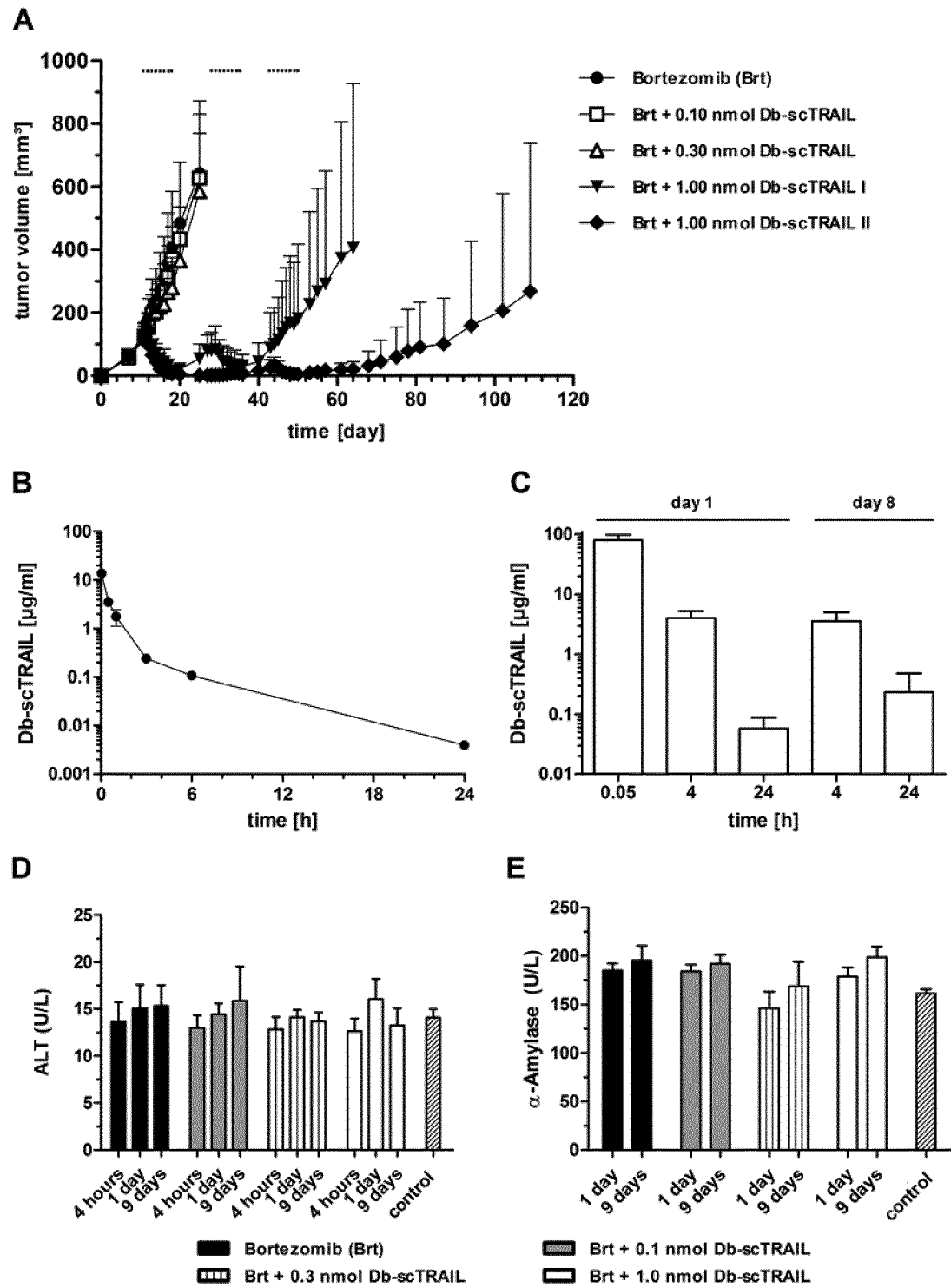


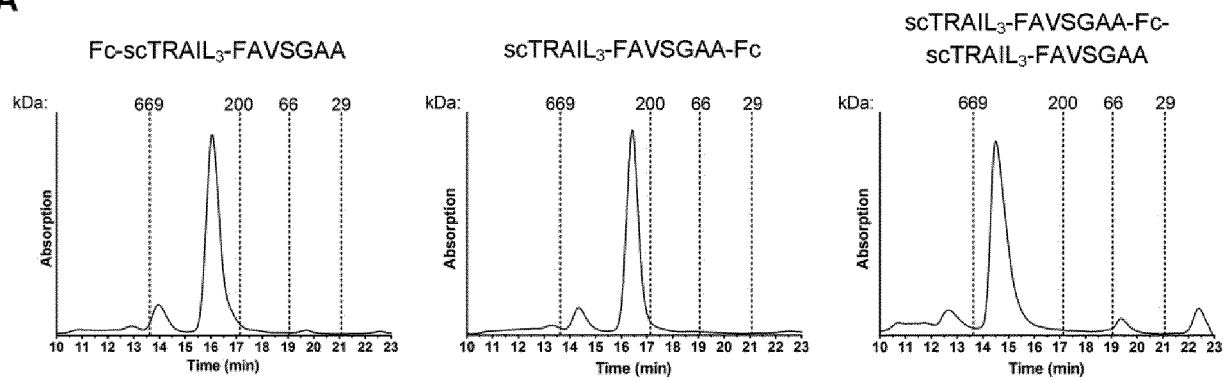
