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(54) Title: TNF SUPERFAMILY COLLECTIN FUSION PROTEINS

(57) Abrégé/Abstract:
The present invention refers to a fusion protein comprising a TNF- superfamily (TNFSF) cytokine or a receptor binding domain thereof fused to a collectin trimerization domain, to a nucleic acid molecule encoding the fusion protein, and to a cell comprising the nucleic acid molecule. The fusion protein is present as a trimeric complex or as an oligomer thereof. The fusion protein, the nucleic acid, and the cell is suitable as pharmaceutical composition or for therapeutic, diagnostic and/or research applications.

Abstract:

The present invention refers to a fusion protein comprising a TNF- superfamily (TNFSF) cytokine or a receptor binding domain thereof fused to a collectin trimerization domain, to a nucleic acid molecule encoding the fusion protein, and to a cell comprising the nucleic acid molecule. The fusion protein is present as a trimeric complex or as an oligomer thereof. The fusion protein, the nucleic acid, and the cell is suitable as pharmaceutical composition or for therapeutic, diagnostic and/or research applications.

# TNF Superfamily Collectin Fusion Proteins

## Description

### Field of Invention

The present invention refers to a fusion protein comprising a TNF-superfamily (TNFSF) cytokine or a receptor binding domain thereof fused to a collectin trimerization domain, to a nucleic acid molecule encoding the fusion protein, and to a cell comprising the nucleic acid molecule. The fusion protein is present as a trimeric complex or as an oligomer thereof. The fusion protein, the nucleic acid, and the cell is suitable as pharmaceutical composition or for therapeutic, diagnostic and/or research applications as described herein.

### State of the Art

Ligands of the tumor necrosis factor (TNF) family fulfill crucial roles in the immune system, but have also been implicated in the development of epithelial and endothelial structures.[1] TNF family ligands are primarily expressed as trimeric type II transmembrane proteins and are often processed into soluble variants that are also organized as trimers.[1,2] While shedding of some TNF ligands does not interfere with their capability to activate their corresponding receptors and might be even important for their physiological function, other TNF ligands become inactivated by proteolytic processing.[2] Soluble TNF ligands that are not or only poorly active still interact with their cognate receptors. For example, the soluble forms of TNF, CD95L, TRAIL and CD40L interact with TNFR2, CD95, TRAILR2 and CD40, respectively, but do not or only poorly activate signaling by these receptors.[3-6] Notably, inactive or poorly active soluble TNF ligands can be converted into highly active molecules by artificially increasing their avidity. For example, soluble Flag-tagged variants of TNF, CD95L, TRAIL and CD40L

stimulate robust signaling by TNFR2, CD95, TRAILR2 and CD40, respectively, provided they were crosslinked with the Flag-specific mAb M2. Likewise, hexameric and dodecameric fusion proteins of soluble CD95L and soluble CD40L as well as non-specifically aggregated preparations of TNF ligands produced in E. coli display high activity.[6-8]

The structural hall mark of the ligands of the TNF family is the carboxy-terminal "TNF 2 homology domain" (THD) or "receptor binding domain" (RBD), both terms are equally used herein, which is part of both the transmembrane and soluble forms of TNF ligands.[1-2] The THDs of the various TNF ligands are composed of a framework of aromatic and hydrophobic residues that adopt an almost identical tertiary fold and cause self association into trimers.[1-2] The THD also mediates receptor binding. In general, trimeric ligands of the TNF family bind to three molecules of their corresponding receptor(s). This interaction alone is not necessarily sufficient to activate receptor-associated intracellular signaling pathways. Several lines of evidence suggest that the initial formation of trimeric signaling competent ligand receptor complexes is followed by secondary multimerization into supramolecular clusters.[9-11] These two steps in TNF receptor activation (1. ligand binding; 2. secondary aggregation of receptor ligand complexes) depend to a varying extent on several factors including lipid raft localization, cytoskeleton support, receptor autoaggregation, receptor associated adapter proteins, but also on affinity and avidity of the ligand receptor interaction and the way how the ligand is presented to the receptor (membrane ligand or immobilized ligand versus soluble ligand, trimers versus higher aggregates).

It is known that trimeric complexes of TNF superfamily cytokines are difficult to prepare from recombinant monomeric units.

For example, WO 01/49866 discloses recombinant fusion proteins comprising a TNF cytokine and a multimerization component. A disadvantage of these fusion proteins is, however, that the trimerization domain usually has a large molecular weight and/or that the trimerization is

rather inefficient.

Schneider et al. (J Exp Med 187 (1989), 1205-1213) describes that trimers of TNF cytokines are stabilized by N-terminally positioned stabilization motifs. In CD95L, the stabilization of the CD95L-receptor binding domain trimer is presumably caused by N-terminal amino acid domains which are located near the cytoplasmic membrane.

Shiraishi et al. (Biochem Biophys Res Commun 322 (2004), 197-202) describes that the receptor binding domain of CD95L may be stabilized by N-terminally positioned artificial α-helical coiled-coil (leucine zipper) motifs. It was found, however, that the orientation of the polypeptide chains to each other, e.g. parallel or antiparallel orientation, can hardly be predicted. Further, the optimal number of hepta-d-repeats in the coiled-coil zipper motif are difficult to determine. In addition, coiled-coil structures have the tendency to form macromolecular aggregates after alteration of pH and/or ionic strength.

Mc Alinden et al. (J of Biol Chem, 2002, 277(43):41274-41281) discloses the preparation of a fusion protein between a human type IIA procollagen amino acid sequence and a 14 amino acid sequence corresponding to the first two heptad repeats of the rat surfactant protein's (SP-D) neck domain.

WO 01/42298 discloses the preparation of a fusion protein between surfactant protein-D comprising the signal sequence, the collagen domain and the neck domain and CD40L. The disadvantage of those fusion proteins is that they lead to multimeric aggregates that are highly immunogenic and that they do not produce functionally defined trimeric ligands.

It was an object of the present invention to provide fusion proteins comprising a TNF cytokine or a receptor binding domain, which allow efficient recombinant manufacture combined with good trimerization properties and improved pharmaceutical properties.

## Summary of the Invention

The present invention relates to a fusion protein comprising
(i) a TNF-superfamily cytokine or a receptor binding domain thereof, and
(ii) a collectin trimerization domain.

The invention further relates to a nucleic acid molecule encoding a fusion protein as described herein and to a cell or a non-human organism transformed or transfected with a nucleic acid molecule as described herein.

The invention also relates to a pharmaceutical or diagnostic composition comprising as an active agent a fusion protein, a nucleic acid molecule, or a cell as described herein.

The invention also relates to a fusion protein, a nucleic acid molecule, or a cell as described herein for use in therapy, e.g., the use of a fusion protein, a nucleic acid molecule, or a cell as described herein for the preparation of a pharmaceutical composition in the prophylaxis and/or treatment of proliferative disorders, particularly disorders caused by, associated with and/or accompanied by dysfunction of TNF cytokines, such as tumors, e.g. solid or lymphatic tumors, infectious diseases, inflammatory diseases, metabolic diseases, autoimmune disorders, e.g. rheumatoid and/or arthritic diseases, degenerative diseases, e.g. neurodegenerative diseases such as multiple sclerosis, apoptosis-associated diseases and transplant rejections.

## Detailed Description of the Invention

The fusion protein may be a monomeric protein or a multimeric protein. Preferably, the fusion protein is present as a trimeric complex consisting of three monomeric units which may be identical or different. Preferably, a trimeric complex consists of three identical fusion proteins. In a further

preferred embodiment, the complex is formed by covalent linkage between three of the fusion proteins described herein, e.g., a covalent linkage of disulfide bridges between cysteines of the collectin trimerization domain (ii) as described herein. The trimeric complex as such shows biological activity. It was found, however, that oligomers of the trimeric complex, e.g. defined complexes wherein the basic trimeric structure is present 2, 3 or 4 times, also have biological activity. Thus, also preferred is an oligomer of the trimeric complex.

One component (i) of the fusion protein is a cytokine of the TNF superfamily or a receptor binding domain thereof. Preferably, component (i) is a mammalian, particularly human cytokine or a receptor binding domain thereof including allelic variants and/or derivatives thereof. Further, it is preferred that the TNF cytokine is a receptor binding domain thereof capable of binding to the corresponding cytokine receptor and preferably capable of receptor activation, whereby apoptotic or proliferative activity may be caused. The cytokine may e.g. be selected from TNF superfamily members, e.g. human TNFSF-1 to -18 as indicated in Table 1, preferably from LTA (SEQ ID NO:1), TNFα (SEQ ID NO:2), LTB (SEQ ID NO:3), OX40L (SEQ ID NO:4), CD40L (SEQ ID NO:5), CD95L (SEQ ID NO:6), CD27L (SEQ ID NO: 7), CD30L (SEQ ID NO:8), CD137L (SEQ ID NO:9), TRAIL (SEQ ID NO:10), RANKL (SEQ ID NO:11), TWEAK (SEQ ID NO:12), APRIL 1 (SEQ ID NO: 13), APRIL 2 (SEQ ID NO:14), BAFF (SEQ ID NO:15), LIGHT (SEQ ID NO: 16), TL1A (SEQ ID NO:17), GITRL (SEQ ID NO:18), EDA-A1 (SEQ ID NO: 19), EDA-A2 (SEQ ID NO:20), or a receptor binding domain thereof. Preferred receptor binding domains of the respective proteins are indicated in Table 1 ($NH_2$-aa to COOH-aa) and comprise, e.g., comprises amino acids 59-205 or 60-205 of LTA (SEQ ID NO:1), 86-233 of TNFα (SEQ ID NO:2), 82-244 or 86-244 of LTB (SEQ ID NO:3), 52-183 or 55-183 of OX40L (SEQ ID NO:4), 112-261 or 117-261 of CD40L (SEQ ID NO:5), 51-193 or 56-193 of CD27L (SEQ ID NO:7), 97-234, 98-234 or 102-234 of CD30L (SEQ ID NO:8), 86-254 of CD137L (SEQ ID NO:9), 161-317 of RANKL (SEQ ID NO: 11), 103-249, 104-249 or 105-249 of TWEAK (SEQ ID NO:12), 112-247 or

113-247 of APRIL 1 (SEQ ID NO:13), 112-250 or 113-250 of APRIL 2 (SEQ ID NO:14), 140-285 of BAFF (SEQ ID NO:15), 91-240 of LIGHT (SEQ ID NO:16), 91-251 or 93-251 of TL1A (SEQ ID NO:17), 52-177 of GITRL (SEQ ID NO:18), 245-391 of EDA-A1 (SEQ ID NO:19), 245-389 of EDA-A2 (SEQ ID NO:20).

More preferably, the cytokine of the TNF superfamily or a receptor binding domain thereof is selected from CD95L or TRAIL or a receptor binding domain thereof. In an especially preferred embodiment, the cytokine of the TNF superfamily or a receptor binding domain thereof comprises the extracellular portion of a TNF cytokine including the receptor binding domain without membrane located domains.

In a preferred embodiment, the cytokine of the TNF superfamily or a receptor binding domain thereof of the fusion protein is selected from human CD95L (SEQ ID NO:6), particularly amino acids 142-281 or 144-281 of human CD95L.

In a further preferred embodiment, the cytokine of the TNF superfamily or a receptor binding domain thereof of the fusion protein is selected from human TRAIL (SEQ ID NO:10), particularly amino acids 95-281, 116-281, 117-281, 118-281, 119-281 or 120-281 of human TRAIL. In another preferred embodiment human TRAIL comprise any amino acid from 95-120 as initial amino acid - amino acid 281 of SEQ ID NO:10.

In a further preferred embodiment of the invention, the cytokine of the TNF superfamily or a receptor binding domain thereof of the fusion protein as described herein comprises a mutant of the cytokine of the TNF superfamily or a receptor binding domain thereof which binds and/or activates TRAIL-receptor 1 (TRAILR1) and/or TRAIL-receptor 2 (TRAILR2). The binding and/or activity of the mutant may be, e.g., determined by the assays as disclosed herein, e.g., in the Examples or by the assays disclosed in van der Sloot et al. (PNAS, 2006, 103:8634-8639), Kelley et al. (J. Biol. Chem., 2005,

280:2205-2215), or MacFarlane et al. (Cancer Res., 2005, 65: 11265-11270).

The mutant may be generated by any technique and is known by the skilled person, e.g., the techniques disclosed in an der Sloot et al. (PNAS, 2006, 103:8634-8639), Kelley et al. (J. Biol. Chem., 2005, 280:2205-2215), or MacFarlane et al. (Cancer Res., 2005, 65: 11265-11270) any may comprise any type of structural mutations, e.g., substitution, deletion, duplication and/or insertion of an amino acid. A preferred embodiment is the generation of substitutions. The substitution may affect at least one amino acid of the cytokine of the TNF superfamily or a receptor binding domain thereof as described herein. In a preferred embodiment, the substitution may affect at least one of the amino acids of TRAIL, e.g., human TRAIL (e.g., SEQ ID NO: 10). Preferred substitutions in this regard affect at least one of the following amino acids of human TRAIL of SEQ ID NO:10: R130, G160, Y189, R191, Q193, E195, N199, K201, Y213, T214, S215, H264, I266, D267, D269. Preferred amino acid substitutions of human TRAIL of SEQ ID NO:10 are at least one of the following substitutions: R130E, G160M, Y189A, Y189Q, R191K, Q193S, Q193R, E195R, N199V, N199R, K201R, Y213W, T214R, S215D, H264R, I266L, D267Q, D269H, D269R, or D269K.

The amino acid substitution(s) may affect the binding and/or activity of TRAIL, e.g., human TRAIL, to or on either the TRAILR1 or the TRAILR2. Alternatively, the amino acid substitution(s) may affect the binding and/or activity of TRAIL, e.g., human TRAIL, to or on both, the TRAILR1 and the TRAILR2. The binding and/or activity of the TRAILR1 and/or TRAILR2 may be affected positively, i.e., stronger, more selective or specific binding and/or more activation of the receptor. Alternatively, the binding and/or activity of the TRAILR1 and/or TRAILR2 may be affected negatively, i.e., weaker, less selective or specific binding and/or less or no activation of the receptor.

Examples of mutants of TRAIL with amino acid substitution(s) that affect binding and/or activity of both TRAILR1 and TRAILR2 may be found, e.g., in

Table 1 of MacFarlane et al. (cf. above) and may comprise human TRAIL mutants with the following two amino acid substitutions of SEQ ID NO:10 Y213W and S215D or the following single amino acid substitution Y189A.

Examples of mutants of TRAIL with amino acid substitution(s) that affect binding and/or activity of TRAILR1 may be found, e.g., in Table 1 of MacFarlane et al. (cf. above) and may comprise human TRAIL mutants with the following four amino acid substitutions of SEQ ID NO:10 N199V, K201R, Y213W and S215D or the following five amino acid substitutions Q193S, N199V, K201R, Y213W and S215D or in Table 2 of Kelley et al. (cf. above) and may comprise human TRAIL mutants with the following six amino acid substitutions Y213W, S215D, Y189A, Q193S, N199V, and K201R or Y213W, S215D, Y189A, Q193S, N199R, and K201R.

Examples of mutants of TRAIL with amino acid substitution(s) that affect binding and/or activity of TRAILR2 may be found, e.g., in Table 1 of MacFarlane et al. (cf. above) or in Table 2 of Kelley et al. (cf. above) and may comprise human TRAIL mutants with the following six amino acid substitutions of SEQ ID NO:14 Y189Q, R191K, Q193R, H264R, I266L, and D267Q or in Table 2 of van der Sloot et al. (cf. above) and may comprise human TRAIL mutants with the following single amino acid substitution D269H, the following two amino acid substitutions D269H and E195R or D269H and T214R.

In a further preferred embodiment, the cytokine portion of the fusion protein is derived from human LIGHT (SEQ ID NO:16), particularly amino acids 91-240 of SEQ ID NO:16.

In a still further preferred embodiment, the cytokine portion of the fusion protein is derived from human APRIL (SEQ ID NO:13 or 14), particularly amino acids 112-247 or 113-247 of SEQ ID NO:13, or 112-250 or 113-250 of SEQ ID NO:14.

A flexible linker element may additionally located between the cytokine of the TNF superfamily or a receptor binding domain thereof (i) and the collectin trimerization domain as described herein (ii). The flexible linker element preferably has a length of 3-20 amino acids, particularly a length of 3, 6, 9, 10, 12, 15 or 18 amino acids. More preferably, the length of the linker is 9-15 amino acids. The linker element is preferably a glycine/serine linker, i.e., a peptide linker substantially consisting of the amino acids glycine and serine. In an especially preferred embodiment, the linker has the amino acid sequence $(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6. It is clear to the skilled person that in cases in which the cytokine of the TNF superfamily or a receptor binding domain thereof already terminates with a G, e.g. human TRAIL (SEQ ID NO:10) such a G may form the first G of the linker in the linker sequence $(GSS)_a(SSG)_b(GSG)_c$.

The collectin trimerization domain (ii) may comprise any collectin family member. Such members and their structures are summarized in, e.g., Hakansson et al. (Protein Science, 2000, 9:1607-1617) and may comprise surfactant protein-D, surfactant protein-A, mannan-binding protein-A, mannan-binding-protein-C, collectin liver 1, collectin placenta 1, or collectin-11. The collectin trimerization domain as described herein may be from a different species than the cytokine of the TNF superfamily or a receptor binding domain thereof as described herein. Alternatively, the collectin trimerization domain as described herein may be from the same species than the cytokine of the TNF superfamily or a receptor binding domain thereof described herein. In a preferred embodiment, the collectin domain as described herein is from human and the cytokine of the TNF superfamily or a receptor binding domain thereof as described herein is from human. In a preferred embodiment, the collectin trimerization domain comprises the neck and carbohydrate binding domain (CRD) domain of the surfactant protein-D, particularly amino acids 217-375, 218-375, 219-375, 220-375, 221-375, 222-375, 223-375, 224-375, 225-375 from human surfactant protein-D of SEQ ID NO:21. In another preferred embodiment, the collectin trimerization domain comprises the neck domain of the surfactant

protein-D, particularly amino acids 217-257, 218-257, 219-257, 220-257, 221-257, 222-257, 223-257, 224-257, or 225-257 from human surfactant protein-D of SEQ ID NO:21. In another preferred embodiment, the collectin trimerization domain comprises the neck and carbohydrate binding domain (CRD) domain of collectin-11, particularly amino acids 110-271, 116-271, or 121-271 of human collectin-11 of SEQ ID NO:22. In another preferred embodiment, the collectin trimerization domain comprises the neck domain of collectin-11, particularly amino acids 110-147, 110-148, 110-149, 110-150, 110-151, 116-147, 116-148, 116-149, 116-150, 116-151, 121-147, 121-148, 121-149, 121-150, or 121-151 of human collectin-11 of SEQ ID NO:22.

The collectin trimerization domain (ii) may comprise a mutant, e.g., a mutant of surfactant protein-D or collectin-11, which does not bind to mannose. Such mutants may be identified by methods known to the skilled person, e.g., the methods disclosed in Crouch et al. (J Biol Chem, 2006, 281(26): 18008-18014). The collectin trimerization domain (ii) may further comprise a mutant which comprise at least one amino acid substitution as is described herein and may be generated as described herein. Such amino acid substitutions may modify the binding of the collectin trimerization domain to its ligand mannose and lead to an alteration of the clearance rate of a fusion protein as described herein when used in therapy and/or as pharmaceutical composition. The modification may result in a decreased or no binding to mannose and a low clearance rate. Such modifications may be achieved by, e.g., amino acid substitution that affect amino acid position F355 of human surfactant protein-D of SEQ ID NO:21, particularly by the amino acid substitutions F355A, F355S, F355T, F355E, F355D, F355K, or F355R. Especially preferred is the substitution F355D. Alternatively, the modification may result in an increased binding to mannose and a high clearance rate. Such modifications may be achieved by, e.g., amino acid substitution that affect amino acid position F355 of human surfactant protein-D of SEQ ID NO:21, particularly by the amino acid substitutions F355L, F355Y, or F355W.

In the fusion protein of the invention as described herein, the collectin trimerization domain (ii) may be located C-terminally of the cytokine of the TNF superfamily or a receptor binding domain thereof (i). Thus, the fusion protein may comprise a cytokine of the TNF superfamily or a receptor binding domain thereof as described herein and a collectin trimerization domain that comprises the neck domain alone or the neck and the CRD domain, e.g., the neck domain and the CRD and/or neck domain of surfactant protein-D or the neck domain and the CRD and/or neck domain of collectin-11 both as described herein wherein those domains are located C-terminally of the TNF superfamily or a receptor binding domain thereof (i). In this embodiment, it is preferred that the collectin trimerization domain comprises the neck domain and the CRD.

In the fusion protein of the invention as described herein, the collectin trimerization domain (ii) may be located N-terminally of the cytokine of the TNF superfamily or a receptor binding domain thereof (i). Thus, the fusion protein may comprise a cytokine of the TNF superfamily or a receptor binding domain thereof as described herein and a collectin trimerization domain that comprises the neck domain, e.g., the neck domain of surfactant protein-D or the neck domain of collectin-11 both as described herein wherein those domains are located N-terminally of the TNF superfamily or a receptor binding domain thereof (i).

In a preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or a receptor binding domain thereof or a mutant of TRAIL as described herein, preferably 95-281, 116-281, 117-281, 118-281, 119-281 or 120-281 of human TRAIL (SEQ ID NO:10) and a collectin trimerization domain or mutant thereof as described herein, particularly the CRD and neck domain of surfactant protein-D, preferably amino acids 217-375, 218-375, 219-375, 220-375, 221-375, 222-375, 223-375, 224-375, 225-375 of human surfactant protein-D of SEQ ID NO:21 wherein the collectin trimerization domain is located C-terminally of TRAIL or mutant TRAIL as described

herein. Preferred fusion proteins in this regard are SEQ ID Nos:26 or 27. Alternatively, the above fusion protein may additionally comprise a linker as described herein, e.g., a linker with the amino acid sequence $(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6. Preferably, the linker has a length of 9-15 amino acids.

In a preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or a receptor binding domain thereof or a mutant of TRAIL as described herein, preferably 95-281, 116-281, 117-281, 118-281, 119-281 or 120-281 of human TRAIL (SEQ ID NO:10) and a collectin trimerization domain or mutant thereof as described herein, particularly the neck domain of surfactant protein-D, preferably amino acids 217-257, 218-257, 219-257, 220-257, 221-257, 222-257, 223-257, 224-257, or 225-257 of human surfactant protein-D of SEQ ID NO:21 wherein the collectin trimerization domain is located C-terminally of TRAIL or mutant TRAIL as described herein. A preferred fusion protein in this regard is SEQ ID NO:28. Alternatively, the above fusion protein may additionally comprise a linker as described herein, e.g., a linker with the amino acid sequence $(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6. Preferably, the linker has a length of 9-15 amino acids.

In another preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or a receptor binding domain thereof or a mutant of TRAIL as described herein, preferably 95-281, 116-281, 117-281, 118-281, 119-281 or 120-281 of human TRAIL (SEQ ID NO:10) and a collectin trimerization domain or mutant thereof as described herein, particularly the CRD and neck domain of collectin-11, preferably amino acids 110-271, 116-271, or 121-271 of human collectin-11 of SEQ ID NO:22 wherein the collectin trimerization domain is located C-terminally of TRAIL or mutant TRAIL as described herein. Preferred fusion proteins in this regard are SEQ ID Nos:29 or 30. Alternatively, the above fusion protein may additionally comprise a linker as described herein, e.g., a linker with the amino acid sequence $(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3,

4, 5 or 6. Preferably, the linker has a length of 9-15 amino acids.

In another preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or a receptor binding domain thereof or a mutant of TRAIL as described herein, preferably 95-281, 116-281, 117-281, 118-281, 119-281 or 120-281 of human TRAIL (SEQ ID NO:10) and a collectin trimerization domain or mutant thereof as described herein, particularly the neck domain of collectin-11, preferably amino acids 110-147, 110-148, 110-149, 110-150, 110-151, 116-147, 116-148, 116-149, 116-150, 116-151, 121-147, 121-148, 121-149, 121-150, or 121-151 of human collectin-11 of SEQ ID NO:22 wherein the collectin trimerization domain is located C-terminally of TRAIL or mutant TRAIL as described herein. A preferred fusion protein in this regard is SEQ ID NO:31. Alternatively, the above fusion protein may additionally comprise a linker as described herein, e.g., a linker with the amino acid sequence $(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6. Preferably, the linker has a length of 9-15 amino acids. Preferred fusion proteins in this regard are SEQ ID Nos:36 or 37.