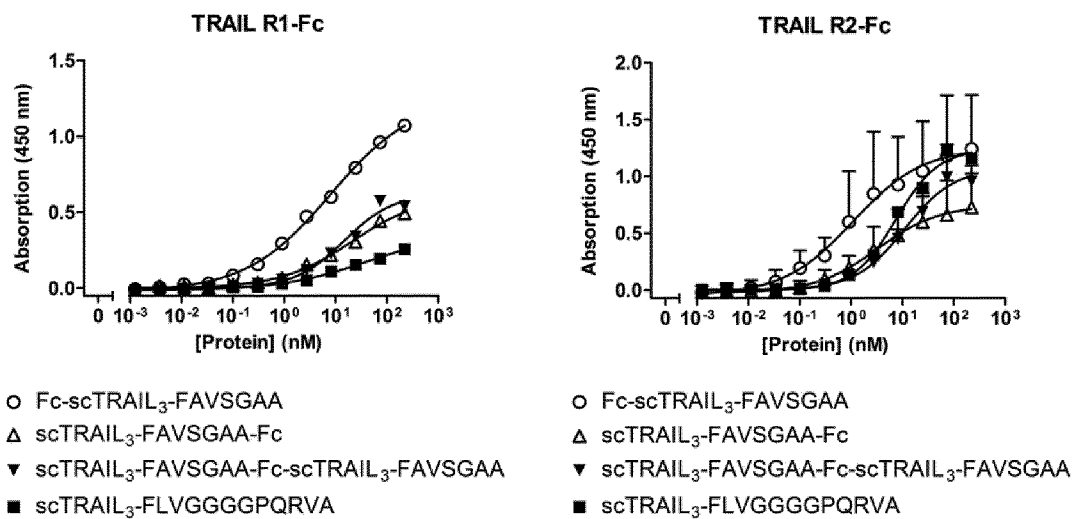
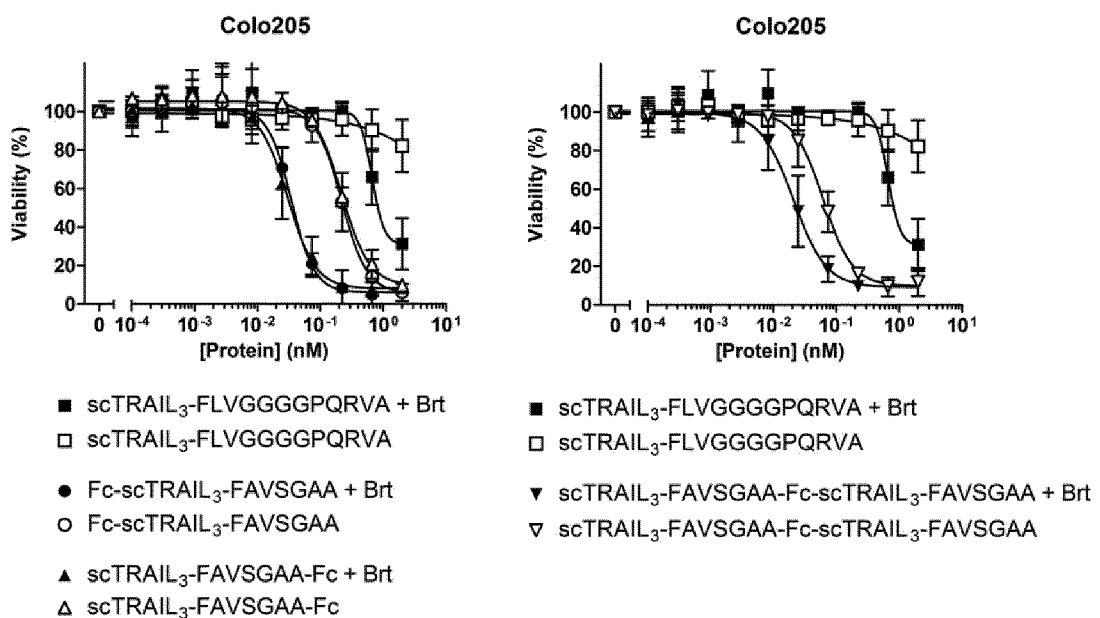
Fig. 38**A****B****C**

Fig. 39

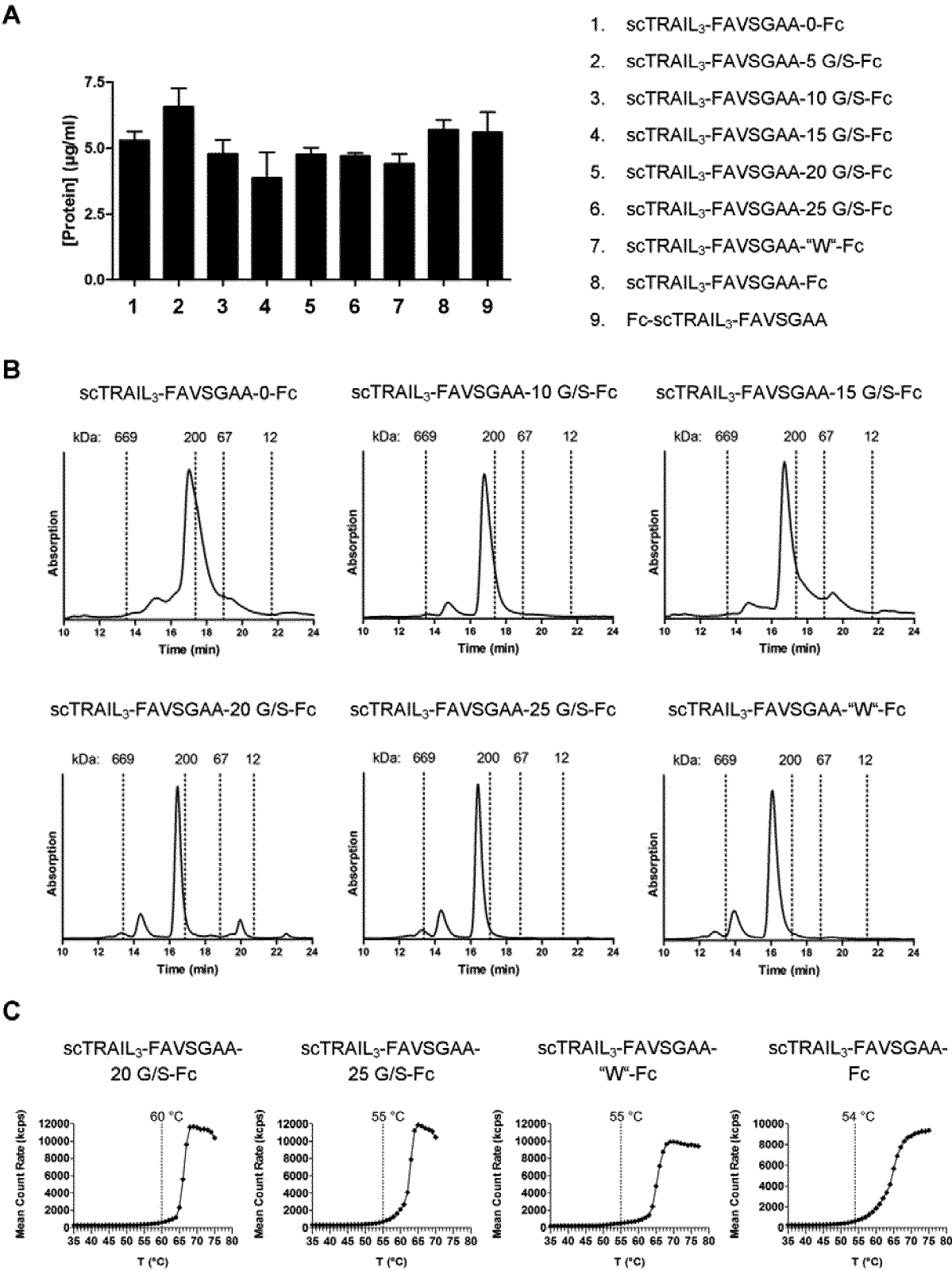


Fig. 40