In a preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or a receptor binding domain thereof or a mutant of TRAIL as described herein, preferably 95-281, 116-281, 117-281, 118-281, 119-281 or 120-281 of human TRAIL (SEQ ID NO:10) and a collectin trimerization domain or mutant thereof as described herein, particularly the neck domain of surfactant protein-D, preferably amino acids 217-257, 218-257, 219-257, 220-257, 221-257, 222-257, 223-257, 224-257, or 225-257 of human surfactant protein-D of SEQ ID NO:21 wherein the collectin trimerization domain is located N-terminally of TRAIL or mutant TRAIL as described herein. Alternatively, the above fusion protein may additionally comprise a linker as described herein, e.g., a linker with the amino acid sequence $(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6. Preferably, the linker has a length of 9-15 amino acids.

In another preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or a receptor binding domain thereof or a mutant of TRAIL as described herein, preferably 95-281, 116-281, 117-281, 118-281, 119-281 or 120-281 of human TRAIL (SEQ ID NO:10) and a collectin trimerization domain or mutant thereof as described herein, particularly the neck domain of collectin-11, preferably amino acids 110-147, 110-148, 110-149, 110-150, 110-151, 116-147, 116-148, 116-149, 116-150, 116-151, 121-147, 121-148, 121-149, 121-150, or 121-151 of human collectin-11 of SEQ ID NO:22 wherein the collectin trimerization domain is located N-terminally of TRAIL or mutant TRAIL as described herein. Preferred fusion proteins in this regard are SEQ ID Nos:32-34. Alternatively, the above fusion protein may additionally comprise a linker as described herein, e.g., a linker with the amino acid sequence $(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6. Preferably, the linker has a length of 9-15 amino acids. Preferred fusion proteins in this regard is SEQ ID NO: 35.

In another preferred embodiment, the fusion protein comprises CD95L, particularly human CD95L, or a receptor binding domain thereof as described herein, e.g. amino acids 21-160 of SEQ ID NO:40, and a collectin trimerization domain comprising the neck domain and optionally the CRD of human SP-D, e.g. amino acids 172-209 and 210-327 of SEQ ID NO:40, respectively, or a mutant thereof as described herein. Preferably, the fusion protein may comprise a linker, e.g. a flexible linker, more preferably a glycine/serine linker as described herein having a length of preferably 9-15 amino acids. A preferred fusion protein in this regard comprises SEQ ID NO: 40, particularly amino acids 21-327 of SEQ ID NO:40.

In another preferred embodiment, the fusion protein comprises LIGHT, particularly human LIGHT or a receptor binding domain thereof as described herein, preferably amino acids 21-170 of SEQ ID NO:41, and a collectin trimerization domain comprising the neck domain and optionally the CRD of human SP-D, e.g. amino acids 182-219, and 220-337 of SEQ ID NO:41,

respectively, or a mutant thereof as described herein. Preferably, the cytokine and the collectin domain are connected by a linker, e.g. a glycine/serine linker as described herein, having a length of preferably 9-15 amino acids. A preferred fusion protein in this regard comprises SEQ ID NO: 41, particularly amino acids 21-327 of SEQ ID NO:41.

In another preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or a receptor binding domain thereof or mutant of TRAIL as described herein, e.g. amino acids 21-181 of SEQ ID NO:43 (wild type TRAIL), amino acids 21-181 of SEQ ID NO:47 (TRAILR1mut) or amino acids 21-181 of SEQ ID NO:48 (TRAILR2mut). Further, the fusion protein comprises a collectin trimerization domain selected from the neck domain and optionally the CRD of human SP-D, e.g. amino acids 193-230, and 231-384 of SEQ ID NO:43, respectively, or a mutant thereof as described herein, e.g. mutants as shown in SEQ ID NO:49 or 50. Preferably, the fusion polypeptide comprises both the neck region and the CRD of human SP-D. The cytokine and collectin domain are preferably connected by a linker, e.g. a glycine/serine linker as described herein. Preferably, the linker has a length of 9-15 amino acids. Preferred fusion proteins in this regard comprise (i) SEQ ID NO:43, particularly amino acids 21-348 of SEQ ID NO:43, (ii) SEQ ID NO:44, particularly amino acids 21-230 of SEQ ID NO:44, (iii) SEQ ID NO: 47, particularly amino acids 21-348 of SEQ ID NO:47, (iv) SEQ ID NO:48, particularly amino acids 21-348 of SEQ ID NO:48, (v) SEQ ID NO: 49, particularly amino acids 21-348 of SEQ ID NO:49 or (vi) SEQ ID NO:50, particularly amino acids 21-348 of SEQ ID NO:50.

In another preferred embodiment, the fusion protein comprises TRAIL, particularly human TRAIL or receptor-binding domain thereof or a mutant of TRAIL as described herein above, and a collectin trimerization domain, which is the neck domain of human collectin 11, and optionally the CRD of human collectin 11, e.g. amino acids 193-224 and 225-347 of SEQ ID NO: 45, respectively. Preferably, the CRD is present. Preferably, the cytokine and the collectin domain are connected by a linker, e.g. a glycine/serine linker as

described above herein, preferably having a length of 9-15 amino acids. Preferred fusion proteins in this regard comprise SEQ ID NO:45 and SEQ ID NO:46, particularly, amino acids 21-347 of SEQ ID NO:45 or amino acids 21-229 of SEQ ID NO:46.

In another preferred embodiment, the fusion protein comprises APRIL, particularly human APRIL or a receptor binding domain thereof as described herein, e.g. amino acids 21-158 of SEQ ID NO:51 and a collectin trimerization domain as described herein, particularly the neck domain and optionally the CRD of human SP-D or a mutant thereof, as described herein, e.g. amino acids 170-207 and 208-325 of SEQ ID NO:51, respectively. The cytokine and the collectin domain are preferably connected by a linker, e.g. a glycine/serine linker as described herein, preferably having a length of 9-15 amino acids. The preferred fusion protein in this regard comprises SEQ ID NO:51, particularly amino acids 21-325 of SEQ ID NO:51.

The fusion protein as described herein may additionally comprise an N-terminal signal peptide domain, which allows processing, e.g., extracellular secretion, in a suitable host cell. Preferably, the N-terminal signal peptide domain comprises a protease, e.g., a signal peptidase cleavage site and thus may be removed after or during expression to obtain the mature protein. In a preferred embodiment, the N-terminal signal peptide domain comprises the sequence SEQ ID NO:23, SEQ ID NO:24, or SEQ ID NO:25.

Further, the fusion protein may comprise comprises a recognition/purification domain, e.g., a Strep-tag domain and/or a poly-His domain, which may be located at the N-terminus or at the C-terminus.

The fusion protein may additionally comprise a C-terminal flexible element, having a length of, e.g., 1-50, preferably 10-30 amino acids which may include and/or connect to a recognition/purification domain as described herein.

A further aspect of the present invention relates to a nucleic acid molecule encoding a fusion protein as described herein. The nucleic acid molecule may be a DNA molecule, e.g., a double-stranded or single-stranded DNA molecule, or an RNA molecule. The nucleic acid molecule may encode the fusion protein or a precursor thereof, e.g., a pro- or pre-proform of the fusion protein which may comprise a signal sequence as described herein or other heterologous amino acid portions for secretion or purification which are preferably located at the N- and/or C-terminus of the fusion protein as described herein. The nucleic acid molecule may encode the fusion protein wherein the heterologous amino acid portions may be linked to the first and/or second domain via a protease cleavage site, e.g., a Factor $X_a$, thrombin or IgA protease cleavage site.

Examples of nucleic acids that comprise the coding sequence of a fusion protein as described herein are SEQ ID Nos:38, 39 or 42.

The nucleic acid molecule may be operatively linked to an expression control sequence, e.g. an expression control sequence which allows expression of the nucleic acid molecule in a desired host cell. The nucleic acid molecule may be located on a vector, e.g. a plasmid, a bacteriophage, a viral vector, a chromosal integration vector, etc. Examples of suitable expression control sequences and vectors are described for example by Sambrook et al. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, and Ausubel et al. (1989), *Current Protocols in Molecular Biology*, John Wiley & Sons or more recent editions thereof.

Various expression vector/host cell systems may be used to express the nucleic acid sequences encoding the fusion proteins of the present invention. Suitable host cells include, but are not limited to, prokaryotic cells such as bacteria, e.g. *E.coli*, eukaryotic host cells such as yeast cells, insect cells, plant cells or animal cells, preferably mammalian cells and, more preferably, human cells. The nucleic acid molecule encoding the fusion protein as described herein may be optimized in view of its *codon-usage* for

the expression in suitable host cells, e.g. E.coli, yeast cells, plant cells, insect cells, animal cells, e.g., mammalian cells or human cells.

Further, the invention relates to a non-human organism, e.g., mouse or rat, transformed or transfected with a nucleic acid molecule as described herein. Such organisms may be comprise knock-out organisms, generated by known methods of genetic transfer including homologous recombination. Alternatively, such organisms may comprise transgenic organisms which comprise several copies of the nucleic acid molecule as described herein. The generation of transgenic organisms is known in the art.

The fusion protein, the nucleic acid coding therefore, the transformed or transfected cell as well as the trimeric complexes or oligomers of the trimeric complexes, all as described herein may be used for pharmaceutical, diagnostic and/or research applications. For these applications it is preferred to use fusion proteins in which both the TNF-superfamily cytokine or receptor binding domain thereof as described herein and the collectin trimerization domain as described herein are from the same species in order to minimize immunological effects, e.g., from human when applying such proteins to humans. In addition, the fusion of a TNF-superfamily cytokine or receptor binding domain thereof as described herein to a neck-collectin trimerization domain as described herein, e.g., neck domain from surfactant protein-D or collectin-11, may lead to fast clearance. Alternatively, the fusion of a TNF-superfamily cytokine or receptor binding domain thereof as described herein to a neck and CRD-collectin trimerization domain as described herein, e.g., neck and CRD domain from surfactant protein-D or collectin-11, may lead to low clearance. The use of mutants of the collectin trimerization domain as described herein may modify the clearance rate of the fusion protein in a way as described herein.

A further aspect of the present invention relates to a pharmaceutical or diagnostic composition comprising as an active agent at least one fusion protein, the nucleic acid coding therefore, the transformed or transfected cell

as well as the trimeric complexes or oligomers of the trimeric complexes, all as described herein.

At least one fusion protein, the nucleic acid coding therefor, the transformed or transfected cell as well as the trimeric complexes or oligomers of the trimeric complexes, all as described herein may be used in therapy, e.g., in the prophylaxis and/or treatment of disorders selected from proliferative disorders, particularly disorders caused by, associated with and/or accompanied by dysfunction of TNF cytokines, such as tumors, e.g. solid or lymphatic tumors, infectious diseases, inflammatory diseases, metabolic diseases, autoimmune disorders, e.g. rheumatoid and/or arthritic diseases, degenerative diseases, e.g. neurodegenerative diseases such as multiple sclerosis, apoptosis-associated diseases and transplant rejections.

The composition may be administered as monotherapy or as combination therapy with further medicaments, e.g. cytostatic or chemotherapeutic agents, corticosteroids and/or antibiotics. Preferably, the composition is administered together with tumor-selective apoptosis sensitizing and/or inducing agents, e.g. as described in Example 2.8.

The fusion protein is administered to a subject in need thereof, particularly a human patient, in a sufficient dose for the treatment of the specific conditions by suitable means. For example, the fusion protein may be formulated as a pharmaceutical composition together with pharmaceutically acceptable carriers, diluents and/or adjuvants. Therapeutic efficacy and toxicity may be determined according to standard protocols. The pharmaceutical composition may be administered systemically, e.g. intraperitoneally, intramuscularly or intravenously or locally, e.g. intranasally, subcutaneously or intrathecally. Preferred is intravenous administration.

The dose of the fusion protein administered will of course be dependent on the subject to be treated, on the subject's weight, the type and severity of the disease, the manner of administration and the judgement of the prescribing

physician. For the administration of fusion proteins, a daily dose of 0.001 to 100 mg/kg is suitable.

Table 1 shows a list of cytokines of the TNF super family which may be used in the present invention.

**Table 1**

| Approved Gene symbol | TNFSF-number | Synonyms | Accession | NH2-aa | COOH-aa | Length |
|---|---|---|---|---|---|---|
| LTA | TNFSF-1 | LTA | gi\|6806893\|ref\|NP_000586.2\| | Ser59<br>Thr60 | Leu205<br>Leu205 | 147aa<br>146aa |
| TNF | TNFSF-2 | TNF-alpha | gi\|25952111\|ref\|NP_000585.2\| | Asp86 | Leu233 | 148aa |
| LTB | TNFSF-3 | LTB | gi\|4505035\|ref\|NP_002332.1\| | Asp82<br>Gly86 | Gly244<br>Gly244 | 163aa<br>159aa |
| TNFSF4 | TNFSF-4 | OX40L/GP34 | gi\|4507603\|ref\|NP_003317.1\| | Val52<br>Arg55 | Leu183<br>Leu183 | 132aa<br>129aa |
| CD40LG | TNFSF-5 | CD40L | gi\|4557433\|ref\|NP_000065.1\| | Asp117<br>Glu112 | Leu261<br>Leu261 | 150aa<br>145aa |
| FASLG | TNFSF-6 | CD95L/APO-L/FAS-L | gi\|4557329\|ref\|NP_000630.1\| | Glu142<br>Arg144 | Leu281<br>Leu281 | 140aa<br>138aa |
| TNFSF7 | TNFSF-7 | CD27L | gi\|4507605\|ref\|NP_001243.1\| | Glu51<br>Asp56 | Pro193<br>Pro193 | 143aa<br>138aa |
| TNFSF8 | TNFSF-8 | CD30L | gi\|4507607\|ref\|NP_001235.1\| | Lys97<br>Ser98<br>Leu102 | Asp234<br>Asp234<br>Asp234 | 138aa<br>137aa<br>133aa |
| TNFSF9 | TNFSF-9 | 4-1BB/CD137L | gi\|4507609\|ref\|NP_003802.1\| | Asp86 | Glu254 | 169aa |
| TNFSF10 | TNFSF-10 | TRAIL | gi\|4507593\|ref\|NP_003801.1\| | Glu116<br>Gly118 | Gly281<br>Gly281 | 166aa<br>164aa |
| TNFSF11 | TNFSF-11 | TRANCE/RANK L | gi\|4507595\|ref\|NP_003692.1\| | Glu161 | Asp317 | 157aa |
| TNFSF12 | TNFSF-12 | TWEAK/Apo-3 | gi\|4507597\|ref\|NP_003800.1\| | Ala103<br>Arg104<br>Arg105 | His249<br>His249<br>His249 | 147aa<br>146aa<br>145aa |
| TNFSF13 | TNFSF-13 | APRIL/TALL-2/TRDL-1 | gi\|26051248\|ref\|NP_742085.1\| | Lys112 | Leu247 | 136aa |
| TNFSF13 | TNFSF-13 | APRIL/TALL-2/TRDL-1 | gi\|4507599\|ref\|NP_003799.1\| | Lys112 | Leu250 | 139aa |
| TNFSF13B | TNFSF-13B | BAFF/Blys | gi\|5730097\|ref\|NP_006564.1\| | Glu140 | Leu285 | 146aa |
| TNFSF14 | TNFSF-14 | LIGHT | gi\|25952144\|ref\|NP_003798.2\| | Glu91 | Val240 | 150aa |
| TNFSF15 | TNFSF-15 | TL1A/VEGI | gi\|23510445\|ref\|NP_005109.2\| | Asp91<br>Asp93 | Leu251<br>Leu251 | 161aa<br>159aa |
| TNFSF18 | TNFSF-18 | GITRL | gi\|4827034\|ref\|NP_005083.1\| | Glu52 | Ser177 | 126aa |
| EDA | | EDA-A1 | gi\|4503449\|ref\|NP_001390.1\| | Glu245 | Ser391 | 147aa |
| EDA | | EDA-A2 | gi\|54112101\|ref\|NP_001005609.1\| | Glu245 | Ser389 | 145aa |

In a different aspect, the present invention refers to novel amino acid

substitution variants of human surfactant protein-D (SP-D) comprising a carbohydrate recognition domain with reduced carbohydrate binding capacity, optionally fused to at least one heterologous polypeptide or polypeptide domain as well as nucleic acid molecules encoding such fusion polypeptides. Preferably, the mutated SP-D polypeptides of the present invention have an amino acid substitutions at position F355 of human surfactant protein-D of SEQ ID NO:21, particularly an amino acid substitution by hydrophilic or charged amino acid, e.g. F355S, F355T, F355E, F355D, F355H or F355R, particularly F355D. The heterologous polypeptide or polypeptide domain is preferably of mammalian, e.g. human origin, e.g. a TNSF cytokine domain as described above. The mutated SP-D polypeptides preferably comprise an SP-D neck domain as described above. The heterologous polypeptide may be fused to N- and/or C-terminus of the SP-D domain. Preferably, a linker, e.g. a linker as described herein above, is present between the SP-D and heterologous polypeptide domain.

**Basic Structure of a Fusion Protein**

In the following, the basic structure of the recombinant proteins of the invention is shown exemplified for the TNF-superfamily cytokines as described herein.

1.1 Sequences of the Signal Peptides

MNFGFSLIFLVLVLKGVQC (SEQ ID NO:23)

METDTLLLWVLLLWVPGSTG (SEQ ID NO:24)

METDTLLLWVLLLWVPAGNG (SEQ ID NO:25)

1.2 Flag-epitope/enterokinase-processing site

DYKDDDDKD

1.3 Human Collectins

Surfactant Protein-D (SEQ ID NO:21)

1 MLLFLLSALV LLTQPLGYLE AEMKTYSHRT TPSACTLVMC SSVESGLPGR
DGRDGREGPR
61 GEKGDPGLPG AAGQAGMPGQ AGPVGPKGDN GSVGEPGPKG DTGPSGPPGP
PGVPGPAGRE
121 GPLGKQGNIG PQGKPGPKGE AGPKGEVGAP GMQGSAGARG LAGPKGERGV
PGERGVPGNA
181 GAAGSAGAMG PQGSPGARGP PGLKGDKGIP GDKGAKGESG LPDVASLRQQ
VEALQGQVQH
241 LQAAFSQYKK VELFPNGQSV GEKIFKTAGF VKPFTEAQLL CTQAGGQLAS
PRSAAENAAL
301 QQLVVAKNEA AFLSMTDSKT EGKFTYPTGE SLVYSNWAPG EPNDDGGSED
CVEIFTNGKW
361 NDRACGEKRL VVCEF

Collectin-11 (SEQ ID NO:22)

1 MRGNLALVGV LISLAFLSLL PSGHPQPAGD DACSVQILVP GLKGDAGEKG
DKGAPGRPGR
61 VGPTGEKGDM GDKGQKGSVG RHGKIGPIGS KGEKGDSGDI GPPGPNGEPG
LPCECSQLRK
121 AIGEMDNQVS QLTSELKFIK NAVAGVRETE SKIYLLVKEE KRYADAQLSC
QGRGGTLSMP
181 KDEAANGLMA AYLAQAGLAR VFIGINDLEK EGAFVYSDHS PMRTFNKWRS
GEPNNAYDEE
241 DCVEMVASGG WNDVACHTTM YFMCEFDKEN M

Various fragments of the human collectins Surfactant protein-D and collectin-11 are conceivable as trimerization domains as described herein.

1.4 Flexible Linker Element

$(GSS)_a(SSG)_b(GSG)_c$ wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6

1.5 TNF-Superfamily Cytokine/ Receptor Binding Domain thereof (see also Table 1)

SEQ-ID-01
SEQ NP_000586_TNFSF1_LTA
KEYWORD PROTEIN
FEATURES

ORIGIN

1 MTPPERLFLP RVCGTTLHLL LLGLLLVLLP GAQGLPGVGL TPSAAQTARQ
HPKMHLAHST
61 LKPAAHLIGD PSKQNSLLWR ANTDRAFLQD GFSLSNNSLL VPTSGIYFVY
SQVVFSGKAY

121 SPKATSSPLY LAHEVQLFSS QYPFHVPLLS SQKMVYPGLQ EPWLHSMYHG
AAFQLTQGDQ
181 LSTHTDGIPH LVLSPSTVFF GAFAL

SEQ-ID-02
SEQ NP_000585_TNFSF2_TNFa
KEYWORD PROTEIN

ORIGIN
1 MSTESMIRDV ELAEEALPKK TGGPQGSRRC LFLSLFSFLI VAGATTLFCL
LHFGVIGPQR
61 EEFPRDLSLI SPLAQAVRSS SRTPSDKPVA HVVANPQAEG QLQWLNRRAN
ALLANGVELR
121 DNQLVVPSEG LYLIYSQVLF KGQGCPSTHV LLTHTISRIA VSYQTKVNLL
SAIKSPCQRE
181 TPEGAEAKPW YEPIYLGGVF QLEKGDRLSA EINRPDYLDF AESGQVYFGI IAL

SEQ-ID-03
SEQ NP_002332_TNFSF3_LTB
KEYWORD PROTEIN

ORIGIN
1 MGALGLEGRG GRLQGRGSLL LAVAGATSLV TLLLAVPITV LAVLALVPQD
QGGLVTETAD
61 PGAQAQQGLG FQKLPEEEPE TDLSPGLPAA HLIGAPLKGQ GLGWETTKEQ
AFLTSGTQFS
121 DAEGLALPQD GLYYLYCLVG YRGRAPPGGG DPQGRSVTLR SSLYRAGGAY
GPGTPELLLE
181 GAETVTPVLD PARRQGYGPL WYTSVGFGGL VQLRRGERVY VNISHPDMVD
FARGKTFFGA
241 VMVG

SEQ-ID-04
SEQ NP_003317_TNFSF4_OX40L
KEYWORD PROTEIN

ORIGIN
1 MERVQPLEEN VGNAARPRFE RNKLLLVASV IQGLGLLLCF TYICLHFSAL
QVSHRYPRIQ
61 SIKVQFTEYK KEKGFILTSQ KEDEIMKVQN NSVIINCDGF YLISLKGYFS
QEVNISLHYQ
121 KDEEPLFQLK KVRSVNSLMV ASLTYKDKVY LNVTTDNTSL DDFHVNGGEL
ILIHQNPGEF
181 CVL

SEQ-ID-05
SEQ NP_000065_TNFSF5_CD40L
KEYWORD PROTEIN

ORIGIN

1 MIETYNQTSP RSAATGLPIS MKIFMYLLTV FLITQMIGSA LFAVYLHRRL
DKIEDERNLH
61 EDFVFMKTIQ RCNTGERSLS LLNCEEIKSQ FEGFVKDIML NKEETKKENS
FEMQKGDQNP
121 QIAAHVISEA SSKTTSVLQW AEKGYYTMSN NLVTLENGKQ LTVKRQGLYY
IYAQVTFCSN
181 REASSQAPFI ASLCLKSPGR FERILLRAAN THSSAKPCGQ QSIHLGGVFE
LQPGASVFVN
241 VTDPSQVSHG TGFTSFGLLK L

SEQ-ID-06
SEQ NP_000630_TNFSF6_CD95L
KEYWORD PROTEIN

ORIGIN
1 MQQPFNYPYP QIYWVDSSAS SPWAPPGTVL PCPTSVPRRP GQRRPPPPPP
PPPLPPPPPP
61 PPLPPLPLPP LKKRGNHSTG LCLLVMFFMV LVALVGLGLG MFQLFHLQKE
LAELRESTSQ
121 MHTASSLEKQ IGHPSPPPEK KELRKVAHLT GKSNSRSMPL EWEDTYGIVL
LSGVKYKKGG
181 LVINETGLYF VYSKVYFRGQ SCNNLPLSHK VYMRNSKYPQ DLVMMEGKMM
SYCTTGQMWA
241 RSSYLGAVFN LTSADHLYVN VSELSLVNFE ESQTFFGLYK L

SEQ-ID-07
SEQ NP_001243_TNFSF7_CD27L
KEYWORD PROTEIN

ORIGIN
1 MPEEGSGCSV RRRPYGCVLR AALVPLVAGL VICLVVCIQR FAQAQQQLPL
ESLGWDVAEL
61 QLNHTGPQQD PRLYWQGGPA LGRSFLHGPE LDKGQLRIHR DGIYMVHIQV
TLAICSSTTA
121 SRHHPTTLAV GICSPASRSI SLLRLSFHQG CTIASQRLTP LARGDTLCTN
LTGTLLPSRN
181 TDETFFGVQW VRP

SEQ-ID-08
SEQ NP_001235_TNFSF8_CD30L
KEYWORD PROTEIN

ORIGIN
1 MDPGLQQALN GMAPPGDTAM HVPAGSVASH LGTTSRSYFY LTTATLALCL
VFTVATIMVL
61 VVQRTDSIPN SPDNVPLKGG NCSEDLLCIL KRAPFKKSWA YLQVAKHLNK
TKLSWNKDGI
121 LHGVRYQDGN LVIQFPGLYF IICQLQFLVQ CPNNSVDLKL ELLINKHIKK
QALVTVCESG
181 MQTKHVYQNL SQFLLDYLQV NTTISVNVDT FQYIDTSTFP LENVLSIFLY SNSD

SEQ-ID-09
SEQ NP_003802_TNFSF9_CD137L
KEYWORD PROTEIN

ORIGIN
1 MEYASDASLD PEAPWPPAPR ARACRVLPWA LVAGLLLLLL LAAACAVFLA
CPWAVSGARA
61 SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY
SDPGLAGVSL
121 TGGLSYKEDT KELVVAKAGV YYVFFQLELR RVVAGEGSGS VSLALHLQPL
RSAAGAAALA
181 LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVHLHTEA RARHAWQLTQ
GATVLGLFRV
241 TPEIPAGLPS PRSE

SEQ-ID-10
SEQ NP_003801_TNFSF10_TRAIL
KEYWORD PROTEIN

ORIGIN
1 MAMMEVQGGP SLGQTCVLIV IFTVLLQSLC VAVTYVYFTN ELKQMQDKYS
KSGIACFLKE
61 DDSYWDPNDE ESMNSPCWQV KWQLRQLVRK MILRTSEETI STVQEKQQNI
SPLVRERGPQ
121 RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
NGELVIHEKG
181 FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI LLMKSARNSC
WSKDAEYGLY
241 SIYQGGIFEL KENDRIFVSV TNEHLIDMDH EASFFGAFLV G

SEQ-ID-11
SEQ NP_003692_TNFSF11_a_RANKL
KEYWORD PROTEIN

ORIGIN
1 MRRASRDYTK YLRGSEEMGG GPGAPHEGPL HAPPPPAPHQ PPAASRSMFV
ALLGLGLGQV
61 VCSVALFFYF RAQMDPNRIS EDGTHCIYRI LRLHENADFQ DTTLESQDTK
LIPDSCRRIK
121 QAFQGAVQKE LQHIVGSQHI RAEKAMVDGS WLDLAKRSKL EAQPFAHLTI
NATDIPSGSH
181 KVSLSSWYHD RGWAKISNMT FSNGKLIVNQ DGFYYLYANI CFRHHETSGD
LATEYLQLMV
241 YVTKTSIKIP SSHTLMKGGS TKYWSGNSEF HFYSINVGGF FKLRSGEEIS
IEVSNPSLLD
301 PDQDATYFGA FKVRDID

SEQ-ID-12
SEQ NP_003800_TNFSF12_TWEAK
KEYWORD PROTEIN
ORIGIN

1 MAARRSQRRR GRRGEPGTAL LVPLALGLGL ALACLGLLLA VVSLGSRASL SAQEPAQEEL
61 VAEEDQDPSE LNPQTEESQD PAPFLNRLVR PRRSAPKGRK TRARRAIAAH YEVHPRPGQD
121 GAQAGVDGTV SGWEEARINS SSPLRYNRQI GEFIVTRAGL YYLYCQVHFD EGKAVYLKLD
181 LLVDGVLALR CLEEFSATAA SSLGPQLRLC QVSGLLALRP GSSLRIRTLP WAHLKAAPFL
241 TYFGLFQVH

SEQ-ID-13
SEQ NP_742085_TNFSF13_APRIL_ver1
KEYWORD PROTEIN
ORIGIN
1 MPASSPFLLA PKGPPGNMGG PVREPALSVA LWLSWGAALG AVACAMALLT QQTELQSLRR
61 EVSRLQGTGG PSQNGEGYPW QSLPEQSSDA LEAWENGERS RKRRAVLTQK QKKQHSVLHL
121 VPINATSKDD SDVTEVMWQP ALRRGRGLQA QGYGVRIQDA GVYLLYSQVL FQDVTFTMGQ
181 VVSREGQGRQ ETLFRCIRSM PSHPDRAYNS CYSAGVFHLH QGDILSVIIP RARAKLNLSP
241 HGTFLGL

SEQ-ID-14
SEQ NP_003799_TNFSF13_APRIL_ver2
KEYWORD PROTEIN

ORIGIN
1 MPASSPFLLA PKGPPGNMGG PVREPALSVA LWLSWGAALG AVACAMALLT QQTELQSLRR
61 EVSRLQGTGG PSQNGEGYPW QSLPEQSSDA LEAWENGERS RKRRAVLTQK QKKQHSVLHL
121 VPINATSKDD SDVTEVMWQP ALRRGRGLQA QGYGVRIQDA GVYLLYSQVL FQDVTFTMGQ
181 VVSREGQGRQ ETLFRCIRSM PSHPDRAYNS CYSAGVFHLH QGDILSVIIP RARAKLNLSP
241 HGTFLGFVKL

SEQ-ID-15
SEQ NP_006564_TNFSF13b_BAFF
KEYWORD PROTEIN

ORIGIN
1 MDDSTEREQS RLTSCLKKRE EMKLKECVSI LPRKESPSVR SSKDGKLLAA TLLLALLSCC
61 LTVVSFYQVA ALQGDLASLR AELQGHHAEK LPAGAGAPKA GLEEAPAVTA GLKIFEPPAP
121 GEGNSSQNSR NKRAVQGPEE TVTQDCLQLI ADSETPTIQK GSYTFVPWLL SFKRGSALEE
181 KENKILVKET GYFFIYGQVL YTDKTYAMGH LIQRKKVHVF GDELSLVTLF RCIQNMPETL

241 PNNSCYSAGI AKLEEGDELQ LAIPRENAQI SLDGDVTFFG ALKLL

SEQ-ID-16
SEQ NP_003798_TNFSF14_LIGHT
KEYWORD PROTEIN

ORIGIN
1 MEESVVRPSV FVVDGQTDIP FTRLGRSHRR QSCSVARVGL GLLLLLMGAG
LAVQGWFLLQ
61 LHWRLGEMVT RLPDGPAGSW EQLIQERRSH EVNPAAHLTG ANSSLTGSGG
PLLWETQLGL
121 AFLRGLSYHD GALVVTKAGY YYIYSKVQLG GVGCPLGLAS TITHGLYKRT
PRYPEELELL
181 VSQQSPCGRA TSSSRVWWDS SFLGGVVHLE AGEKVVVRVL DERLVRLRDG
TRSYFGAFMV

SEQ-ID-17
SEQ NP_005109_TNFSF15_TL1A
KEYWORD PROTEIN

ORIGIN
1 MAEDLGLSFG ETASVEMLPE HGSCRPKARS SSARWALTCC LVLLPFLAGL
TTYLLVSQLR
61 AQGEACVQFQ ALKGQEFAPS HQQVYAPLRA DGDKPRAHLT VVRQTPTQHF
KNQFPALHWE
121 HELGLAFTKN RMNYTNKFLL IPESGDYFIY SQVTFRGMTS ECSEIRQAGR
PNKPDSITVV
181 ITKVTDSYPE PTQLLMGTKS VCEVGSNWFQ PIYLGAMFSL QEGDKLMVNV
SDISLVDYTK
241 EDKTFFGAFL L

SEQ-ID-18
SEQ NP_005083_TNFSF18_GITRL
KEYWORD PROTEIN

ORIGIN
1 MCLSHLENMP LSHSRTQGAQ RSSWKLWLFC SIVMLLFLCS FSWLIFIFLQ
LETAKEPCMA
61 KFGPLPSKWQ MASSEPPCVN KVSDWKLEIL QNGLYLIYGQ VAPNANYNDV
APFEVRLYKN
121 KDMIQTLTNK SKIQNVGGTY ELHVGDTIDL IFNSEHQVLK NNTYWGIILL
ANPQFIS

SEQ-ID-19
SEQ NP_001390_EDA-A1
KEYWORD PROTEIN
ORIGIN
1 MGYPEVERRE LLPAAAPRER GSQGCGCGGA PARAGEGNSC LLFLGFFGLS
LALHLLTLCC
61 YLELRSELRR ERGAESRLGG SGTPGTSGTL SSLGGLDPDS PITSHLGQPS

PKQQPLEPGE
121 AALHSDSQDG HQMALLNFFF PDEKPYSEEE SRRVRRNKRS KSNEGADGPV
KNKKKGKKAG
181 PPGPNGPPGP PGPPGPQGPP GIPGIPGIPG TTVMGPPGPP GPPGPQGPPG
LQGPSGAADK
241 AGTRENQPAV VHLQGQGSAI QVKNDLSGGV LNDWSRITMN PKVFKLHPRS
GELEVLVDGT
301 YFIYSQVEVY YINFTDFASY EVVVDEKPFL QCTRSIETGK TNYNTCYTAG
VCLLKARQKI
361 AVKMVHADIS INMSKHTTFF GAIRLGEAPA S

SEQ-ID-20
SEQ NP_001005609_EDA-A2
KEYWORD PROTEIN

ORIGIN
1 MGYPEVERRE LLPAAAPRER GSQGCGCGGA PARAGEGNSC LLFLGFFGLS
LALHLLTLCC
61 YLELRSELRR ERGAESRLGG SGTPGTSGTL SSLGGLDPDS PITSHLGQPS
PKQQPLEPGE
121 AALHSDSQDG HQMALLNFFF PDEKPYSEEE SRRVRRNKRS KSNEGADGPV
KNKKKGKKAG
181 PPGPNGPPGP PGPPGPQGPP GIPGIPGIPG TTVMGPPGPP GPPGPQGPPG
LQGPSGAADK
241 AGTRENQPAV VHLQGQGSAI QVKNDLSGGV LNDWSRITMN PKVFKLHPRS
GELEVLVDGT
301 YFIYSQVYYI NFTDFASYEV VVDEKPFLQC TRSIETGKTN YNTCYTAGVC
LLKARQKIAV
361 KMVHADISIN MSKHTTFFGA IRLGEAPAS

Various fragments, e.g., receptor binding domains, of TNF-superfamily cytokines are conceivable as described herein.

## 1.6 Examples of Fusion Proteins

**SEQ ID NO:26** SP-hsTrailsyn-SPD-Konstrukt-1_PRO.PRO
KEYWORD PROTEIN

ORIGIN
1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR
KINSWESSRS
61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY
IYKYTSYPDP
121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD
HEASFFGAFL
181 VGSGLPDVAS LRQQVEALQG QVQHLQAAFS QYKKVELFPN GQSVGEKIFK
TAGFVKPFTE
241 AQLLCTQAGG QLASPRSAAE NAALQQLVVA KNEAAFLSMT DSKTEGKFTY
PTGESLVYSN

301 WAPGEPNDDG GSEDCVEIFT NGKWNDRACG EKRLVVCEF

**SEQ ID NO:27** SP-hsTrailsyn-SPD-Konstrukt-2_PRO.PRO
KEYWORD PROTEIN

ORIGIN
1 METDTLLLWV LLLWVPGSTG ERGPQRVAAH ITGTRGRSNT LSSPNSKNEK ALGRKINSWE
61 SSRSGHSFLS NLHLRNGELV IHEKGFYYIY SQTYFRFQEE IKENTKNDKQ MVQYIYKYTS
121 YPDPILLMKS ARNSCWSKDA EYGLYSIYQG GIFELKENDR IFVSVTNEHL IDMDHEASFF
181 GAFLVGSGLP DVASLRQQVE ALQGQVQHLQ AAFSQYKKVE LFPNGQSVGE KIFKTAGFVK
241 PFTEAQLLCT QAGGQLASPR SAAENAALQQ LVVAKNEAAF LSMTDSKTEG KFTYPTGESL
301 VYSNWAPGEP NDDGGSEDCV EIFTNGKWND RACGEKRLVV CEF

**SEQ ID NO:28**
ORIGIN
1 METDTLLLWV LLLWVPGSTG ERGPQRVAAH ITGTRGRSNT LSSPNSKNEK ALGRKINSWE
61 SSRSGHSFLS NLHLRNGELV IHEKGFYYIY SQTYFRFQEE IKENTKNDKQ MVQYIYKYTS
121 YPDPILLMKS ARNSCWSKDA EYGLYSIYQG GIFELKENDR IFVSVTNEHL IDMDHEASFF
181 GAFLVGSGLP DVASLRQQVE ALQGQVQHLQ AAFSQYKKVE LFPNG

**SEQ ID NO:29** SP-hsTrailsyn-coll11-Konstrukt-1.pro
KEYWORD PROTEIN

ORIGIN
1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR KINSWESSRS
61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY IYKYTSYPDP
121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD HEASFFGAFL
181 VGSQLRKAIG EMDNQVSQLT SELKFIKNAV AGVRETESKI YLLVKEEKRY ADAQLSCQGR
241 GGTLSMPKDE AANGLMAAYL AQAGLARVFI GINDLEKEGA FVYSDHSPMR TFNKWRSGEP
301 NNAYDEEDCV EMVASGGWND VACHTTMYFM CEFDKENM

**SEQ ID NO:30** SP-hsTrailsyn-coll-11-Konstrukt-2.pro
KEYWORD PROTEIN

ORIGIN
1 METDTLLLWV LLLWVPGSTG ERGPQRVAAH ITGTRGRSNT LSSPNSKNEK ALGRKINSWE

61 SSRSGHSFLS NLHLRNGELV IHEKGFYYIY SQTYFRFQEE IKENTKNDKQ
MVQYIYKYTS
121 YPDPILLMKS ARNSCWSKDA EYGLYSIYQG GIFELKENDR IFVSVTNEHL
IDMDHEASFF
181 GAFLVGSQLR KAIGEMDNQV SQLTSELKFI KNAVAGVRET ESKIYLLVKE
EKRYADAQLS
241 CQGRGGTLSM PKDEAANGLM AAYLAQAGLA RVFIGINDLE KEGAFVYSDH
SPMRTFNKWR
301 SGEPNNAYDE EDCVEMVASG GWNDVACHTT MYFMCEFDKE NM

**SEQ ID NO:31** SP-hsTrailsyn-coll-11-Konstrukt-3.pro
KEYWORD PROTEIN

ORIGIN
1 METDTLLLWV LLLWVPGSTG ERGPQRVAAH ITGTRGRSNT LSSPNSKNEK
ALGRKINSWE
61 SSRSGHSFLS NLHLRNGELV IHEKGFYYIY SQTYFRFQEE IKENTKNDKQ
MVQYIYKYTS
121 YPDPILLMKS ARNSCWSKDA EYGLYSIYQG GIFELKENDR IFVSVTNEHL
IDMDHEASFF

181 GAFLVGSQLR KAIGEMDNQV SQLTSELKFI KNAVAGVRET ES

**SEQ ID NO:32** FLAG-hCol11-hTRAIL_Glu116_Gly281.pro
KEYWORD PROTEIN
ORIGIN
1 MNFGFSLIFL VLVLKGVQCD YKDDDDKGLP CECSQLRKAI GEMDNQVSQL
TSELKFIKNA
61 VAGVRETESE RGPQRVAAHI TGTRGRSNTL SSPNSKNEKA LGRKINSWES
SRSGHSFLSN
121 LHLRNGELVI HEKGFYYIYS QTYFRFQEEI KENTKNDKQM VQYIYKYTSY
PDPILLMKSA
181 RNSCWSKDAE YGLYSIYQGG IFELKENDRI FVSVTNEHLI DMDHEASFFG
AFLVG

**SEQ ID NO:33** FLAG-hCol11s-hTRAIL_Glu116_Gly281.pro
KEYWORD PROTEIN
ORIGIN
1 MNFGFSLIFL VLVLKGVQCD YKDDDDKGLP CECSQLRKAI GEMDNQVSQL
TSELKFIKNA
61 VAGVRETERG PQRVAAHITG TRGRSNTLSS PNSKNEKALG RKINSWESSR
SGHSFLSNLH
121 LRNGELVIHE KGFYYIYSQT YFRFQEEIKE NTKNDKQMVQ YIYKYTSYPD
PILLMKSARN

181 SCWSKDAEYG LYSIYQGGIF ELKENDRIFV SVTNEHLIDM DHEASFFGAF LVG

**SEQ ID NO:34** hCol11s-hTRAIL_Glu116_Gly281.pro
KEYWORD PROTEIN
ORIGIN
1 MNFGFSLIFL VLVLKGVQCG LPCECSQLRK AIGEMDNQVS QLTSELKFIK
NAVAGVRETE
61 RGPQRVAAHI TGTRGRSNTL SSPNSKNEKA LGRKINSWES SRSGHSFLSN
LHLRNGELVI

121 HEKGFYYIYS QTYFRFQEEI KENTKNDKQM VQYIYKYTSY PDPILLMKSA RNSCWSKDAE

181 YGLYSIYQGG IFELKENDRI FVSVTNEHLI DMDHEASFFG AFLVG

**SEQ ID NO:35** FLAG-hCol11-GSS-hTRAIL_Glu116_Gly281.pro

KEYWORD PROTEIN

ORIGIN

1 MNFGFSLIFL VLVLKGVQCD YKDDDDKGLP CECSQLRKAI GEMDNQVSQL TSELKFIKNA

61 VAGVRETESG SSGSSGSSGS GERGPQRVAA HITGTRGRSN TLSSPNSKNE KALGRKINSW

121 ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE EIKENTKNDK QMVQYIYKYT

181 SYPDPILLMK SARNSCWSKD AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF

241 FGAFLVG

**SEQ ID NO:36** Sp1-hTRAIL_Glu116_Gly281-GSS-coll11.pro

KEYWORD PROTEIN

ORIGIN

1 MNFGFSLIFL VLVLKGVQCE RGPQRVAAHI TGTRGRSNTL SSPNSKNEKA LGRKINSWES

61 SRSGHSFLSN LHLRNGELVI HEKGFYYIYS QTYFRFQEEI KENTKNDKQM VQYIYKYTSY

121 PDPILLMKSA RNSCWSKDAE YGLYSIYQGG IFELKENDRI FVSVTNEHLI DMDHEASFFG

181 AFLVGSSGSS GSSGSGLPCE CSQLRKAIGE MDNQVSQLTS ELKFIKNAVA GVRETES

**SEQ ID NO:37** Sp3-hTRAIL_Glu116_Gly281-GSS-coll11.pro

KEYWORD PROTEIN

ORIGIN

1 METDTLLLWV LLLWVPAGNG ERGPQRVAAH ITGTRGRSNT LSSPNSKNEK ALGRKINSWE

61 SSRSGHSFLS NLHLRNGELV IHEKGFYYIY SQTYFRFQEE IKENTKNDKQ MVQYIYKYTS

121 YPDPILLMKS ARNSCWSKDA EYGLYSIYQG GIFELKENDR IFVSVTNEHL IDMDHEASFF

181 GAFLVGSSGS SGSSGSGLPC ECSQLRKAIG EMDNQVSQLT SELKFIKNAV AGVRETES

**SEQ ID NO:38** SP-hsTrailsyn-SPD-Konstrukt-1_DNA.seq: 1045 bp

KEYWORD DNA (DNA coding sequence corresponding to SEQ ID NO:26 starts at base position 16)

ORIGIN

1 AAGCTTGCCG CCACCATGGA GACCGATACA CTGCTCTTGT GGGTGCTCTT GCTGTGGGTT

61 CCTGCAGGTA ATGGTCAAAG AGTCGCAGCT CACATCACTG GGACTAGAGG CAGGAGTAAC

121 ACCCTGAGTT CTCCCAATTC CAAGAACGAG AAAGCCCTGG GTAGGAAGAT CAACTCCTGG

181 GAAAGCTCCA GAAGCGGCCA TAGCTTTCTT AGCAACCTCC ACTTGAGGAA

TGGCGAACTT
241 GTGATCCATG AGAAGGGCTT CTACTACATC TACAGCCAGA CGTACTTCAG
GTTCCAGGAG
301 GAAATCAAGG AGAACACCAA GAACGACAAG CAGATGGTGC AATACATCTA
CAAGTACACG
361 TCATACCCTG ATCCTATACT GCTGATGAAG TCCGCCAGAA ACAGTTGCTG
GAGCAAAGAC
421 GCTGAATACG GCCTGTATTC CATCTATCAG GGCGGTATCT TTGAACTCAA
GGAGAACGAC
481 AGGATCTTCG TGTCTGTGAC AAACGAGCAT CTGATCGACA TGGACCATGA
AGCGTCTTTC
541 TTCGGTGCCT TCTTGGTGGG ATCCGGTTTG CCAGATGTTG CTTCTTTGAG
ACAACAGGTT
601 GAGGCTTTGC AGGGTCAAGT CCAGCACTTG CAGGCTGCTT TCTCTCAATA
CAAGAAGGTT
661 GAGTTGTTCC CAAATGGTCA ATCTGTTGGC GAAAAGATTT TCAAGACTGC
TGGTTTCGTC
721 AAACCATTCA CGGAGGCACA ATTATTGTGT ACTCAGGCTG GTGGACAGTT
GGCCTCTCCA
781 CGTTCTGCCG CTGAGAACGC CGCCTTGCAA CAATTAGTCG TAGCTAAGAA
CGAGGCTGCT
841 TTCTTGAGCA TGACTGATTC CAAGACAGAG GGCAAGTTCA CCTACCCAAC
AGGAGAATCC
901 TTGGTCTATT CTAATTGGGC ACCTGGAGAG CCCAACGATG ATGGCGGCTC
AGAGGACTGT
961 GTGGAAATCT TCACCAATGG CAAGTGGAAT GACAGAGCTT GTGGAGAGAA
GCGTTTGGTG
1021 GTCTGTGAGT TCTAATAGCG GCCGC

**SEQ ID NO:39** SP-hsTrailsyn-SPD-Konstrukt-2_DNA.seq: 1057 bp
KEYWORD DNA (DNA coding sequence corresponding to SEQ ID NO:27 starts at base position 16)

ORIGIN
1 AAGCTTGCCG CCACCATGGA GACCGATACA CTGCTCTTGT GGGTACTCTT
GCTGTGGGTT
61 CCGGGATCTA CCGGTGAACG TGGTCCTCAA AGAGTCGCAG CTCACATCAC
TGGGACTAGA
121 GGCAGGAGTA ACACCCTGAG TTCTCCCAAT TCCAAGAACG AGAAAGCCCT
GGGTAGGAAG
181 ATCAACTCCT GGGAAAGCTC CAGAAGCGGC CATAGCTTTC TTAGCAACCT
CCACTTGAGG
241 AATGGCGAAC TTGTGATCCA TGAGAAGGGC TTCTACTACA TCTACAGCCA
GACGTACTTC
301 AGGTTCCAGG AGGAAATCAA GGAGAACACC AAGAACGACA AGCAGATGGT
GCAATACATC
361 TACAAGTACA CGTCATACCC TGATCCTATA CTGCTGATGA AGTCCGCCAG
AAACAGTTGC
421 TGGAGCAAAG ACGCTGAATA CGGCCTGTAT TCCATCTATC AGGGCGGTAT
CTTTGAACTC
481 AAGGAGAACG ACAGGATCTT CGTGTCTGTG ACAAACGAGC ATCTGATCGA
CATGGACCAT
541 GAAGCGTCTT TCTTCGGTGC CTTCTTGGTG GGATCCGGTT TGCCAGATGT
TGCTTCTTTG
601 AGACAACAGG TTGAGGCTTT GCAGGGTCAA GTCCAGCACT TGCAGGCTGC
TTTCTCTCAA

661 TACAAGAAGG TTGAGTTGTT CCCAAATGGT CAATCTGTTG GCGAAAAGAT
TTTCAAGACT
721 GCTGGTTTCG TCAAACCATT CACGGAGGCA CAATTATTGT GTACTCAGGC
TGGTGGACAG
781 TTGGCCTCTC CACGTTCTGC CGCTGAGAAC GCCGCCTTGC AACAATTAGT
CGTAGCTAAG
841 AACGAGGCTG CTTTCTTGAG CATGACTGAT TCCAAGACAG AGGGCAAGTT
CACCTACCCA
901 ACAGGAGAAT CCTTGGTCTA TTCTAATTGG GCACCTGGAG AGCCCAACGA
TGATGGCGGC
961 TCAGAGGACT GTGTGGAAAT CTTCACCAAT GGCAAGTGGA ATGACAGAGC
TTGTGGAGAG
1021 AAGCGTTTGG TGGTCTGTGA GTTCTAATAG CGGCCGC

## Examples

### 1. Materials and methods

### 1.1 Construction of TNF-SF-proteins stabilised by a C-terminal positioned Collectin derived trimerization domain

The trimerization motifs (Tables 2 and 3) derived from human Collectin-11 (Col11), the "coiled coil" of Collectin-11 (CC11), human pulmonary surfactant protein-D (SP-D), the "coiled coil" of SP-D (CCSPD) were fused C-terminally to the human receptor binding domain (RBD) of CD95L ("CD95L-RBD"; Glu142-Leu281), human TRAIL-RBD (Gln120-Gly281), human LIGHT-RBD (Glu91-Val240) and human APRIL-RBD (Lys113-Leu250), respectively.

| Trimerization motif | Amino acids of the unprocessed wt sequences used for motif construction | Swiss-Prot entry |
|---|---|---|
| SPD | 220 - 375 | P35247 |
| SPD_F335A | 220 – 375; Phe355 -> Ala355 | P35247 |
| SPD_F335D | 220 – 375; Phe355 -> Asp355 | P35247 |
| CCSPD | 220 – 257 | P35247 |
| Col11 | 117 – 271 | Q9BWP8 |
| CC11 | 116 – 151 | Q9BWP8 |

Table 2: List of the used regions from wild type (wt) sequences for the construction of trimerizing motifs.

| Trimerization motif | Explanation |
|---|---|
| SPD | human Surfactant protein-D (coiled-coiled "neck" + Carbohydrate Recognition Domain, CRD) |
| SPD_F335A | as in 1, but with the mutation Phe -> Ala at position 335 (numbering referring to processed wild type SP-D) |
| SPD_F335D | as in 1, but with the mutation Phe -> Asp at position 335 (numbering referring to processed wild type SP-D) |
| CCSPD | coiled-coiled "neck" of human SP-D |
| Col11 | human Collectin-11 (coiled-coiled "neck" + CRD of human Collectin-11) |
| CC11 | coiled-coiled "neck" of human Collectin-11 |
| T4 | Bacteriophage T4 Whisker protein (WO2008025516) |
| 69 | Bacteriophage 69 Whisker protein (WO2008025516) |

Table 3: Explanation of C-terminal trimerization motifs used to generate stable TNFSF fusion proteins.