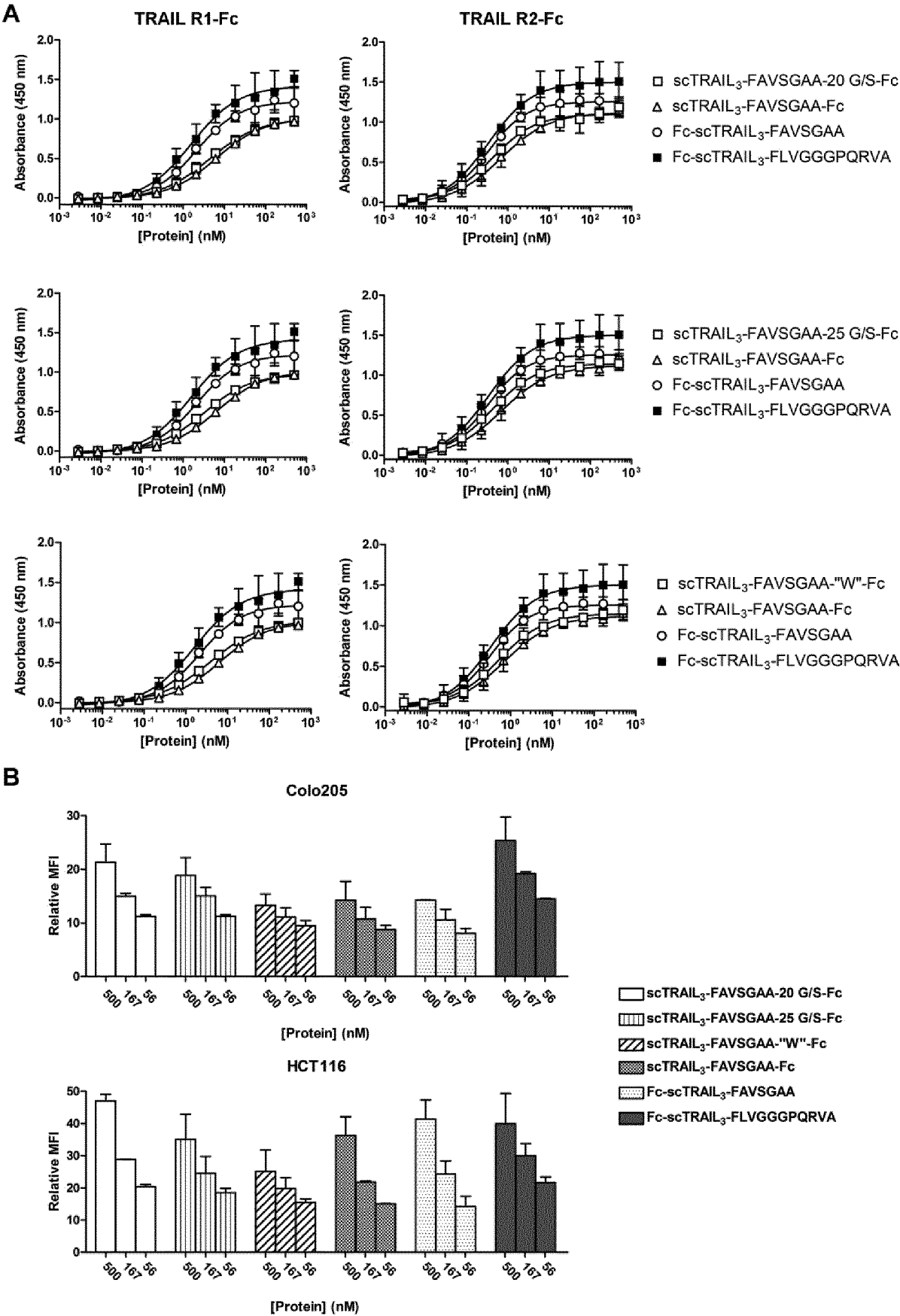


Fig. 41

Colo205

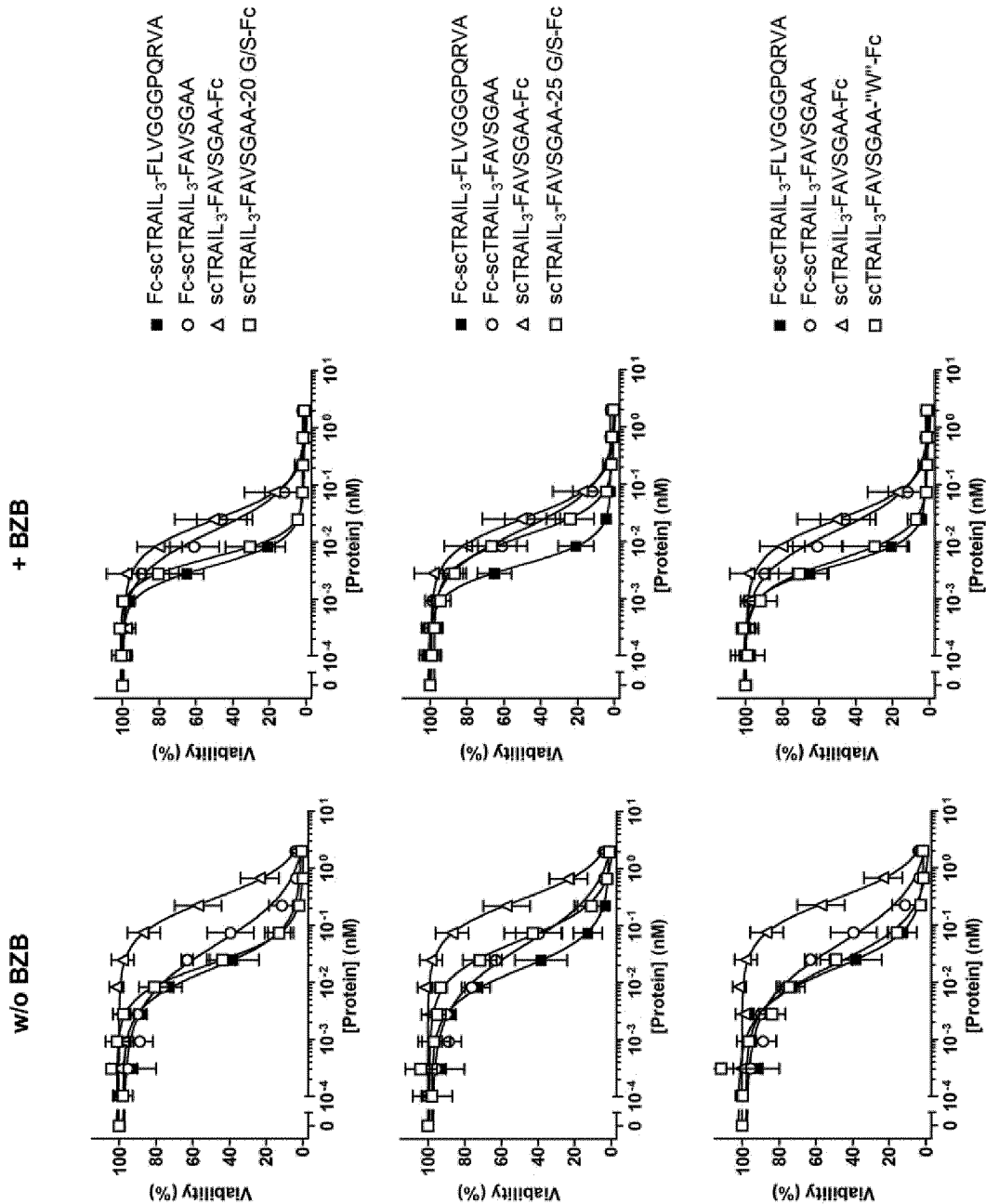