Between the TNFSF-RBD and the trimerization domain, a flexible linker element was placed with varying lengths (Table 4):

| Linker name | Amino-acid sequence |
|---|---|
| A | GSS GSS GSS GS |
| B | GSS GSS GS |
| C | GSS GS |
| D | GS |

Table 4: Linker names and amino acid sequence (G = glycine; S = serine)

## 1.2 Generation of Expression Constructs

The nucleic acid molecule encoding the fusion protein as described herein may be cloned into a suitable vector for expressing the fusion protein. The molecular tools necessary in order to generate such a vector are known to the skilled person and comprise restriction enzymes, vectors, and suitable host for propagating the vectors.

For purification and analytical strategies, a Strep-tag II (amino acid sequence WSHPQFEK) was added C-terminally. This affinity tag was linked to the trimerization domain by a flexible linker element (amino acid sequence PSSSSSSA). To allow for secretory based expression, signal peptides derived from human Igκ were fused to the N-termini of said proteins. The amino acid sequences of the fusion proteins were backtranslated and their codon usage optimised for mammalian cell-based expression. Gene synthesis was done by ENTELECHON GmbH (Regensburg, Germany). The final expression cassettes were subcloned into pCDNA4-HisMax-backbone, using unique Hind-III- and Not-I-sites of the plasmid. All expression cassettes were routinely verified by DNA sequencing.

Data will be presented herein for the following constructs (Table 5a and 5b):

| | **TRAIL (wild-type)** | | | | **TRAIL Mutein (R1-specific)** | | | | **TRAIL Mutein (R2-specific)** | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Linker: / Motif | A | B | C | D | A | B | C | D | A | B | C | D |
| SPD | • | • | • | • | • | n.s. | n.s. | • | • | n.s. | n.s. | • |
| SPD_F335A | • | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| SPD_F335D | • | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| CCSPD | • | • | • | • | • | n.s. | n.s. | • | • | n.s. | n.s. | • |
| Col11 | • | • | • | • | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| CC11 | • | • | • | • | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| T4 | • | • | • | • | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
| 69 | • | • | • | • | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

Table 5a: Overview of TRAIL fusion proteins with shown data. Filled circles indicate that data are presented. N.s., not shown.

| | **LIGHT** | **APRIL** | **CD95L** |
|---|---|---|---|
| Linker: / Motif | A | A | A |
| SPD | • | • | • |
| CCSPD | • | • | n.s. |
| Col11 | • | • | n.s. |
| 69 | • | • | n.s. |

Table 5b: Overview of LIGHT-, APRIL-, and CD95L-constructs with shown data. Filled circles indicate that data are presented. N.s., not shown.

## 1.3 Expression and purification of engineered ligands of the TNF Superfamily

Hek 293T cells grown in DMEM + GlutaMAX (GibCo) supplemented with 10% FBS, 100 units/ml Penicillin and 100 µg/ml Streptomycin were transiently transfected with plasmids encoding a fusion protein as described herein. Cell culture supernatant containing recombinant proteins were harvested three days post transfection and clarified by centrifugation at 300xg followed by filtration through a 0.22 µm sterile filter. For affinity purification, 4 ml of 50% Streptactin Sepharose (IBA GmbH, Göttingen, Germany) were packed to a 2 ml column and equilibrated with 30 ml phosphate buffered saline, pH 7.4 (PBS; Invitrogen Cat. 10010) or buffer W (100 mM Tris-HCl, 150 mM NaCl pH 8.0). The cell culture supernatant was applied to the column at 4°C with a flow rate of 2 ml/min. Subsequently, the column was washed with PBS or buffer W and specifically bound proteins were eluted stepwise by addition of 5 x 2 ml buffer E (PBS or buffer W with 2.5 mM Desthiobiotin, pH 7.4). The protein content of the eluate fractions was analysed by absorption spectroscopy and by silver-stained SDS-PAGE. Postitive fractions were subsequently concentrated by ultrafiltration (Sartorius, Vivaspin, 10,000 Da cut-off) and further analysed by size exclusion chromatography (SEC).

SEC was performed on a Superdex 200 column using an Äkta chromatography system (GE-Healthcare). The column was equilibrated with PBS (Invitrogen Cat. 10010) and the concentrated, streptactin purified proteins were loaded onto the SEC column at a flow rate of 0.5 ml/min. The elution of was monitored by absorbance at 280 nm. The apparent molecular weight of purified proteins were determined based on calibration of the Superdex 200 column with gel filtration standard proteins (Bio-Rad GmbH, München, Germany).

### 1.4. Cell death assays

To analyze caspase activation, a cellular assay with the Jurkat A3 permanent human T-cell line (cat. no. CRL2570, ATCC) was used. Jurkat cells were grown in flasks with RPMI 1640-medium + GlutaMAX (GibCo) supplemented with 10 % FBS (Biochrom), 100 units/ml Penicillin and 100 µg/ml Streptomycin (GibCo). Prior to the assay, 100,000 cells were seeded per well into a 96-well microtiterplate. The addition of different solutions containing the protein with or without a crosslinking antibody to the wells (final volume: 200 µl) was followed by a 3 hour incubation at 37°C. Cells were lysed by adding 20 µl lysis buffer (250 mM HEPES, 50 mM $MgCl_2$, 10 mM EGTA, 5 % Triton-X-100, 100 mM DTT, 10 mM AEBSF, pH 7.5) and plates were incubated on ice for 30 minutes to 2 hours. Apoptosis is paralleled by an increased activity of Caspases. Hence, cleavage of the specific Caspase substrate Ac-DEVD-AFC (Biomol) was used to determine the extent of apoptosis. For the Caspase activity assay, 20 µl cell lysate was transferred to a black 96-well microtiterplate. After the addition of 80 µl buffer containing 50 mM HEPES, 1 % Sucrose, 0.1 % CHAPS, 50 µM Ac-DEVD-AFC, and 25 mM DTT, pH 7.5, the plate was transferred to a Tecan Infinite F500 microtiterplate reader and the increase in fluorescence intensity was monitored (excitation wavelength 400 nm, emission wavelength 505 nm).

For the determination of cell death in HT1080 fibrosarcoma, HeLa cervix carcinoma and WM35 melanoma cells, 15,000 cells were plated in 96-well plates over night in RPMI 1640-medium + GlutaMAX (GibCo) supplemented with 10 % FBS (Biochrom). For Colo205 cells, 50,000 cells were plated over night. Cells were stimulated the following day with indicated ligand and incubated for an additional 18 hours. For HeLa and HT1080 cells, cycloheximide (Sigma) at a final concentration of 2.5 µg/ml was used during stimulation with ligands. Cell death of HT1080, HeLa and WM35 was quantified by staining with buffer KV (0.5% crystal violet, 20% methanol). After staining, the wells were washed with water and air-dried. The dye was eluted with methanol and optical density at 595 nm was measured with an

ELISA reader. Viability of Colo205 cells was quantified by MTS assay (Promega).

### 1.5 Hepatocellular cytotoxicity assay

To determine the effect of TRAIL fusion proteins, primary human hepatocytes were prepared from healthy donors and cultured in Williams E medium using 25,000 cells per well in 96-well plates. At day two, medium was changed to DMEM-F12 supplemented with 10% FCS, human insulin, Pen/Strep, minimum essential medium (MEM), sodium pyruvate and 10 mM Hepes and cultured for another day. Cells were stimulated at day three with varying concentrations of indicated proteins in presence or absence of cross-linking antibodies (StrepMabImmo, IBA GmbH). To evaluate the potential hepatotoxic effect of a cotreatment of ligands with chemotherapeutic agents, TRAIL-ASPD_F335D was coincubated at varying concentrations together with 5 mM of doxorubicin or 5 mM gemcitabine. Cells were incubated for 5 or 24 hours at 37°C and 5% $CO_2$ and were then lysed for determination of caspase activity as described in section „Cell death assays".

### 1.6 Streptactin-ELISA

To determine the binding of receptors to constructed ligands, streptactin-coated 96-well microplates were used. Therefore, supernatants from transiently transfected HEK293 cells, mouse sera or purified proteins were immobilized on streptactin-plates (IBA GmbH) for 1-3 hours in PBS. Samples were diluted in ELISA binding/blocking buffer (PBS, 0.1% Tween-20, 20% SuperBlock T20-PBS (Pierce)). Plates were washed with PBS + 0.1% Tween-20 and incubated with mouse-anti-TRAIL antibody (Pharmingen, clone RIK-2), TRAIL-Receptor 1-Fc (R&D Systems), TRAIL-Receptor 2-Fc (R&D Systems), TACI-Fc (R&D Systems) or HVEM-Fc (R&D Systems) for one hour at room temperature. Plates were again washed and Fc-proteins were detected with anti-human- or anti-mouse-Fc-specific peroxidase-conjugated antibodies (Sigma). Colour reaction was done by addition of 100 µl per well of TMB substrate (Kem-En-Tec Diagnostics) and the absorbance at 450 nm and 630 nm was determined

with an ELISA reader after addition of 25 µl of 25% $H_2SO_4$ as stop-solution. Values were calculated as 450 nm – 630 nm with MS Excel.

### 1.7 Mannan-binding assay

ELISA plates (Nunc Maxisorp) were incubated over night at 4°C with 10 µg/well of yeast mannan (Sigma) in sterile coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, 0.025% $NaN_3$, pH 9.6). Plates were first incubated for one hour at room temperature with buffer BB (20 mM Tris, 140 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA and 20% SuperBlock T20-PBS (Pierce)) and secondly for additional 90 minutes with varying concentrations of indicated ligands in buffer BB. Plates were washed with buffer WB (20 mM Tris, 140 mM NaCl, 5 mM $CaCl_2$, 0.05% Tween-20) and detection was done by using streptactin-HRP (IBA GmbH) in buffer BB. Plates were washed and developed with TMB substrate (Kem-En-Tec Diagnostics). The absorption at 450 nm and 630 nm was determined with an ELISA reader after addition of 25 µl of 25% $H_2SO_4$ as stop-solution. Values were calculated as 450 nm – 630 nm with MS Excel.

### 1.8 Pharmacokinetics of TRAIL-SPD fusion proteins

Male CD1 mice (Charles River) were intravenously injected with 10 µg protein dissolved in 300 µl PBS (Invitrogen). Blood was collected after 0 min (predose), 5 min, 30 min, 2 hours, 6 hours and 24 hours. For each time point, two samples were collected. Blood samples were processed to obtain serum and were stored at -15°C. The concentration of TRAIL-fusion proteins was determined using an ELISA as described below (chapter 1.9) and half-lives were calculated (GraphPad Prism v4.0).

### 1.9 ELISA for the quantitation of TRAIL-constructs in mouse sera

To quantitate the concentration of TRAIL proteins in mouse sera (originating from pharmacokinetic studies), an ELISA method employing 96-well microplates was used.

ELISA plates were coated for 1 h at 37°C with 2 µg/ml mouse-anti-TRAIL

(clone RIK-2; Pharmingen). After washing with PBS + 0.1% Tween-20 and blocking the plate for 30 min at 37°C with StartingBlock™ (Pierce), serum samples at a concentration of 0.2 % and 5 %, calibration samples and control samples were added and incubated for 1 h at 37°C. Calibration and control samples were prepared from the respective TRAIL batch (TRAIL-ASPD or TRAIL-ASPD-F335A or TRAIL-ASPD-F335D) and were supplemented with 0.2 % or 5 % non-treated pooled CD1-mouse serum to account for potential matrix effects. Control samples (high, medium and low concentration of the TRAIL-construct) were added as quality controls to ensure precision and accuracy of the TRAIL-quantitation in the given assay window. Plates were again washed and the StrepTag-containing TRAIL-constructs were detected with 1:1000 diluted StrepTactin-POD (IBA). All samples and proteins were diluted with ELISA buffer (PBS, 0.1% Tween-20, 5% StartingBlock (Pierce)). The colour reaction started after addition of 100 µl per well TMB substrate (Kem-En-Tec Diagnostics). the absorbance at 450 nm and 630 nm was determined with an ELISA reader after addition of 25 µl of 25% $H_2SO_4$ as stop-solution. Values were calculated as 450 nm – 630 nm with MS Excel.

## 2. Results

### 2.1 Characterization of CD95L fusion protein (CD95L-ASPD)

From the Streptactin-affinity purified CD95L-ASPD 0.5 ml (0.86 mg protein) were loaded with a flow rate of 0.5 ml/min onto a Superdex200 column using PBS as running buffer. Fractions of 0.5 ml were collected (A1 to A11 are indicated). The retention volume of the major peak at 11.92 ml corresponded to 170 kDa as determined from size exclusion standard. This indicated that the protein is a trimer composed of glycosylated monomers. The calculated molecular weight of the monomeric polypeptide is 38 kDa. An aliquot of fractions A1 to A11 was used for SDS-PAGE and caspase activity. Only the defined trimeric peak (fractions A7 to A10) was used for final analyses. The results are shown in Fig. 1.

An aliquot from size exclusion chromatography of affinity purified CD95L-ASPD was used for reducing SDS-PAGE followed by silver staining. The band detected at approximately 40-45 kDa (indicated by an arrow) corresponded to CD95L-ASPD. The trimeric species was present in fractions A7 to A10. The results are shown in Fig. 2.

Jurkat cells were incubated with aliquots at a final 8-fold dilution from fractions A1 to A15 from SEC with affinity purified CD95L-ASPD. Cells were lysed after 3h incubation and the caspase activity was determined with a fluorogenic assay. The fractions corresponding to the trimeric peak (fractions A7-A10) induced clear but weak caspase activity in Jurkat as these cells are known to require extensively cross-linked ligand. The aggregated and undefined species in fractions A1-A6 is therefore a potent inducer of caspase activation (not used further). Importantly, only the defined trimeric species (A7 to A10) was collected and used for final analyses. The results are shown in Fig. 3.

The human cancer cell lines HT1080 (A), HeLa (B) or WM35 (C) were incubated with indicated concentrations of purified, trimeric CD95L-ASPD in the presence or absence of cross-linking antibody (2.5 microgram/ml of anti-Strep-tag II). Cells were incubated for 18h and cytotoxicity was analyzed by crystal violet staining. As a result, CD95L-ASPD induced cell death in HeLa cervix cacinoma and HT1080 fibrosarcoma, but not in WM35 melanoma cells. The results are shown in Fig. 4.

The amino acid sequence of CD95L-ASPD is shown below.

**SEQID 40 Sp-CD95L-ASPD**

Total amino acid number: 346, MW=37682

ORIGIN

1 METDTLLLWV LLLWVPGSTG ELRKVAHLTG KSNSRSMPLE WEDTYGIVLL SGVKYKKGGL

61 VINETGLYFV YSKVYFRGQS CNNLPLSHKV YMRNSKYPQD LVMMEGKMMS YCTTGQMWAR

121 SSYVGAVFNL TSADHLYVNV SELSLVNFEE SQTFFGLYKL *GSSGSSGSSG* *SGLPDVASLR*

181 QQVEALQGQV QHLQAAFSQY KKVELFPNGQ SVGEKIFKTA GFVKPFTEAQ
LLCTQAGGQL
241 ASPRSAAENA ALQQLVVAKN EAAFLSMTDS KTEGKFTYPT GESLVYSNWA
PGEPNDDGGS
301 EDCVEIFTNG KWNDRACGEK RLVVCEFGGS PSSSSSSAWS HPQFEK

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 160: CD95L-receptor binding domain
161 - 171: Flexible linker element (A-linker; italic)
172 - 209: Coiled coil "neck" region of human SP-D
210 - 327: C-type lectin domain of human SP-D
328 - 338: Linker element (GGSPSSSSSSA)

339 - 346: Strep-tag II (WSHPQFEK)

**2.2 Characterization of LIGHT Fusion Proteins (LIGHT-ASPD)**

From affinity purified LIGHT-ASPD 0.5 ml (1.56 mg) were loaded onto a Superdex 200 column and resolved at 0.5 ml/min using PBS as running buffer. The major peak detected at 11.96 ml corresponded to a size of 170-180 kDa indicating that LIGHT-ASPD is a trimer composed of three glycosylated monomers. The trimeric peak (fractions A7 to A10) was collected and used for final analyses. The inset shows the silver stained SDS-PAGE of two independent purified and trimeric LIGHT-ASPD batches (designated 0917 and 0918). The results are shown in Fig. 5.

Varying concentrations (0 – 10 microgram/ml) of affinity and SEC purified, trimeric LIGHT-ASPD were used for immobilized via the Strep-tag II on Streptactin-coated microplates. LIGHT-ASPD was then detected in a ELISA set-up using 100 ng/ml of Fc-fusion proteins of the receptors HVEM and TRAIL-Receptor 1, respectively. Whereas the ELISA signal increased for HVEM-Fc with increasing amounts of immobilized ligand, no signal was detected for TRAIL-Receptor 1-Fc over the whole range analyzed. This indicated that LIGHT-ASPD is a functional molecule that could bind to its receptor HVEM. The results are shown in Fig. 6.

The amino acid sequence of the LIGHT-ASPD fusion protein is shown below:

**SEQID 41 Sp-LIGHT-ASPD**
Total amino acid number: 356, MW=37931

ORIGIN
1 METDTLLLWV LLLWVPGSTG EVNPAAHLTG ANSSLTGSGG PLLWETQLGL
AFLRGLSYHD
61 GALVVTKAGY YYIYSKVQLG GVGCPLGLAS TITHGLYKRT PRYPEELELL
VSQQSPCGRA
121 TSSSRVWWDS SFLGGVVHLE AGEEVVVRVL DERLVRLRDG TRSYFGAFMV
*GSSGSSGSSG*
181 *S*GLPDVASLR QQVEALQGQV QHLQAAFSQY KKVELFPNGQ SVGEKIFKTA
GFVKPFTEAQ
241 LLCTQAGGQL ASPRSAAENA ALQQLVVAKN EAAFLSMTDS KTEGKFTYPT
GESLVYSNWA
301 PGEPNDDGGS EDCVEIFTNG KWNDRACGEK RLVVCEFGGS PSSSSSSAWS
HPQFEK

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 170: LIGHT-receptor binding domain
171 - 181: Flexible linker element (A-linker; italic)
182 - 219: Coiled coil "neck" region of human SP-D
220 - 337: C-type lectin domain of human SP-D
338 - 348: Linker element (GGSPSSSSSSA)
349 - 356: Strep-tag II (WSHPQFEK)

## 2.3 Characterization of TRAIL Fusion Proteins

HEK293 cells were transiently transfected with 24 different expression vectors encoding for TRAIL fusion proteins (Table 6).

| No | Ligand | Linker | Trimerization motif |
|---|---|---|---|
| 1 | TRAIL | A/B/C/D | 69 |
| 2 | TRAIL | A/B/C/D | T4 |
| 3 | TRAIL | A/B/C/D | SPD |
| 4 | TRAIL | A/B/C/D | CCSPD |
| 5 | TRAIL | A/B/C/D | Col11 |
| 6 | TRAIL | A/B/C/D | CC11 |

Table 6: Overview fusion proteins produced by transient transfection of expression vecors. The ligand TRAIL was transfected as fusion proteins comprising one of six stabilzing trimerization motifs and the linker element (A, B, C and D linker).

Supernatants were used for SDS-PAGE and TRAIL-constructs were detected by Western Blot analysis employing an antibody specific for Strep-tag II.

Specific bands detected are indicated by an arrow. The expression strength depended on the type of the trimerization motif employed for construction,

(SPD> 69/T4/Collectin11/CCSPD/CC11) as well as on the length of the linker element (A>B>C>D). The results are shown in Fig. 7.

Jurkat cells were incubated for three hours in the presence (filled bars, anti-Strep-tag II) or absence (clear bars) of a cross-linking antibody (2.5 micrograms/ml anti-Strep-tag II) with supernatants from transiently transfected HEK cells. Supernatants contained TRAIL-fusion proteins with different trimerization motifs (T4, 69, SPD, CCSPD, Col11, CC11) fused through varying linker elements (A, B, C and D linker). As negative control, cell supernatant from untransfected cells was used. Jurkat cells were lysed and analyzed for caspase activity with a fluorogenic assay.

As a result, the caspase activity decreased with the type of linker element employed (A>B>C>D) and on the Fold-On employed. Collectin-11 or coiled coil of Collectin-11 (CCCol11) containing TRAIL constructs are expressed (shown by Western Blot analyses), however were not functional, whereas SPD-derived fold-on motifs yielded functional TRAIL-ligands. The results are shown in Fig. 8.

Affinity purified TRAIL-ASPD was subjected to SEC by loading 0.5 ml (0.4 mg protein) to a Superdex200 column at 0.5 ml/min with PBS as running buffer. Protein elution was monitored by absorption at 280 nm and 0.5 ml fractions were collected. The retention volume of 12.28 ml corresponds to 135-140 kDa as determined from size exclusion standard. This indicated that TRAIL-ASPD is a homotrimer, as the calculated molecular weight of the monomeric polypeptide is 40 kDa. Importantly, for all fusion proteins analyzed by SEC consisting of the wild-type TRAIL-RBD sequence, an additional peak at around 8 ml corresponding to aggregated and non-active TRAIL-fusion protein was observed. From the collected fractions A1-A14 only the trimeric peak (A8 – A10) was used for further analyses. The results are shown in Fig. 9.