Fig. 42

HCT116

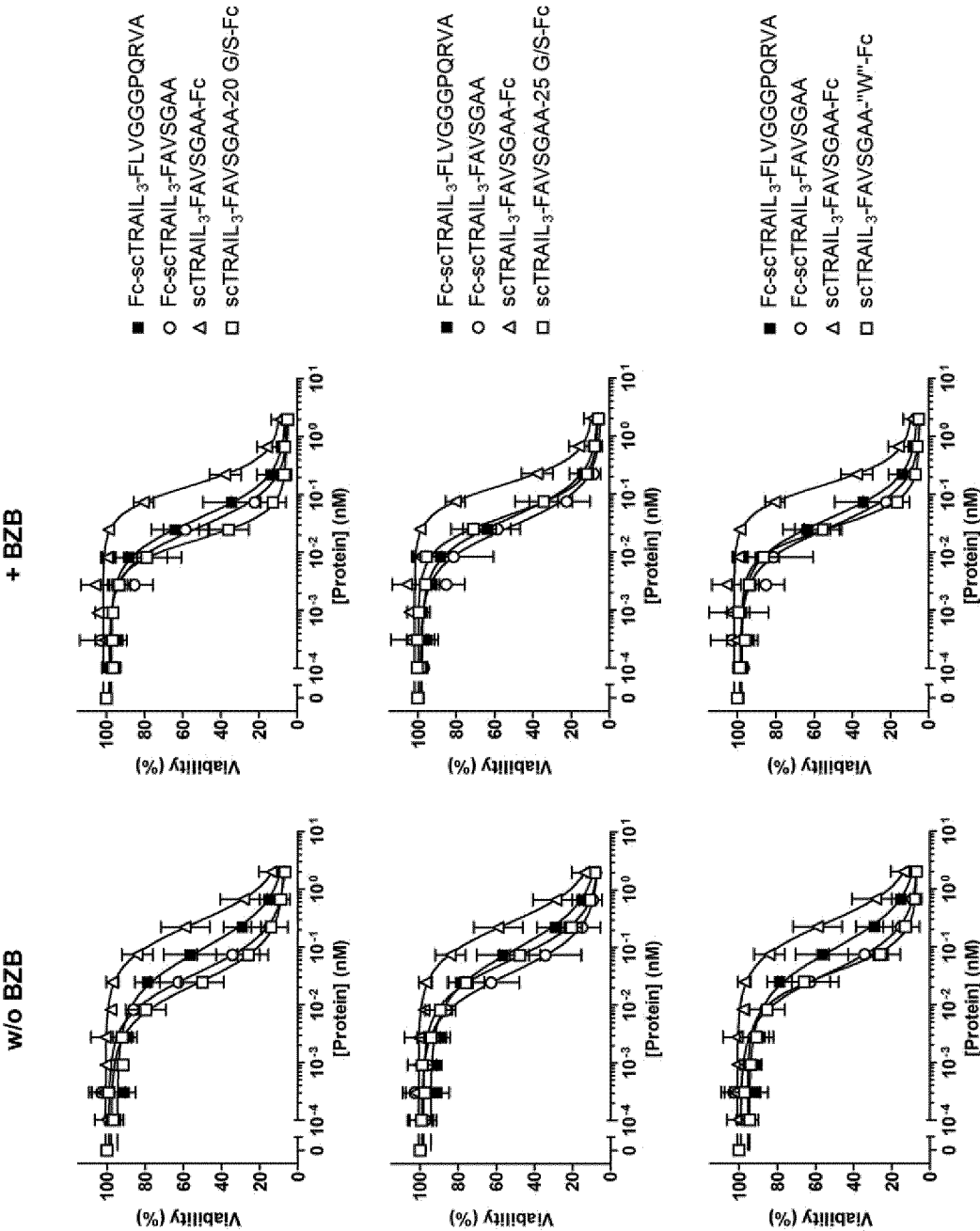


Fig. 43

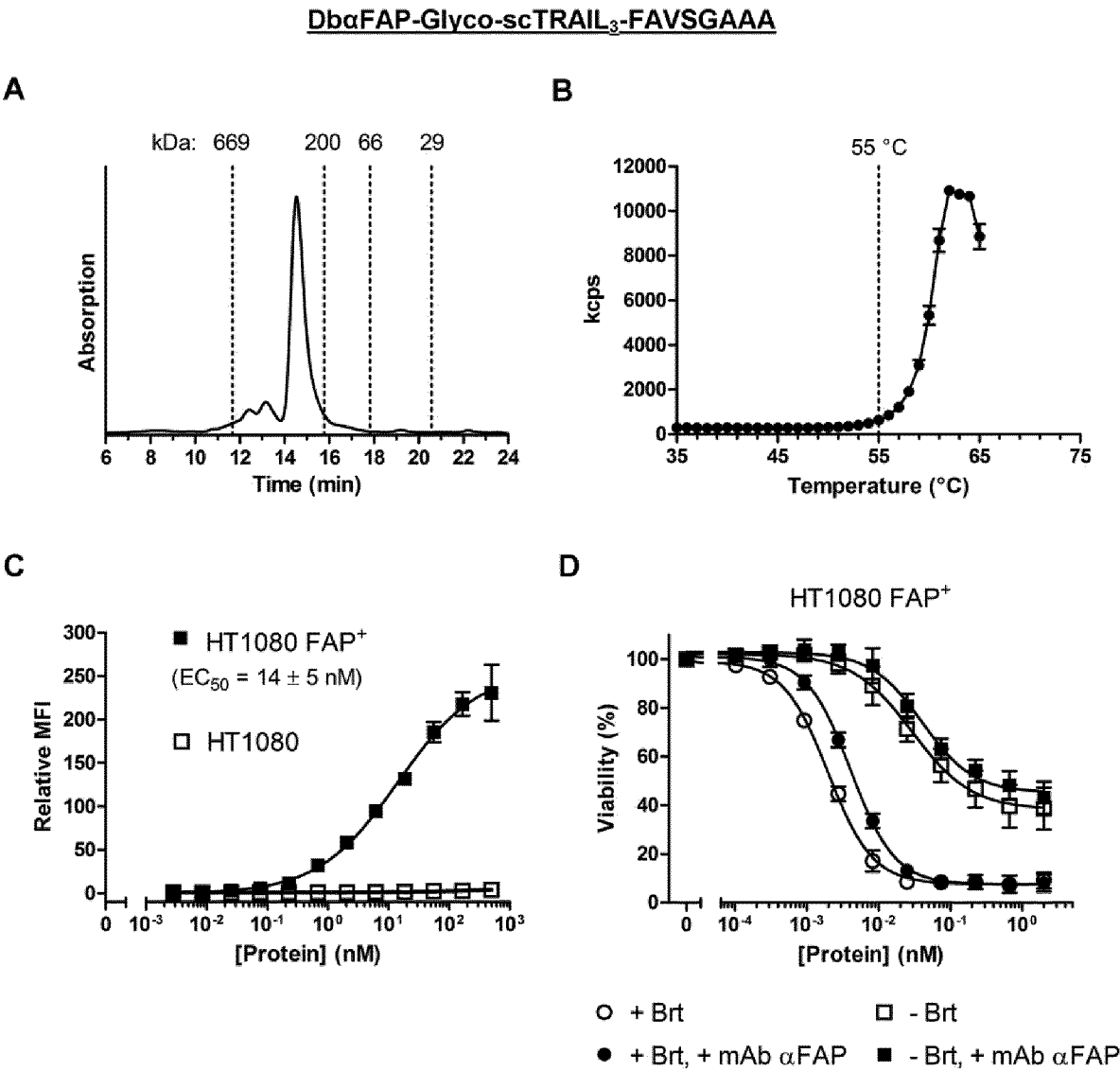


Fig. 44