The human cancer cell lines HeLa, HT1080, Colo205 or WM35 were

incubated for 18 hours with indicated concentrations of purified, trimeric TRAIL-ASPD in the presence or absence of cross-linking antibody (2.5 microgram/ml of anti-Strep-tag II). Cell death was quantified by crystal violet staining (HeLa, WM35 and HT1080) or by MTS assay (Colo205). The rise in the viability of Colo205 cells at high ligand concentration is likely due to limitation of cross-linking antibody. The results are shown in Fig. 10.

Varying (**A**) or a constant (**B**) concentration of affinity and SEC purified, trimeric TRAIL-ASPD was used for immobilization on Streptactin-coated 96-well plates. Plates were then incubated for 5h with 100,000 Jurkat cells per well at 37°C, 5% CO2 and the caspase activity was determined with a fluorogenic assay. To analyze specificity, plate (B) was incubated for 30 minutes with indicated varying concentrations of an antagonistic anti-TRAIL antibody (clone RIK-2, Pharmingen) prior addition of cells. The results are shown in Fig. 11.

HT1080 cells were incubated on the same 96-well plate with purified and trimeric TRAIL-ASPD or TRAIL-DSPD at indicated concentrations. Cell death was quantified the following day by crystal violet staining. The use of the D-linker reduced the bioactivity approximately 4.5-fold, as indicated by the EC50 values of 27 ng/ml and 6 ng/ml for TRAIL-DSPD and TRAIL-ASPD, respectively. The results are shown in Fig. 12.

The nucleic acid and amino sequences of TRAIL fusion polypeptides are shown below.

**SEQID 42: Expression cassette of Sp-TRAIL-ASPD**

Endonuclease restriction sites are underlined (HindIII, AAGCTT; BamHI, GGATCC; NotI, GCGGCCGC). The translational start codon is in boldface.

ORIGIN

1 AAGCTTGCCG CCACC**ATG**GA GACCGATACA CTGCTCTTGT GGGTGCTCTT GCTGTGGGTT

61 CCTGCAGGTA ATGGTCAAAG AGTCGCAGCT CACATCACTG GGACTAGAGG CAGGAGTAAC

121 ACCCTGAGTT CTCCCAATTC CAAGAACGAG AAAGCCCTGG GTAGGAAGAT CAACTCCTGG

181 GAAAGCTCCA GAAGCGGCCA TAGCTTTCTT AGCAACCTCC ACTTGAGGAA TGGCGAACTT
241 GTGATCCATG AGAAGGGCTT CTACTACATC TACAGCCAGA CGTACTTCAG GTTCCAGGAG
301 GAAATCAAGG AGAACACCAA GAACGACAAG CAGATGGTGC AATACATCTA CAAGTACACG
361 TCATACCCTG ATCCTATACT GCTGATGAAG TCCGCCAGAA ACAGTTGCTG GAGCAAAGAC
421 GCTGAATACG GCCTGTATTC CATCTATCAG GGCGGTATCT TTGAACTCAA GGAGAACGAC
481 AGGATCTTCG TGTCTGTGAC AAACGAGCAT CTGATCGACA TGGACCATGA AGCGTCTTTC
541 TTCGGTGCCT TCTTGGTGGG ATCCTCTGGT TCGAGTGGTT CGAGTGGTTC TGGATTGCCA
601 GACGTTGCTT CTTTGAGACA ACAGGTTGAG GCTTTGCAGG GTCAAGTCCA GCACTTGCAG
661 GCTGCTTTCT CTCAATACAA GAAGGTTGAG TTGTTCCCAA ACGGTCAATC TGTTGGCGAA
721 AAGATTTTCA AGACTGCTGG TTTCGTCAAA CCATTCACGG AGGCACAATT ATTGTGTACT
781 CAGGCTGGTG GACAGTTGGC CTCTCCACGT TCTGCCGCTG AGAACGCCGC CTTGCAACAG
841 TTGGTCGTAG CTAAGAACGA GGCTGCTTTC TTGAGCATGA CTGATTCCAA GACAGAGGGC
901 AAGTTCACCT ACCCAACAGG AGAATCCTTG GTCTATTCTA ATTGGGCACC TGGAGAGCCC
961 AACGATGATG GCGGCTCAGA GGACTGTGTG GAAATCTTCA CCAATGGCAA GTGGAATGAC
1021 AGAGCTTGTG GAGAGAAGCG TTTGGTGGTC TGTGAGTTCG GAGGCAGTCC TTCATCTTCA
1081 TCTAGCTCTG CCTGGTCGCA TCCACAATTC GAGAAATAAT AGCGGCCGC

**SEQID 43 Sp-TRAIL-ASPD**

Total amino acid number: 367, MW=40404

ORIGIN

1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR KINSWESSRS
61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY IYKYTSYPDP
121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD HEASFFGAFL
181 V*GSSGSSGSS* *GS*GLPDVASL RQQVEALQGQ VQHLQAAFSQ YKKVELFPNG QSVGEKIFKT
241 AGFVKPFTEA QLLCTQAGGQ LASPRSAAEN AALQQLVVAK NEAAFLSMTD SKTEGKFTYP
301 TGESLVYSNW APGEPNDDGG SEDCVEIFTN GKWNDRACGE KRLVVCEFGG SPSSSSSSAW
361 SHPQFEK

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 181: TRAIL-receptor binding domain
182 - 192: Flexible linker element (A-linker; italic)
193 - 230: Coiled coil "neck" region of human SP-D
231 - 348: C-type lectin domain of human SP-D
349 - 359: Linker element (GGSPSSSSSSA)
360 - 367: Strep-tag II (WSHPQFEK)

**SEQID 44 Sp-TRAIL-ACCSPD**
Total amino acid number: 246, MW=27534
ORIGIN
1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR
KINSWESSRS
61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY
IYKYTSYPDP
121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD
HEASFFGAFL
181 V*GSSGSSGSS* *GS*GLPDVASL RQQVEALQGQ VQHLQAAFSQ YKKVELFPNG
PSSSSSSAWS
241 HPQFEK

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 181: TRAIL-receptor binding domain
182 - 192: Flexible linker element (A-linker; italic)
193 - 230: Coiled coil "neck" region of human SP-D
231 - 238: Linker element (PSSSSSSA)
239 - 246: Strep-tag II (WSHPQFEK)

**SEQID 45 Sp-TRAIL-ACol11**
Total amino acid number: 365, MW=40806
ORIGIN
1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR
KINSWESSRS
61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY
IYKYTSYPDP
121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD
HEASFFGAFL
181 V*GSSGSSGSS* *GS*QLRKAIGE MDNQVSQLTS ELKFIKNAVA GVRETESKIY
LLVKEEKRYA
241 DAQLSCQGRG GTLSMPKDEA ANGLMAAYLA QAGLARVFIG INDLEKEGAF
VYSDHSPMRT
301 FNKWRSGEPN NAYDEEDCVE MVASGGWNDV ACHTTMYFMC EFDKENMGSP
SSSSSSAWSH
361 PQFEK

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 181: TRAIL-receptor binding domain
182 - 192: Flexible linker element (A-linker; italic)
193 - 224: Coiled coil "neck" region of human Collectin-11
225 - 347: C-type lectin domain of human Collectin-11
348 - 357: Linker element (GSPSSSSSSA)
358 - 365: Strep-tag II (WSHPQFEK)

**SEQID 46 Sp-TRAIL-ACC11**
Total amino acid number: 246, MW=27431
ORIGIN
1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR
KINSWESSRS
61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY
IYKYTSYPDP
121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD

HEASFFGAFL
181 *VGSSGSSGSS GSGS*QLRKAI GEMDNQVSQL TSELKFIKNA VAGVRETESG
PSSSSSSAWS
241 HPQFEK

1 - 20: Secretion signal peptide (underlined)
21 - 181: TRAIL-receptor binding domain
182 - 193: Flexible linker element (A-linker; *GSS GSS GSS GSG* italic)
194 - 229: Coiled coil "neck" region of human Collectin-11
230 - 238: Linker element (GPSSSSSSA)
239 - 246: Strep-tag II (WSHPQFEK)

## 2.4 Characterization of Receptor-selective TRAIL ('mutein') fusion proteins

HEK293 cells were transiently transfected with expression plasmids encoding for different TRAIL receptor-selective SPD constructs:

| No. | Transfected Expression Vector |
|---|---|
| 1 | TRAILR1mut-A-SPD |
| 2 | TRAILR1mut-A-CCSPD |
| 3 | TRAILR1mut-D-SPD |
| 4 | TRAILR1mut-D-CCSPD |
| 5 | TRAILR2mut-A-SPD |
| 6 | TRAILR2mut-A-CCSPD |
| 7 | TRAILR2mut-D-SPD |
| 8 | TRAILR2mut-D-CCSPD |
| 9 | TRAIL-A-SPD |
| 10 | TRAIL-A-CCSPD |
| 11 | TRAIL-D-SPD |
| 12 | TRAIL-D-CCSPD |

Supernatants were collected three days post-transfection and an aliquot was used for SDS-PAGE and Western Blotting employing an antibody specifc for Strep-tag II. Specific bands were detected at around 38 kDa (SPD-fusion proteins) and 28 kDa (coiled-coil-SPD fusion proteins). The amount of expressed protein depended on the ligand itself

(TRAILR1mutein>TRAILR2mutein>TRAIL), secondly the linker length used (A>D) and third the trimerization motif used (SPD>CCSPD). Apparent molecular weights were as expected from the calculated sizes (40 kDa and 27 kDa for SPD and CCSPD fusion proteins, respectively). The results are shown in Fig. 13.

The selectivity of TRAIL-Receptor 1 or TRAIL-Receptor 2 towards fusion proteins of SPD/ccSPD and TRAIL, TRAILR1mut and TRAILR2mut was shown by Streptactin-ELISA. Therefore, TRAIL-SPD-fusion proteins in *supernatants from transiently transfected HEK293 cells were immobilized on* Streptactin coated microplates. Cell supernatant from untransfected cells served as negative control. The results are shown in Fig. 14. Specifically bound proteins were detected with constant (**A, B**) or varying (**C, D**) concentrations of either TRAIL-Receptor 1-Fc or TRAIL-Receptor 2-Fc. As shown in (A), the ligand TRAILR1mut fused to SPD variants is deteced by TRAIL-Receptor 1, whereas the ligand TRAILR2mut is not. As shown in (B), the ligand TRAILR2mut is preferentially detected by TRAIL-Receptor 2, whereas TRAILR1mut- and TRAIL wild-type constructs are equally well detected. As shown in C, TRAIL-Receptor 1-Fc bound to TRAIL-R1mut-ASPD and TRAIL-ASPD equally well over the whole receptor *titration range, whereas TRAIL-R2mut-ASPD is not detected. As shown in D,* TRAIL-Receptor 2-Fc bound to TRAIL-R2mut-ASPD and TRAIL-ASPD equally well over the receptor titration range analyzed, whereas the signal for TRAIL-R1mut-ASPD decreased rapidely with decreasing concentrations of receptor.

One microgram/ml of affinity purified, trimeric TRAIL-ASPD, TRAILR1mut-ASPD or TRAILR2mut-ASPD in 100 microliter of PBS were used for immobilization *via* the Strep-tag II on Streptactin-coated microplates. Bound ligands were detected in a ELISA set-up using Fc-fusion proteins of TRAIL-Receptor 1 (**A**) or TRAIL-Receptor 2 (**B**). As shown in (A), TRAIL-Receptor 1 *bound preferentially to the receptor-selective TRAILR1mut-ASPD* as compared to TRAILR2mut-ASPD. As shown in (B), TRAIL-Receptor 2

preferentially bound to TRAILR2mut-ASPD as compared to TRAILR1mut-ASPD. In conclusion, the constructed TRAIL variants fused to SPD are receptor selective. The results are shown in Fig. 15.

Affinity purified TRAILR1mut-ASPD was subjected to SEC by loading 0.5 ml (0.95 mg protein) on a Superdex200 column. The results are shown in Fig. 16. Proteins were resolved at 0.5 ml/minute with PBS as running buffer and 0.5 ml fractions were collected (fractions A1 to A14 are indicated). The retention volume of 12.46 ml corresponded to 140 -145 kDa as determined by size exclusion standard. A minor peak at 10.83 ml indicated some aggregated species, importantly however, no peak was detected at the running front (8ml) indicating that this molecule is much more soluble as compared to proteins containing parts of the wild-type TRAIL amino acid sequence.

An aliquot from size exclusion chromatography of affinity purified TRAILR1mut-ASPD was used for non-reducing (**A**) or reducing (**B**) SDS-PAGE followed by silver staining as shown in Fig. 17. Under non-reducing conditions, two bands were detected at 35 and 70 kDa, whereas a single band of 40kDa (indicated by an arrow) was detected under reducing conditions. This indicated the formation of disulphide bridged molecules. The trimeric species was present in fractions A8 to A11 and was used for later analyses.

Jurkat cells were incubated in the absence (open bars) or presence (filled bars) of 2.5 microgram/ml of cross-linking antibody with aliquots at a final 80-fold dilution from fractions A1 to A14 from SEC of affinity purified TRAILR1mut-ASPD. The results are shown in Fig. 18. As negative control, Jurkat cells were incubated with medium only. Jurkat cells were lysed after 3h incubation and the caspase activity was determined with a fluorogenic assay. As Jurkat cells have been shown to mainly express TRAIL-Receptor 2, no fraction induced significant caspase activity, even when TRAILR1mit-ASPD was cross-linked by Strep-tag II specific antibody. This indicated that

TRAILR1mut-ASPD does not bind to TRAIL-Receptor 2.

Affinity purified TRAILR2mut-ASPD was subjected to size exclusion chromatography by loading 0.5 ml (0.5 mg protein) to a Superdex 200 column as shown in Fig. 19. Proteins were resolved at 0.5 ml/minute with PBS as running buffer and 0.5 ml fractions were collected (fractions A1 to A14 are indicated). The retention volume of 12.60 ml corresponds to 130 – 135 kDa as determined from size exclusion standard. This indicated that TRAILR2mut-ASPD is a homotrimer as calulated from the expected monomeric weight of 40 kDa. Importantly, more than 95% was present in the trimeric peak fraction and no aggregates were detected. The trimeric peak was used for later analyses.

An aliquot from size exclusion chromatography of affinity purified TRAILR2mut-ASPD was used for non-reducing (**A**) or reducing (**B**) SDS-PAGE followed by silver staining as shown in Fig. 20. Under non-reducing conditions, two bands were detected at 35 and 70 kDa, whereas a single band of approximately 40kDa (indicated by an arrow) was detected under reducing conditions. This indicated the formation of disulphide bridged molecules. The trimeric species was present in fractions A9 to A11 and was used for later analyses.

The results from a Jurkat cell kill assay with TRAILR2-mut-ASPD are shown in Fig. 21. Jurkat cells were incubated in th absence (clear bars) or presence (filled bars) of cross-linking antibodies (2.5 microgram/ml anti-Strep-tag II) with aliquots from fractions A1 to A14 from SEC of affinity purified TRAILR2mut-ASPD. Samples were used at at final 640-fold dilution. Cells were lysed after 3h of incubation and the caspase activity was determined with a fluorogenic assay. As Jurkat cells have been shown to mainly express TRAIL-Receptor 2 that requires multimerized ligand forms for efficient signalling, TRAILR2mut-ASPD induced caspase activity when cross-linked. This indicated that TRAILR2mut-ASPD is a functional molecule.

The cytotoxic activity of TRAIL-ASPD, TRAILR1mut-ASPD and TRAILR2mut-ASPD on different human cancer cells is shown in Fig. 22. The indicated cell lines HT1080 (A and B), Hela (C and D) or Colo205 (E and F) were treated with varying concentrations of purified and trimeric TRAIL-ASPD, TRAILR1mut-ASPD or TRAILR2mut-ASPD in the absence (A, C and E) or presence (B, D and F) of cross-linking antibody (anti-Strep-tag II). Cells were incubated for 18 hours with indicated concentrations of ligands and cell death was quantified by crystal violet staining (HT1080 and HeLa) or MTS assay (Colo205). As a result, the ligand TRAIL-ASPD induced cell death on the three cell lines tested and TRAILR2mut-ASPD showed superior cell killing activity. In contrast, TRAIL-Receptor 1 selective TRAILR1mut-ASPD was not active on any cell line tested.

Affinity purified TRAILR2mut-ASPD was concentrated 20-fold in PBS by centrifugation through a 10 kDa membrane to give a solution of 2.5 mg/ml. From the concentrate, 0.1 ml were subjected to size exclusion chromatography. As a result, only the trimeric peak and no aggregates were detected, indicating that this composition has improved production capabilities (Fig. 23). Similar results were achieved for TRAILR1mut-ASPD, where a concentrated solution of even 5.4 mg/ml showed no signs of aggregation (not shown). In contrast, all fusion proteins tested containing the receptor binding domain composed of the wild type TRAIL sequence showed aggregation with 40% aggregates at concentrations as low as 0.4 mg/ml.

The amino acid sequences of receptor-selective TRAIL mutein fusion polypeptides are shown in the following.

**SEQID 47 Sp-TRAILR1mut-ASPD**

Total amino acid number: 367, MW=40335

ORIGIN

1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR KINSWESSRS

61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTA FRFSEEIKEV TRNDKQMVQY IYKWTDYPDP

121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD HEASFFGAFL

181 V*GSSGSSGSS* *GS*GLPDVASL RQQVEALQGQ VQHLQAAFSQ YKKVELFPNG

QSVGEKIFKT
241 AGFVKPFTEA QLLCTQAGGQ LASPRSAAEN AALQQLVVAK NEAAFLSMTD
SKTEGKFTYP
301 TGESLVYSNW APGEPNDDGG SEDCVEIFTN GKWNDRACGE KRLVVCEFGG
SPSSSSSSAW
361 SHPQFEK

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 181: TRAILR1mut-receptor binding domain
182 - 192: Flexible linker element (A-linker; italic)
193 - 230: Coiled coil "neck" region of human SP-D
231 - 348: C-type lectin domain of human SP-D
349 - 359: Linker element (GGSPSSSSSSA)
360 - 367: Strep-tag II (WSHPQFEK)

**SEQID 48 Sp-TRAILR2mut-ASPD**
Total amino acid number: 367, MW=40401
ORIGIN
1 METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR
KINSWESSRS
61 GHSFLSNLHL RNGELVIHEK GFYYIYSQTQ FKFREEIKEN TKNDKQMVQY
IYKYTSYPDP
121 ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNERLLQMD
HEASFFGAFL
181 *VGSSGSSGSS GS*GLPDVASL RQQVEALQGQ VQHLQAAFSQ YKKVELFPNG
QSVGEKIFKT
241 AGFVKPFTEA QLLCTQAGGQ LASPRSAAEN AALQQLVVAK NEAAFLSMTD
SKTEGKFTYP
301 TGESLVYSNW APGEPNDDGG SEDCVEIFTN GKWNDRACGE KRLVVCEFGG
SPSSSSSSAW
361 SHPQFEK

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 181: TRAILR2mut-receptor binding domain
182 - 192: Flexible linker element (A-linker; italic)
193 - 230: Coiled coil "neck" region of human SP-D
231 - 348: C-type lectin domain of human SP-D
349 - 359: Linker element (GGSPSSSSSSA)
360 - 367: Strep-tag II (WSHPQFEK)

## 2.5 Characterization of SPD Carbohydrate-variants

Affinity purified TRAIL-ASPD_F335A was subjected to Size Exclusion Chromatography by loading 0.5 ml PBS solution (0.4 mg protein) to a Superdex 200 column as shown in Fig. 24. Proteins were resolved at 0.5 ml/ minute with PBS as running buffer and 0.5 ml fractions were collected (A1 to A13 are indicated). The retention volume of 12.27 ml corresponds to 135-145 kDa as determined from size exclusion standard. This indicated that TRAIL-ASPD_F335A is a homotrimer as calulated from the expected

monomeric weight of 40 kDa. Two additional peaks at 8.32 and 10.68 ml indicated the formation of TRAIL-ASPD_F335A aggregates. Only the trimeric peak was used for later analyses.

From Size exclusion chromatography an aliquot from collected fractions A1 to A13 was resolved by reducing SDS-PAGE and the gel was silver stained (Fig. 25). The band detected at approximately 40 kDa corresponded to the calculated molecular weight of 40 kDa for TRAIL-ASPD_F335A. Positive fractions corresponding the trimeric molecule (A8, A9, A10) of the SEC run were pooled and used for further analyses.

The amino acid sequences of TRAIL-SPD carbohydrate variant fusion proteins is shown in the following.

**SEQID 49: Sp-TRAIL-ASPD_F335A**

Total amino acid number: 367, MW=40328
ORIGIN

```
1           METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR
KINSWESSRS
61          GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY
IYKYTSYPDP
121         ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD
HEASFFGAFL
181         VGSSGSSGSS GSGLPDVASL RQQVEALQGQ VQHLQAAFSQ YKKVELFPNG
QSVGEKIFKT
241         AGFVKPFTEA QLLCTQAGGQ LASPRSAAEN AALQQLVVAK NEAAFLSMTD
SKTEGKFTYP
301         TGESLVYSNW APGEPNDDGG SEDCVEIATN GKWNDRACGE KRLVVCEFGG
SPSSSSSSAW
361      SHPQFEK
```

1 - 20: Secretion signal peptide (Sp; underlined)
21 - 181: TRAIL-receptor binding domain
182 - 192: Flexible linker element (A-linker; italic)
193 - 230: Coiled coil "neck" region of human SP-D
231 - 348: C-type lectin domain of human SP-D (Phe mutation in bold-face)
349 - 359: Linker element (GGSPSSSSSSA)
360 - 367: Strep-tag II (WSHPQFEK)

**SEQID 50: Sp-TRAIL-ASPD_F335D**

Total amino acid number: 367, MW=40372
ORIGIN

```
1           METDTLLLWV LLLWVPAGNG QRVAAHITGT RGRSNTLSSP NSKNEKALGR
KINSWESSRS
```

```
61          GHSFLSNLHL RNGELVIHEK GFYYIYSQTY FRFQEEIKEN TKNDKQMVQY
IYKYTSYPDP
121         ILLMKSARNS CWSKDAEYGL YSIYQGGIFE LKENDRIFVS VTNEHLIDMD
HEASFFGAFL
181         VGSSGSSGSS GSGLPDVASL RQQVEALQGQ VQHLQAAFSQ YKKVELFPNG
QSVGEKIFKT
241         AGFVKPFTEA QLLCTQAGGQ LASPRSAAEN AALQQLVVAK NEAAFLSMTD
SKTEGKFTYP
301         TGESLVYSNW APGEPNDDGG SEDCVEIDTN GKWNDRACGE KRLVVCEFGG
SPSSSSSSAW
361         SHPQFEK


1   -  20: Secretion signal peptide (Sp; underlined)
21  - 181: TRAIL-receptor binding domain
182 - 192: Flexible linker element (A-linker; italic)
193 - 230: Coiled coil "neck" region of human SP-D
231 - 348: C-type lectin domain of human SP-D (Asp mutation in bold-
face)
349 - 359: Linker element (GGSPSSSSSSA)
360 - 367: Strep-tag II (WSHPQFEK)
```

The cytotoxic effect of TRAIL-ASPD_F335A on human cancer cells is shown in Fig. 26. Indicated human cancer cell lines were incubated over night with varying concentrations of affinity and SEC purified, trimeric TRAIL-ASPD_F335A in the presence or absence of cross-linking antibody (2.5 microgram/ml of anti Strep-tag II). Cell viability was quantified by crystal violet staining (HT1080, HeLa and WM35) or MTS (Colo205). The rise of Colo205 cell viability at high ligand concentrations is likely due to limitation of cross-linking antibody.

Affinity purified TRAIL-ASPD_F335D was subjected to Size Exclusion Chromatography by loading 0.5 ml (0.2 mg protein) to a Superdex 200 column as shown in Fig. 27. Proteins were resolved at 0.5 ml/minute with PBS as running buffer and 0.5 ml fractions were collected (A1 to A13 are indicated). The retention volume of 12.29 ml corresponds to 135-145 kDa as determined from size exclusion standard. This indicated that TRAIL-ASPD_F335D is a homotrimer as calulated from the expected monomeric weight of 40 kDa. The peak at 8.35 corresponded to inactive TRAIL-ASPD_F335D aggregates typically found for all fusion proteins containing parts of the wild type TRAIL amino acid sequence.

From Size exclusion chromatography aliquots of affinity purified TRAIL-ASPD_F335D from the collected fractions A1 to A13 were resolved by reducing SDS-PAGE and the gel was silver stained (Fig. 28). The bands detected at approximately 40 kDa (indicated by an arrow) corresponded to the calculated molecular weight of 40 kDa for TRAIL-ASPD_F335D. Fractions containing trimeric protein (fractions A8 to A10) were pooled and used for further analyses.

The human cancer cell lines HT1080 (A), HeLa (B), WM35 (C) or Colo205 (D) were incubated over night with varying concentrations of affinity purified, trimeric TRAIL-ASPD_F335D in the presence or absence of cross-linking antibodies (anti-Strep-tag II). Cell viability was quantified by crystal violet staining (HT1080, HeLa and WM35) or MTS (Colo205). The data show that TRAIL-ASPD_F335D is capable of inducing cell death in exemplified cancer cell lines (Fig. 29). The rise of Colo205 cell viability at high concentrations of ligand is likely due to limitation of cross-linking antibody.

**2.6 Analysis of Carbohydrate binding characteristics of the SPD trimerization motif variants**

It has been shown that wild-type, full length and oligomeric SP-D protein from several species, as well as the trimeric neck+CRD of human SP-D bind to several different carbohydrates. In addition, the neck+CRD of human SP-D also has been shown to excert immunomodulatory effects by serving as a chemotactic factor for immuno cells such as neutrophils (Cai et al., 1999, *Am J Physiol Lung Cell Mol Physiol* 276:131-136). Other cells may also be recruited by SP-D. The chemotactic effect of neck+CRD of human SP-D has been shown to depend on the glycobinding function, as the addition of maltose inhibited the chemotactic function. Thus, a ligand of the TNFSF with a SP-D-mediated chemotactic function may be of superior activity as compared to ligands or constructs thereof with natural amino acid sequences. For instance, in a scenario where cellular effects are desirable such as in cancer treatment such a described ligand may be desirable.

In addition, a ligand where SP-D has no carbohydrate function may be desirable in other settings. For human SP-D a mutant has been described in which amino acid phenylalanine 335 (corresponding to amino acid 355 of SEQ ID NO:21) has been mutated to alanine (SPD_F335A, Crouch et al., *JBC 281: 18008–18014*). This mutant showed very weak carbohydrate binding. However, introducing a charged amino acid (e.g. an acidic amino acid) may be even better as compared to F335A if no carbohydrate binding is desired. Therefore the mutant SPD_F335D may be superior towards F335A mutant.

To analyze the binding of TRAIL-fusion proteins to carbohydrates, mannan from yeast was immobilized on microplates and the binding of TRAIL-SPD, TRAIL-SPD_F335A or TRAIL-SPD_F335D was detected by ELISA. The results are shown in Fig. 30. As expected, the ELISA signal increased with increasing concentrations of TRAIL-ASPD. In contrast, the carbohydrate-mutant form TRAIL-ASPD_F335A showed a very low ELISA signal. In addition, the new constructed variant TRAIL-ASPD_F335D displayed the lowest ELISA signal (see inset and arrow). This indicated that the mutant F335D has a lower mannan-binding affinity as compared to the previously described SP-D mutant form F335A.

**2.7 Pharmacokinetics of TRAIL-SPD Fusion Proteins**

To determine the half-lifes of TRAIL-SPD fusion protein, ten micrograms of TRAIL-ASPD (A) or TRAIL-ASPD_F335D (B) were injected intraveneously into male CD1 mice and serum samples were collected after several time points (predose, 5 min., 30 min., 2h, 6h and 24h). TRAIL proteins in sera of mice were quantified by an ELISA and the data was used to calculate halflifes. The results are shown in Fig. 31. For the two proteins analyzed, a halflife of 7 to 14 hours for TRAIL-ASPD (A) and TRAIL-ASPD_F335D (B) were calculated. No animal died or showed signs of intolerance during the period observed. The data indicate an at least 80-fold improvement of the serum halftime as compared to wild type TRAIL that was reported to have a

half time in the range of three to five minutes in rodents (Kelley et. al 2001).

## 2.8 Cytotoxicity of TRAIL-ASPD Fusion Proteins

To analyze potential hepatotoxic effects of TRAIL-ASPD, TRAIL-ASPD_F335A or TRAIL-ASPD_F335D, primary human hepatocytes (PHH) were incubated with varying concentrations of indicated TRAIL-SPD-fusion proteins, with or without cross-linking antibodies (anti-Strep-tag II). As a control, a stabilized variant of CD95L, CD95L-T4 (described in WO2008/025516) was used. The results are shown in Fig. 32.

In addition, the effect of a simultaneous incubation of PHH with 5 mM of chemotherapeutic drugs was analyzed for TRAIL-ASPD_F335D. After 5h (A,B and E) or 24h (C, D and F) of incubation, cells were lysed and caspase activity was assessed with a fluorogenic assay.

As a result, all analyzed TRAIL-SPD fusion proteins induced no hepatotoxic effects, even if ligands were secondarily cross-linked by antibodies. In contrast, CD95L-T4 is hepatotoxic as indicated by an increase of active caspase (A to D). Five hours of co-incubation of primary human hepatocytes with trimeric TRAIL-ASPD_F335D together with chemotherapeutic drugs induced no caspase activity (E). However, after 24h of co-incubation with doxorubicin, soluble TRAIL-ASPD_F335D induced a strong caspase activity signal (F).

This indicates that TRAIL fusion proteins of the present invention may not show undesired hepatotoxicity in medical use. Thus, TRAIL fusion proteins are preferably administered in combination with drugs, which are apoptosis sensitizers and/or apoptosis inducers, e.g. a chemotherapeutic drug such as oxaliplatin, cisplatin, 5-fluorouracil, etoposide, gemcitabine, irinotecan and others, or Bcl2 binding molecules, e.g. small molecules or peptidic compounds, which bind to polypeptides of the Bcl2 family, particularly Bcl2 or Bclxl.

## 2.9 Characterization of APRIL Fusion Proteins

HEK293 cells were transiently transfected with expression vectors encoding for APRIL-A69 (WO2008025516), APRIL-ASPD, APRIL-ACCSPD or APRIL-ACol11. After three days supernatants were analyzed for secreted proteins by Western Blotting. The results are shown in Fig. 33. For the detection of APRIL-fusion proteins an antibody specifc for Strep-tag II was used. Arrows indicate specific bands that were detected around 40 kDa (APRIL-ASPD and APRIL-ACol11, respectively), as well as at around 25 kDa (APRIL-A69 and APRIL-ACCSPD, respectively). Thus APRIL expression cassettes are functional and the secretion of protein indicated that the proteins are properly folded. As for other TNFSF proteins analyzed, the highest secreted protein levels were found for APRIL fused to the trimerization motif composed of coiled coil "neck" + CRD of human SP-D (APRIL-ASPD, lane No. 2). APRIL-ASPD was used to analyze the binding to the receptor TACI.

To show that the constructed APRIL-ASPD fusion protein is functional, the binding to a known receptor of APRIL, namely TACI, was assessed (Fig. 34). Therefore, APRIL-ASPD in supernatant from transiently transfected HEK293 cells was immobilized on Streptactin coated microplates. Cell supernatant from untransfected HEK293 cells served as negative control. Specifically bound proteins were detected with varying concentrations of TACI-Fc followed by incubation with an anti-human, Fc-specific antibody conjugated with peroxidase. As a result, the ELISA signal increased with increasing concentrations of TACI-Fc, indicating that APRIL-ASPD is a functional molecule.

The amino acid sequence of an APRIL fusion protein is shown below.

**SEQID 51: Sp-APRIL-ASPD**

Total amino acid number: 344, MW=37120

ORIGIN

1 METDTLLLWV LLLWVPAGNG KQHSVLHLVP INATSKDDSD VTEVMWQPAL RRGRGLQAQG

61 YGVRIQDAGV YLLYSQVLFQ DVTFTMGQVV SREGQGRQET LFRCIRSMPS HPDRAYNSCY

121 SAGVFHLHQG DILSVIIPRA RAKLNLSPHG TFLGFVKL*GS SGSSGSSGSG*
LPDVASLRQQ
181 VEALQGQVQH LQAAFSQYKK VELFPNGQSV GEKIFKTAGF VKPFTEAQLL
CTQAGGQLAS
241 PRSAAENAAL QQLVVAKNEA AFLSMTDSKT EGKFTYPTGE SLVYSNWAPG
EPNDDGGSED
301 CVEIFTNGKW NDRACGEKRL VVCEFGGSPS SSSSSAWSHP QFEK

1 - 20: Signal secretion peptide (underlined)
21 - 158: APRIL-RBD
159 - 169: Flexible linker element (A-linker; *GSS GSS GSS GS* italic)
170 - 207: Coiled coil "neck" region of human SP-D
208 - 325: C-type lectin domain of human SP-D
326 - 336: Linker element (GGSPSSSSSSA)
337 - 344: Strep-tag II (WSHPQFEK)

## References

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What is claimed is:

1. A fusion protein comprising: (i) a TNF (tumor necrosis factor)-superfamily cytokine that is CD40 ligand (CD40L) or a receptor binding domain thereof, and (ii) a collectin trimerization domain comprising a neck domain or a neck and carbohydrate binding domain of surfactant protein-D, wherein (ii) is located C-terminally of (i), wherein (i) comprises amino acids 112-261 or 117-261 of a human CD40L of SEQ ID NO:5, wherein (ii) comprises amino acids 217-257, 218-257, 219-257, 220-257, 221-257, 222-257, 223-257, 224-257, or 225-257 of a human surfactant protein-D of SEQ ID NO:21.

2. The fusion protein of claim 1, additionally comprising a flexible linker between (i) and (ii), wherein the flexible linker is a glycine/serine linker and has a length of 3-20 amino acids.

3. The fusion protein of claim 2, wherein the flexible linker has the amino acid sequence of SEQ ID NO: 53, which is (GSS)a(SSG)b(GSG)c wherein a, b, c is each 0, 1, 2, 3, 4, 5 or 6.

4. The fusion protein of claim 2, wherein the flexible linker has a length of 9-15 amino acids.

5. The fusion protein of claim 1, wherein the CD40L has the sequence of SEQ ID NO:5.

6. The fusion protein of claim 1, wherein (ii) comprises amino acids 217-375, 218-375, 219-375, 220-375, 221-375, 222-375, 223-375, 224-375, or 225-375 of a human surfactant protein-D of SEQ ID NO:21.

7. The fusion protein of claim 1, wherein (ii) comprises a mutant of human surfactant protein-D of SEQ ID NO:21 having only one amino acid substitution.

8. The fusion protein of claim 7, wherein the amino acid substitution affects amino acid position F355 of SEQ ID NO:21, and the amino acid substitution is one of the following: F355A, F355S, F355T, F355E, F355D, F355K, or F355R.

9. The fusion protein of claim 7, wherein (ii) comprises a mutant which does not bind to mannose.

10. The fusion protein of claim 1, which additionally comprises an N-terminal signal peptide

domain.

11. The fusion protein of claim 1, wherein the fusion protein further comprises a recognition/purification domain located at the N-terminus or at the C-terminus.

12. The fusion protein of claim 11, wherein the recognition/purification domain is a Strep-tag or a poly His-domain.

13. The fusion protein of claim 1, which additionally comprises a C-terminal flexible element.

14. A trimeric complex comprising three fusion proteins of claim 1.

15. The trimeric complex of claim 14, wherein the complex is formed by covalent linkage between the three fusion proteins.

16. The trimeric complex of claim 15, wherein the complex consists of three identical fusion proteins.

17. A nucleic acid molecule encoding the fusion protein of claim 1.

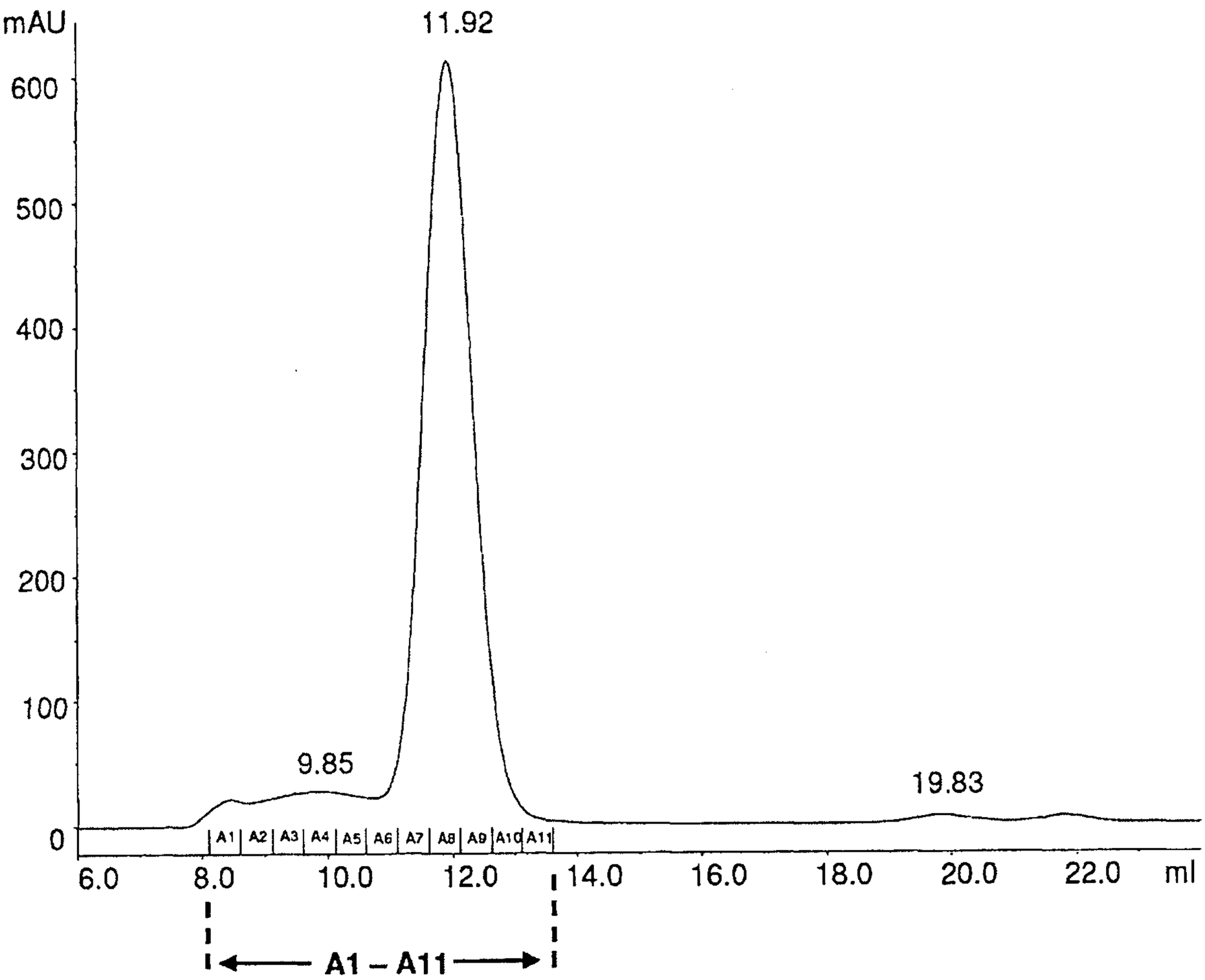
18. The nucleic acid molecule of claim 17, which is operatively linked to an expression control sequence.

19. A vector comprising the nucleic acid molecule of claim 18.

20. An isolated cell transformed or transfected with the nucleic acid molecule of claim 18.

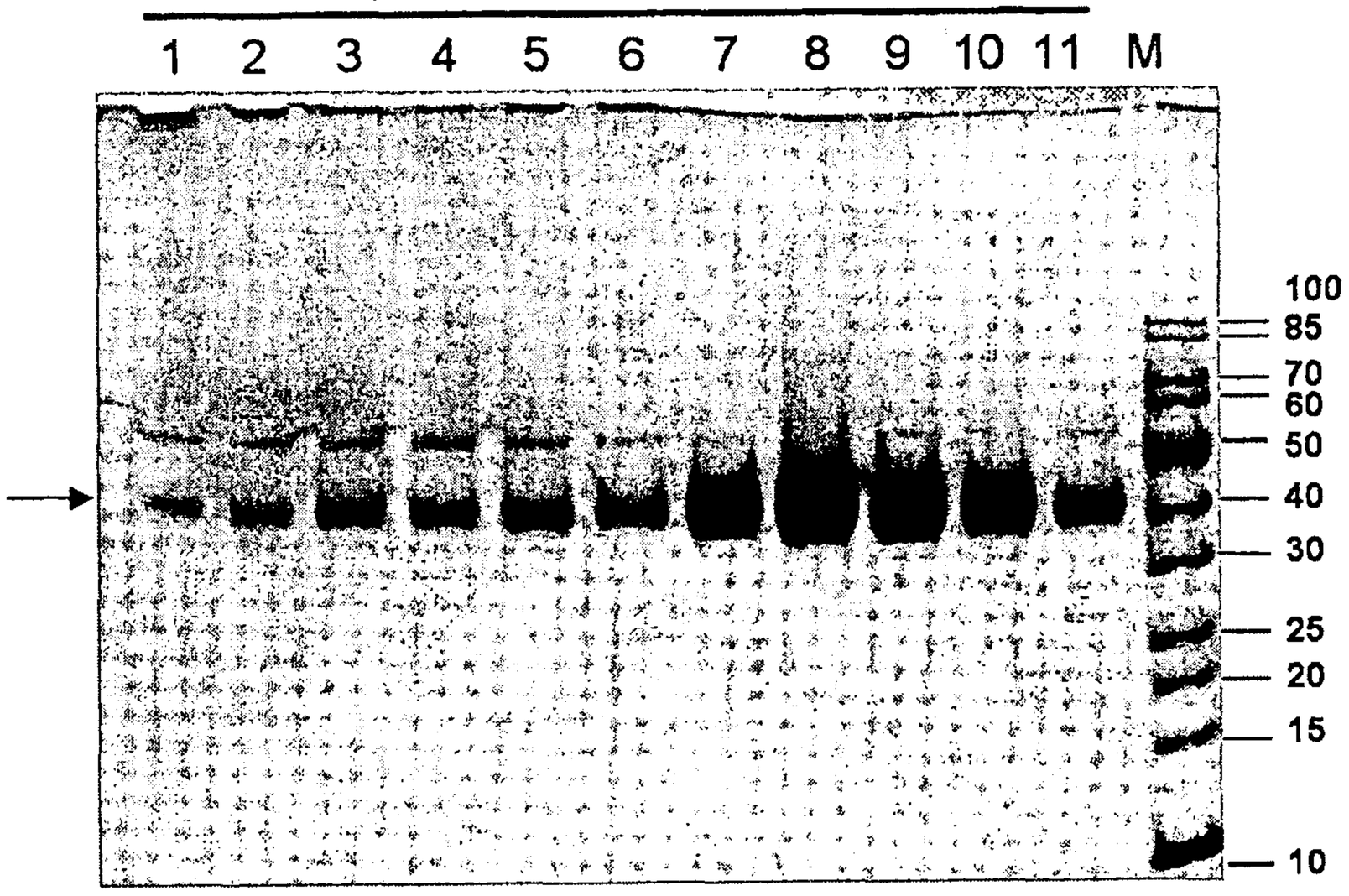
# Figure 1

## SEC of affinity purified CD95L-ASPD



# Figure 2

## Silver gel of SEC fractions A1-A11 from affinity purified CD95L-ASPD



## Figure 3

**Caspase activity on Jurkat cells induced by SEC fractions A1-A15 from affinity purified CD95L-ASPD**

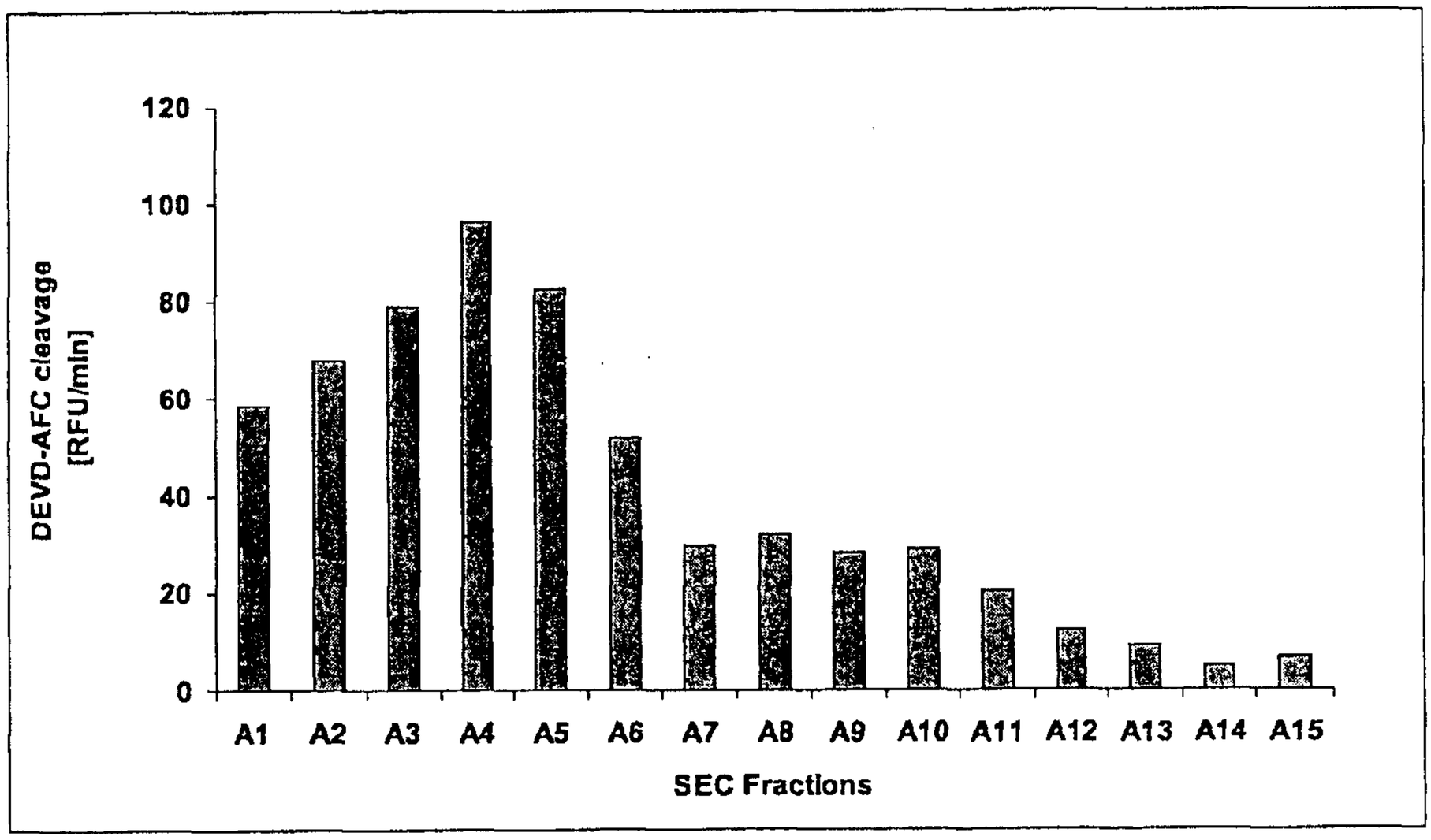
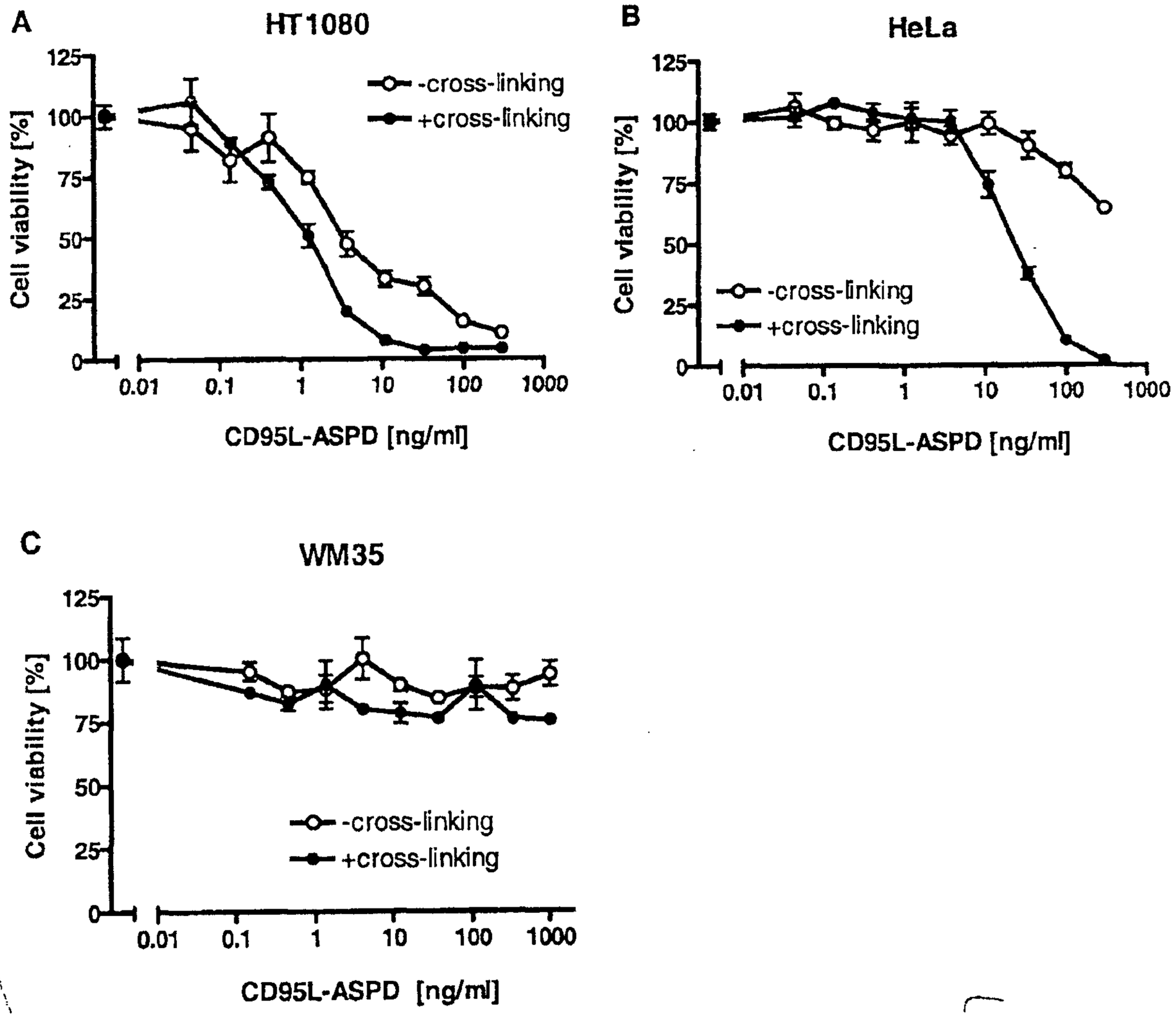