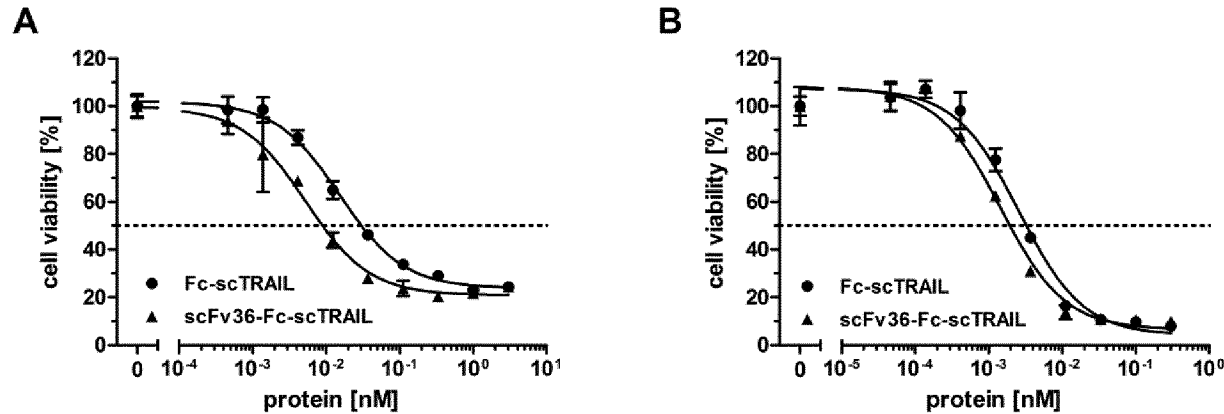
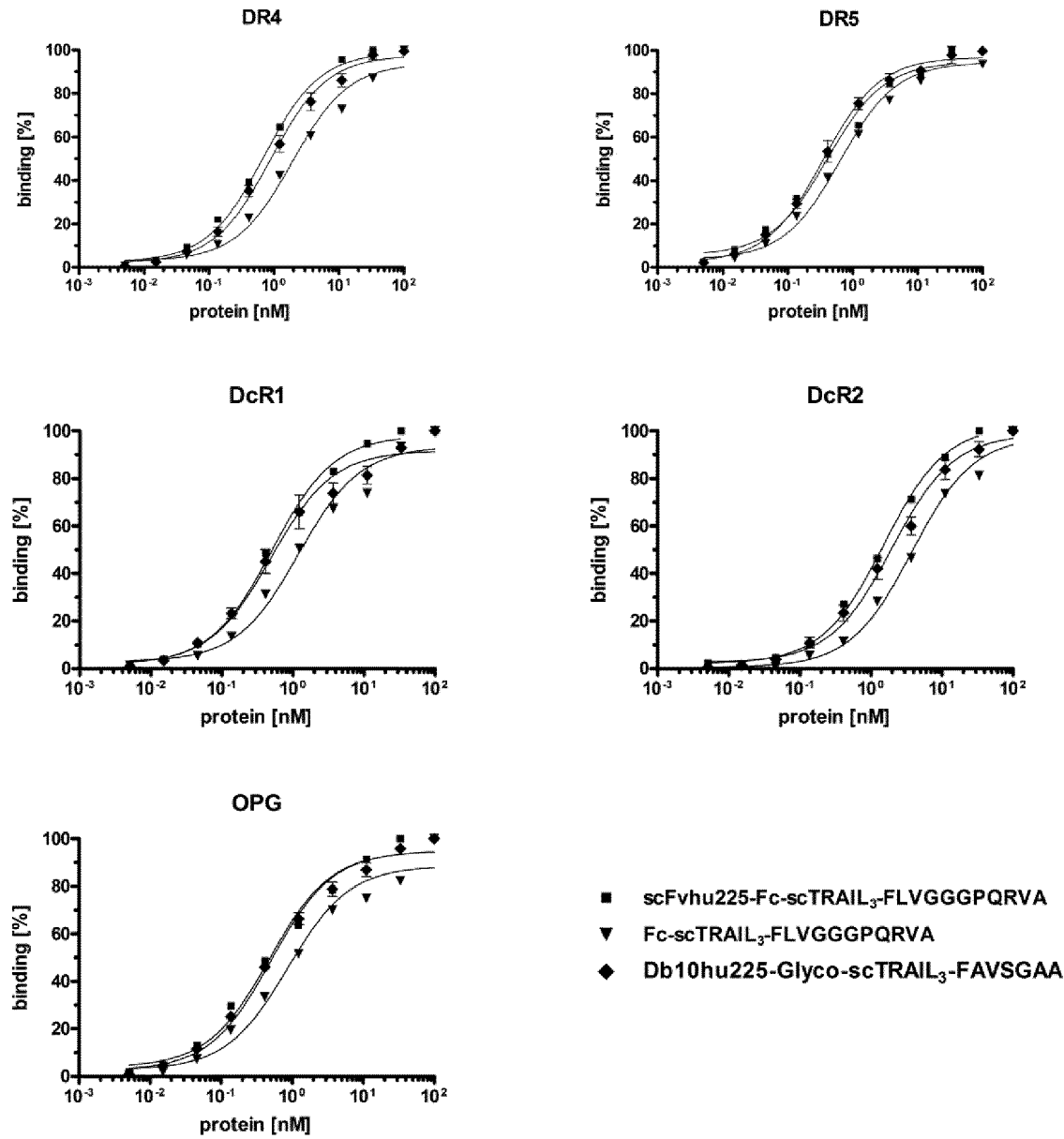