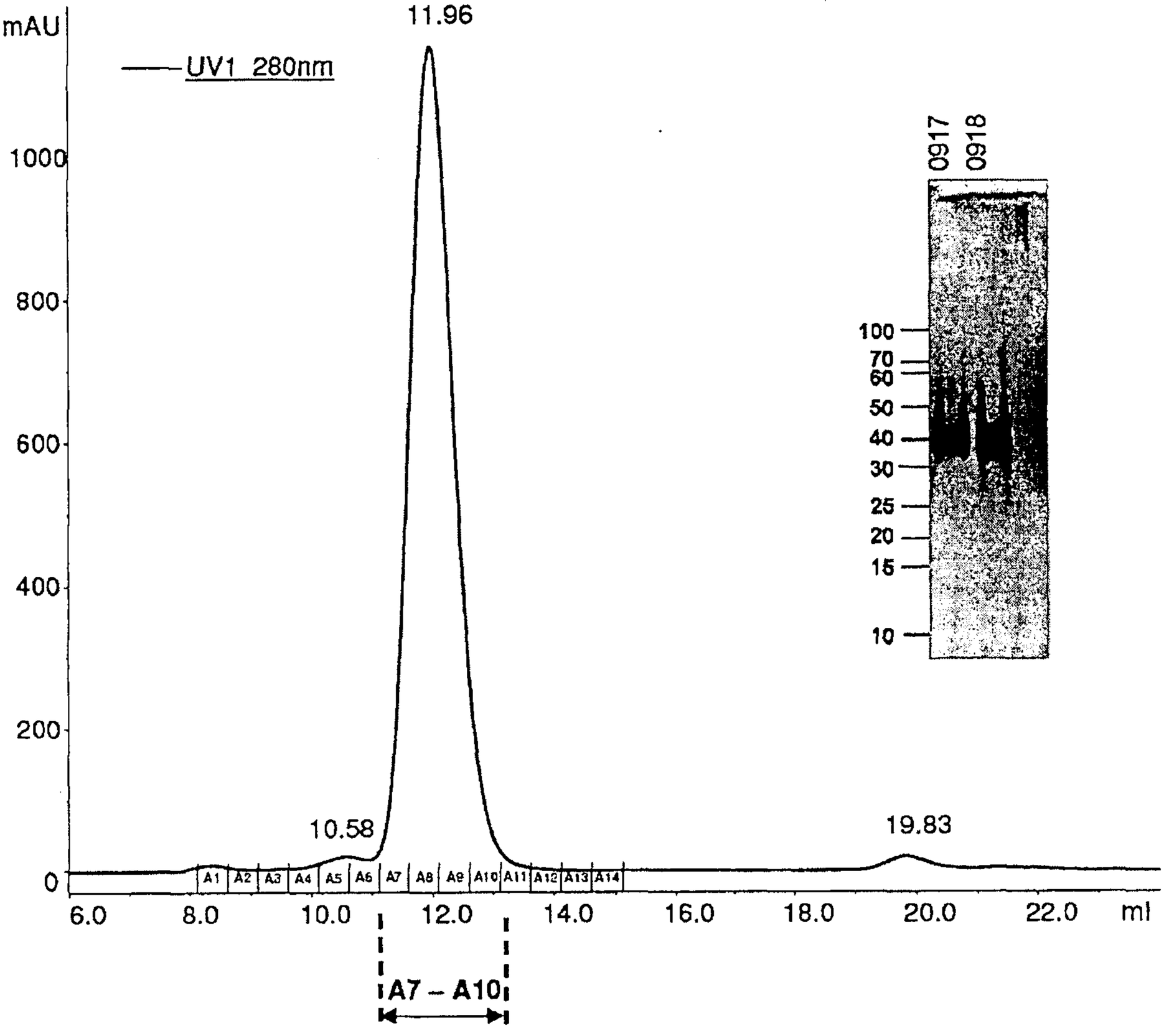
**Figure 4**

**Cytotoxicity of CD95L-ASPD on WM35, HT1080 and HeLa cells**



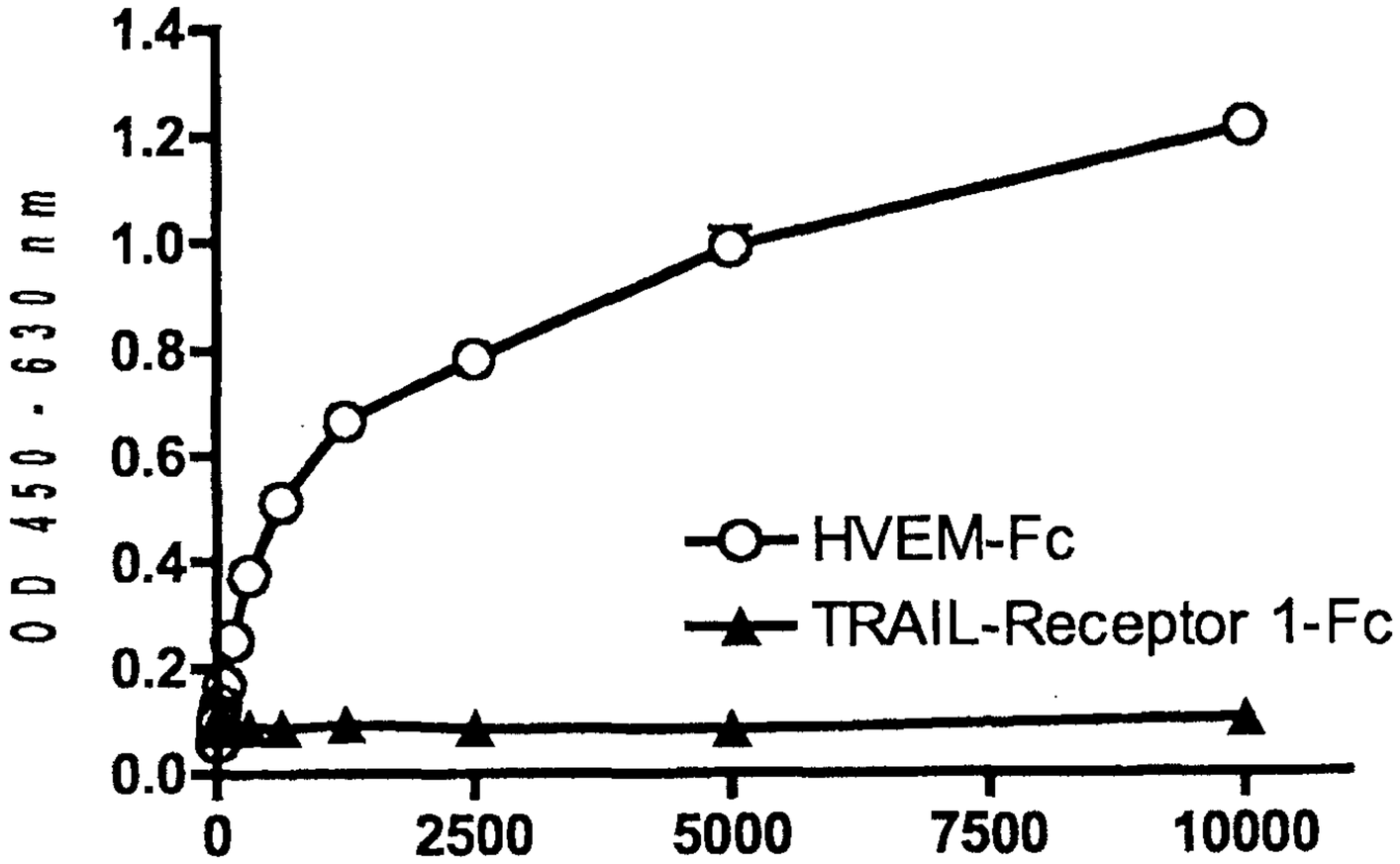
# Figure 5

## SEC of affinity purified LIGHT-ASPD



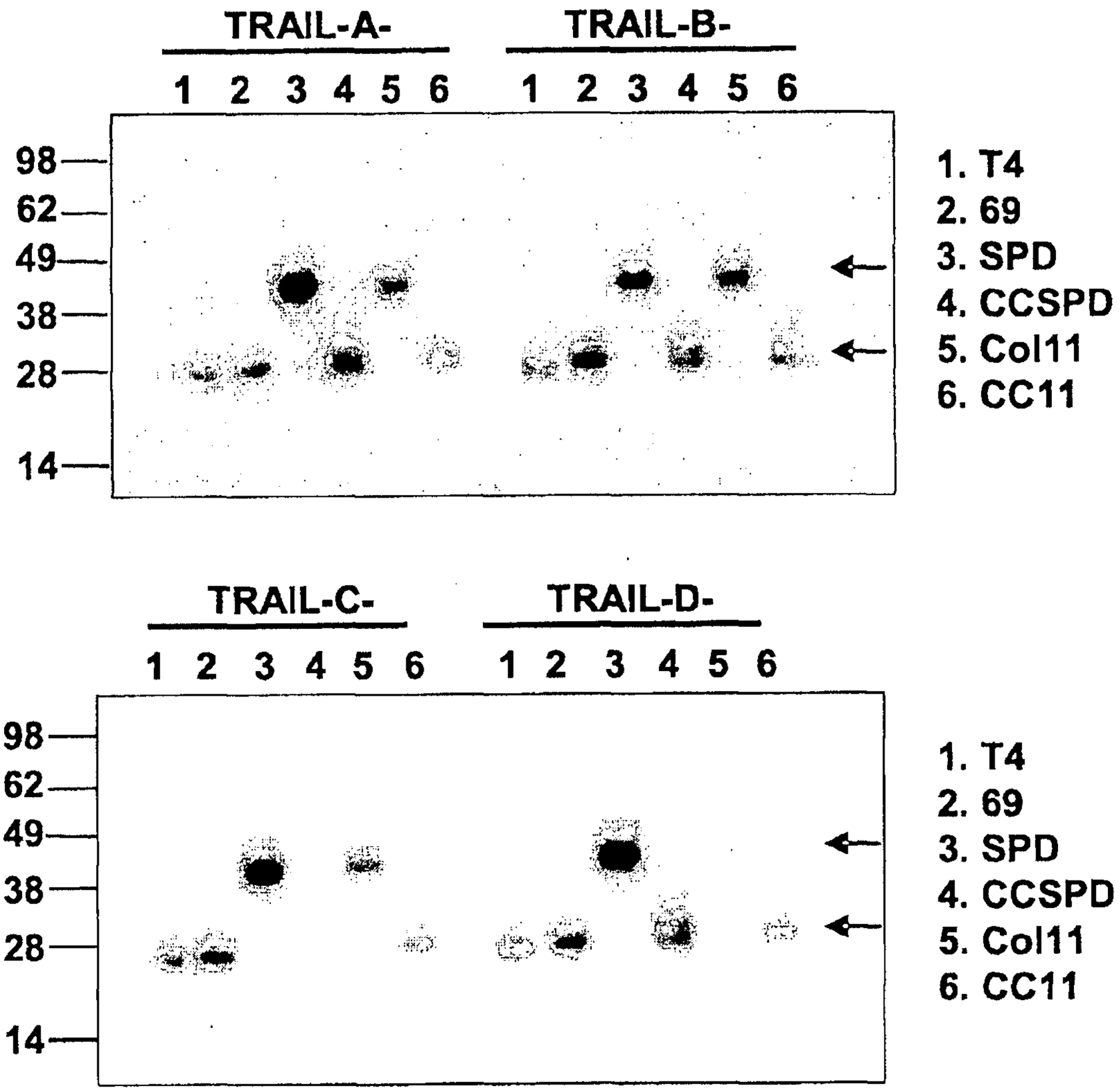
# Figure 6

## Binding of HVEM-Fc to immobilized LIGHT-ASPD



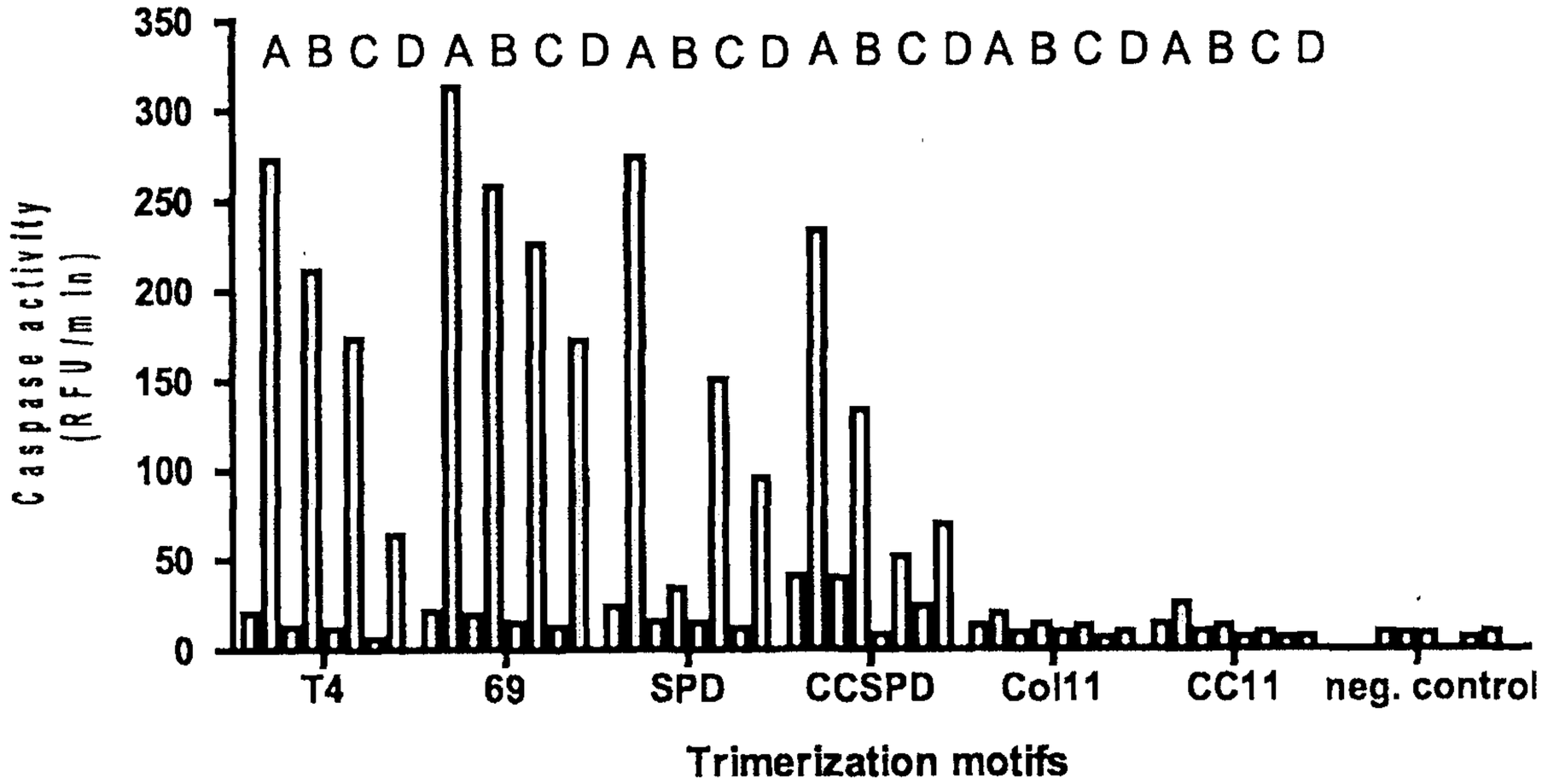
## Figure 7

**Western blot from transiently transfected HEK cells transiently transfected with TRAIL-constructs**



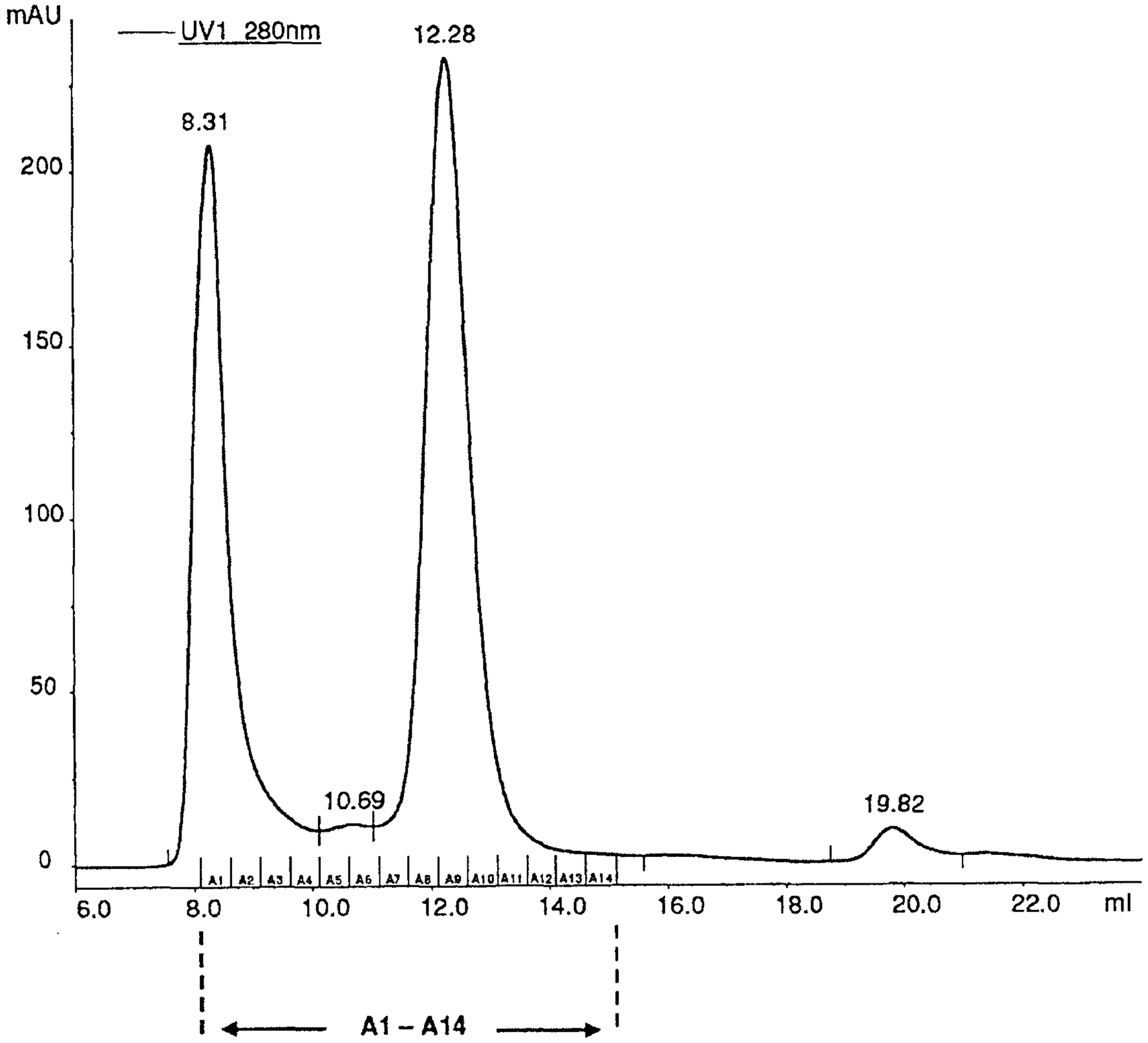
# Figure 8

## Caspase activity in Jurkat T-cells



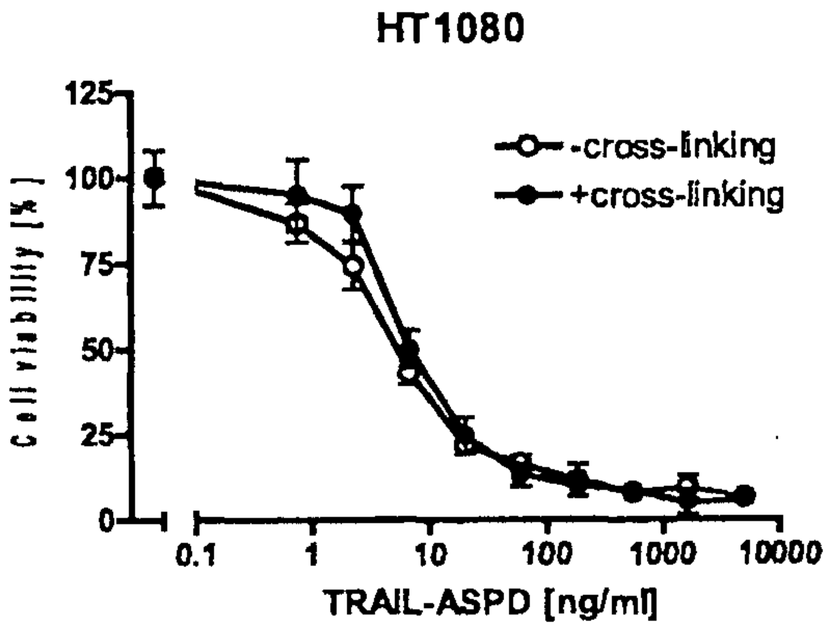
# Figure 9

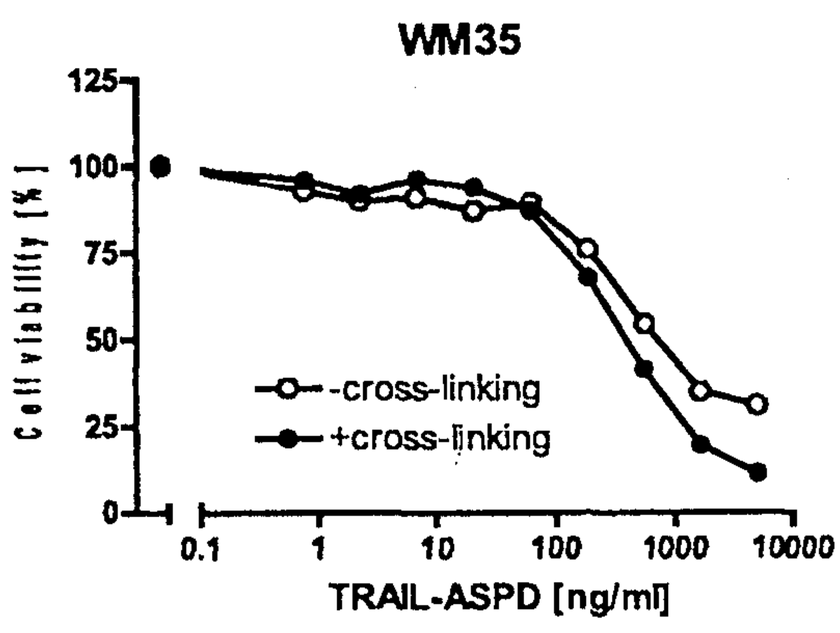
## Size exclusion chromatography of TRAIL-ASPD