Fig. 45



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/055974

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-3, 8-17(all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055974

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 C07K16/32 C07K14/705
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	M SIEGEMUND ET AL: "Superior antitumoral activity of dimerized targeted single-chain TRAIL fusion proteins under retention of tumor selectivity", CELL DEATH AND DISEASE, vol. 3, no. 4, 1 April 2012 (2012-04-01), page e295, XP055200421, DOI: 10.1038/cddis.2012.29 e.g. abstract; page 2, right-hand column, paragraph 1; the whole document ----- -/--	1-3,8-17



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 May 2016

Date of mailing of the international search report

29/07/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Gruber, Andreas

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055974

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	O. SEIFERT ET AL: "Tetravalent Antibody-scTRAIL Fusion Proteins with Improved Properties", MOLECULAR CANCER THERAPEUTICS, vol. 13, no. 1, 1 January 2014 (2014-01-01), pages 101-111, XP055201437, ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-13-0396 e.g. abstract; the whole document -----	1-3,8-17
A	WO 2010/010051 A1 (APOGENIX GMBH [DE]; HILL OLIVER [DE]; GIEFFERS CHRISTIAN [DE]; THIEMAN) 28 January 2010 (2010-01-28) page 2, lines 10-16; page 14, line 18 - page 15, line 4; the whole document -----	1-3,8-17
Y	HOLLIGER P ET AL: "DIABODIES: SMALL BIVALENT AND BISPECIFIC ANTIBODY FRAGMENTS", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 90, 1 July 1993 (1993-07-01), pages 6444-6448, XP002008022, ISSN: 0027-8424, DOI: 10.1073/PNAS.90.14.6444 e.g. fig. 1 and table 1; the whole document -----	1-3,8-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/055974

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2010010051	A1	28-01-2010	
		CA 2731388 A1	28-01-2010
		CA 2910512 A1	28-01-2010
		DK 2310509 T3	27-04-2015
		DK 2604693 T3	30-05-2016
		EP 2310509 A1	20-04-2011
		EP 2604693 A2	19-06-2013
		EP 2806029 A1	26-11-2014
		ES 2538122 T3	17-06-2015
		ES 2571879 T3	27-05-2016
		HR P20150425 T1	05-06-2015
		JP 5674155 B2	25-02-2015
		JP 2011528562 A	24-11-2011
		JP 2014230545 A	11-12-2014
		JP 2014230546 A	11-12-2014
		SI 2310509 T1	31-07-2015
		SM T201500135 B	09-07-2015
		US 2011162095 A1	30-06-2011
		US 2014056843 A1	27-02-2014
		US 2015110734 A1	23-04-2015
		US 2015125419 A1	07-05-2015
		WO 2010010051 A1	28-01-2010

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-3, 8-17(all partially)

polypeptide comprising at least three TNF homology domains of TNF-ligand family member proteins (THD), wherein the TNF-ligand family member is Trail, and wherein the polypeptide comprises a dimerization domain

2. claims: 4-17(partially)

polypeptide comprising at least three TNF homology domains of TNF-ligand family member proteins (THD), wherein the TNF-ligand family member is Trail, and wherein Xc has a linker length of 3 to 5 amino acids

3-11. claims: 1-17(partially)

polypeptide comprising at least three TNF homology domains of TNF-ligand family member proteins (THD), wherein the TNF-ligand family member is:

- Invention 3: SEQ ID N: 7 (FasL)
- Invention 4: SEQ ID NO: 8 (LIGHT),
- Invention 5: SEQ ID NO: 9 (LTa),
- Invention 6: SEQ ID NO: 10 (Lt(3),
- Invention 7: SEQ ID NO: 11 (TNF),
- Invention 8: SEQ ID NO: 12 (CD40L),
- Invention 9: SEQ ID NO: 13 (TWEAK),
- Invention 10: SEQ ID NO: 14 (CD27L),
- Invention 11 SEQ ID NO: 15 (BAFF).