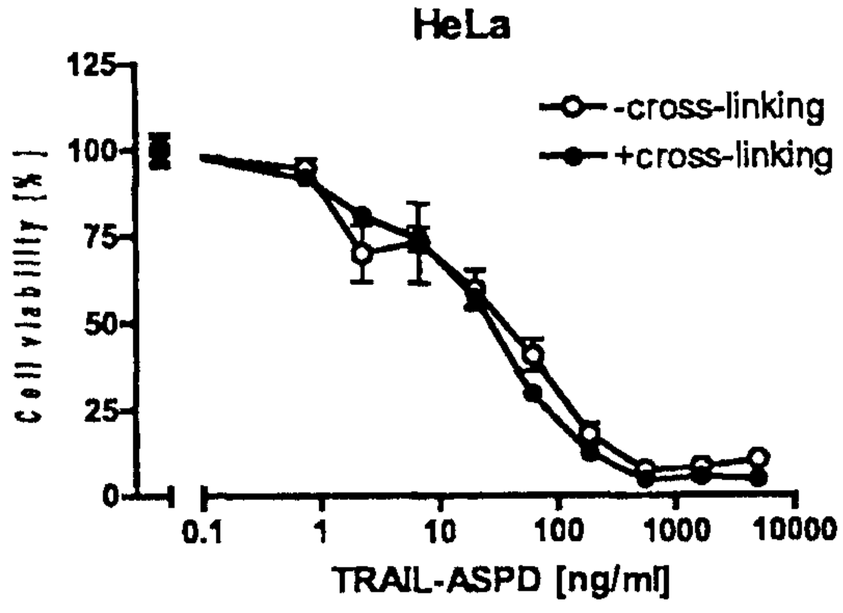


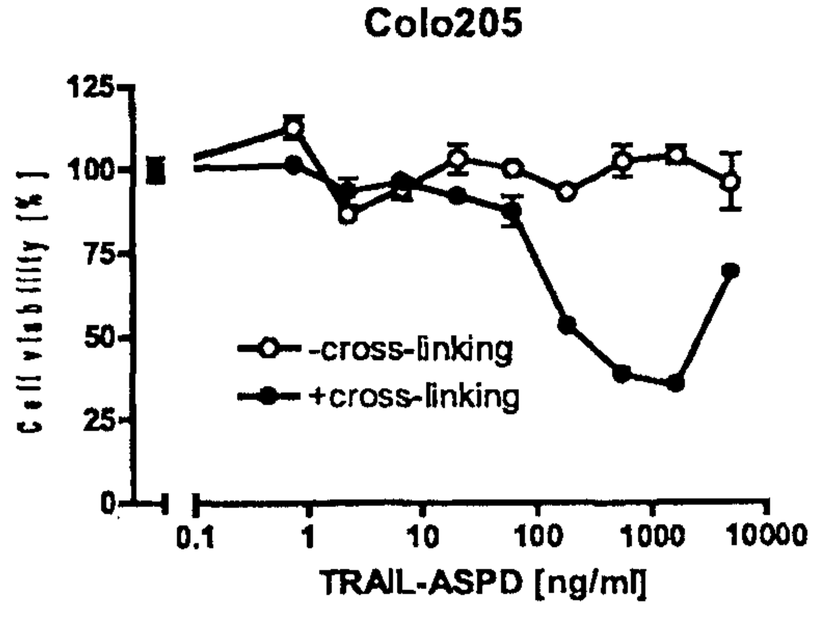
# Figure 10

## Cytotoxic activity of TRAIL-ASPD against human cancer cells



# Figure 11

## TRAIL-ASPD induced caspase activity in Jurkat

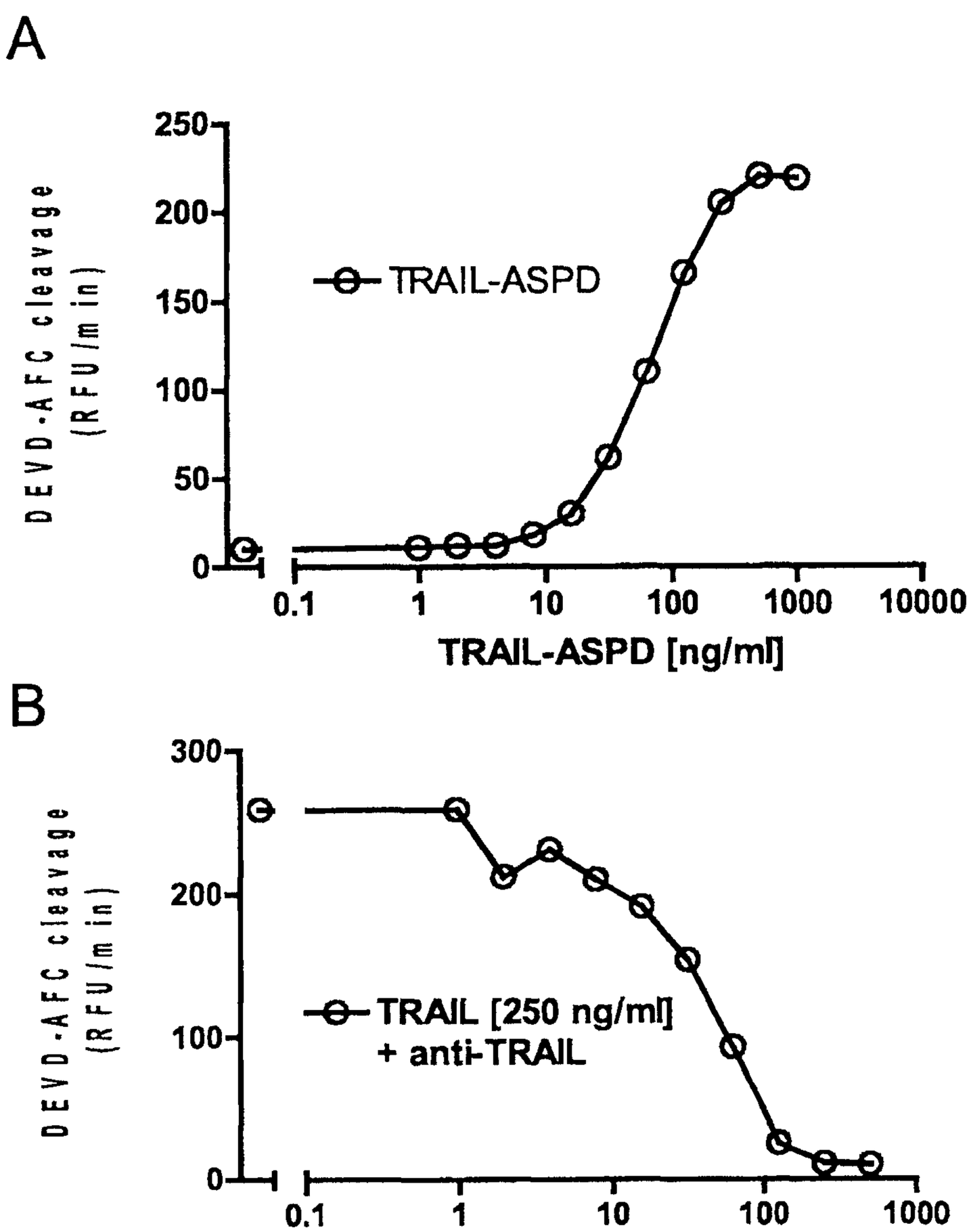
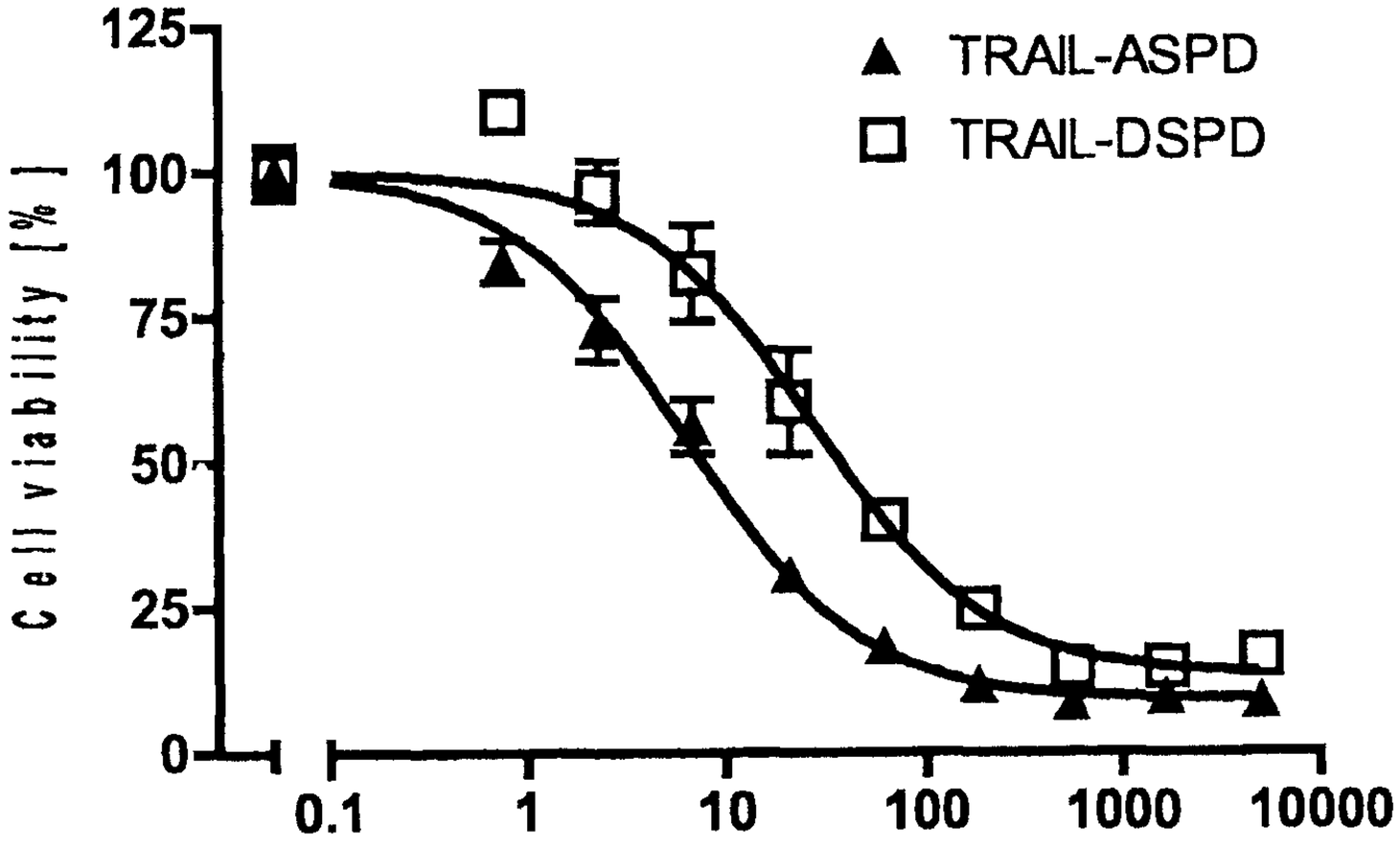


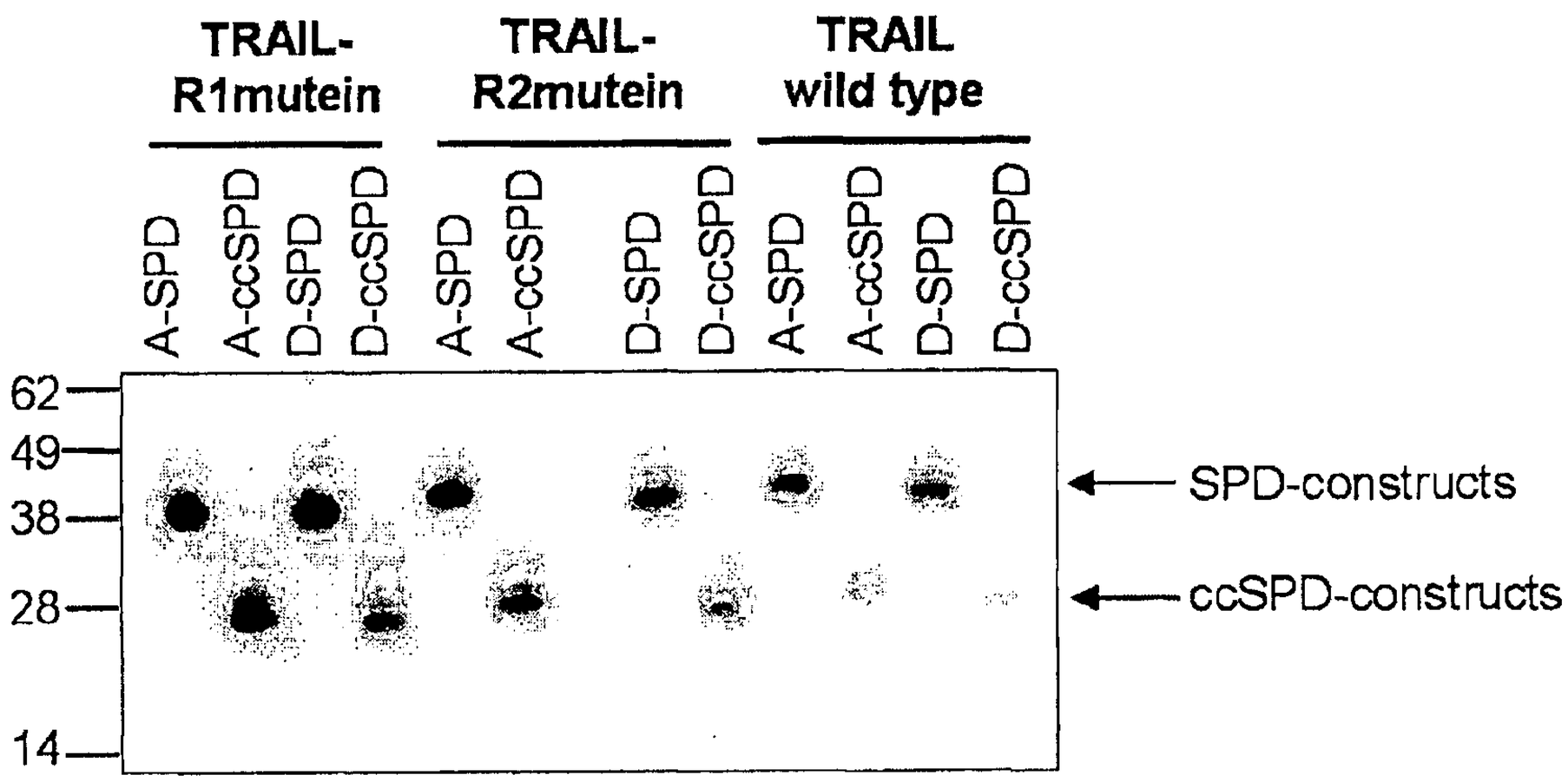
**Figure 12**

**Cytotoxicity assay with TRAIL-ASPD or TRAIL-DSPD on HT1080 cells**



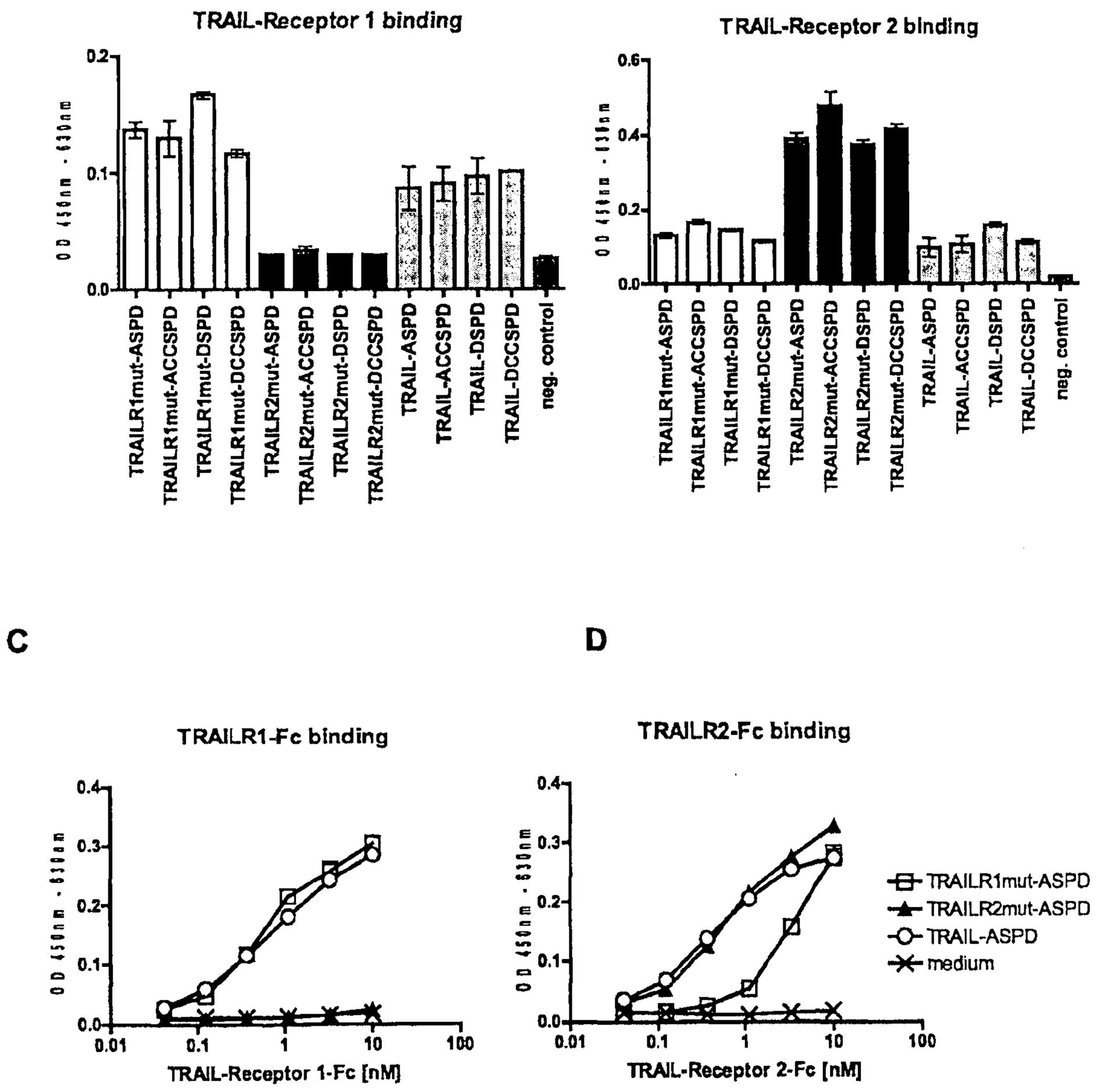
## Figure 13

**Western blot from transiently transfected HEK cells transiently transfected with TRAIL-SPD-constructs or TRAIL-receptor selective SPD constructs.**



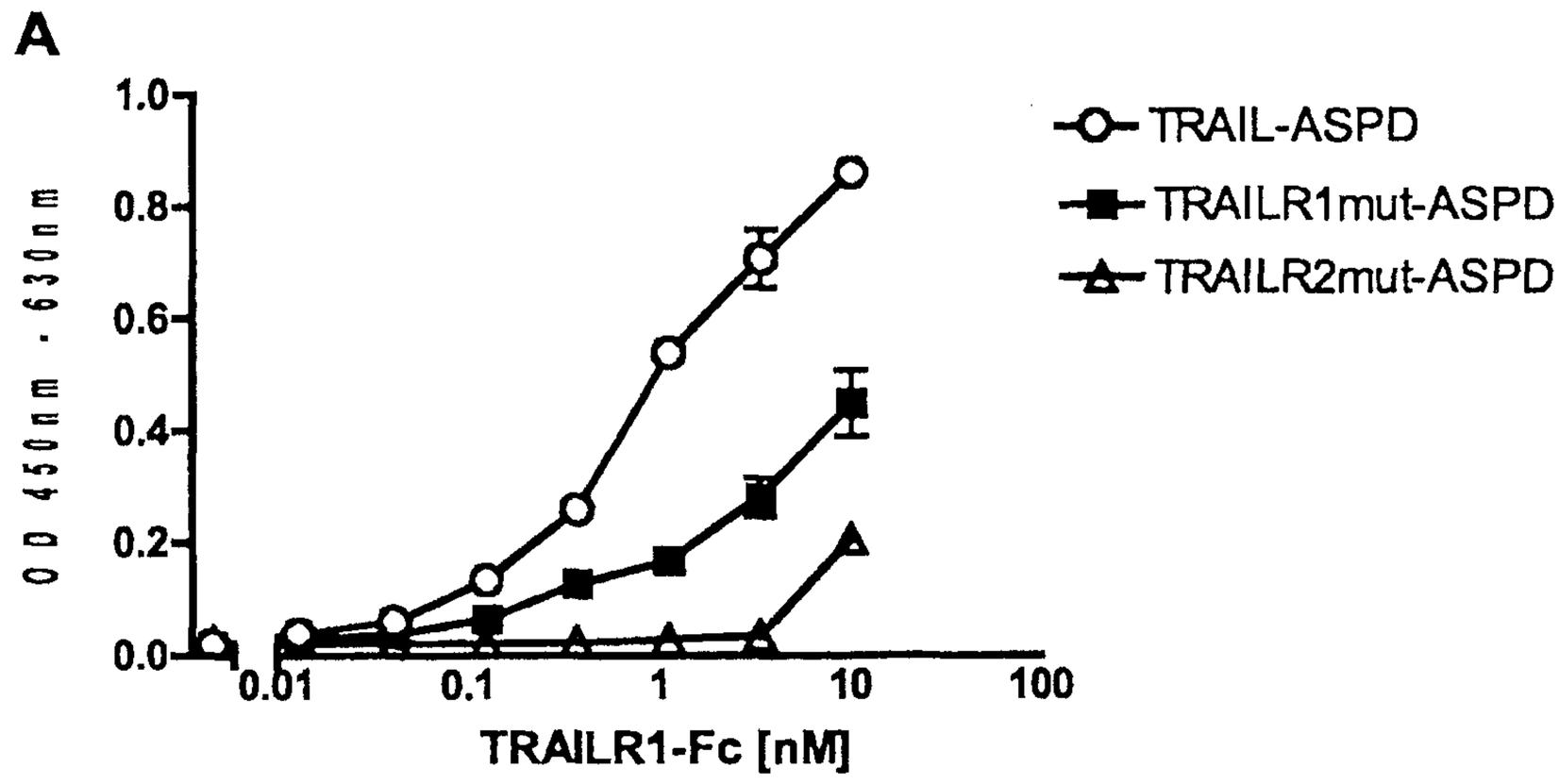
## Figure 14

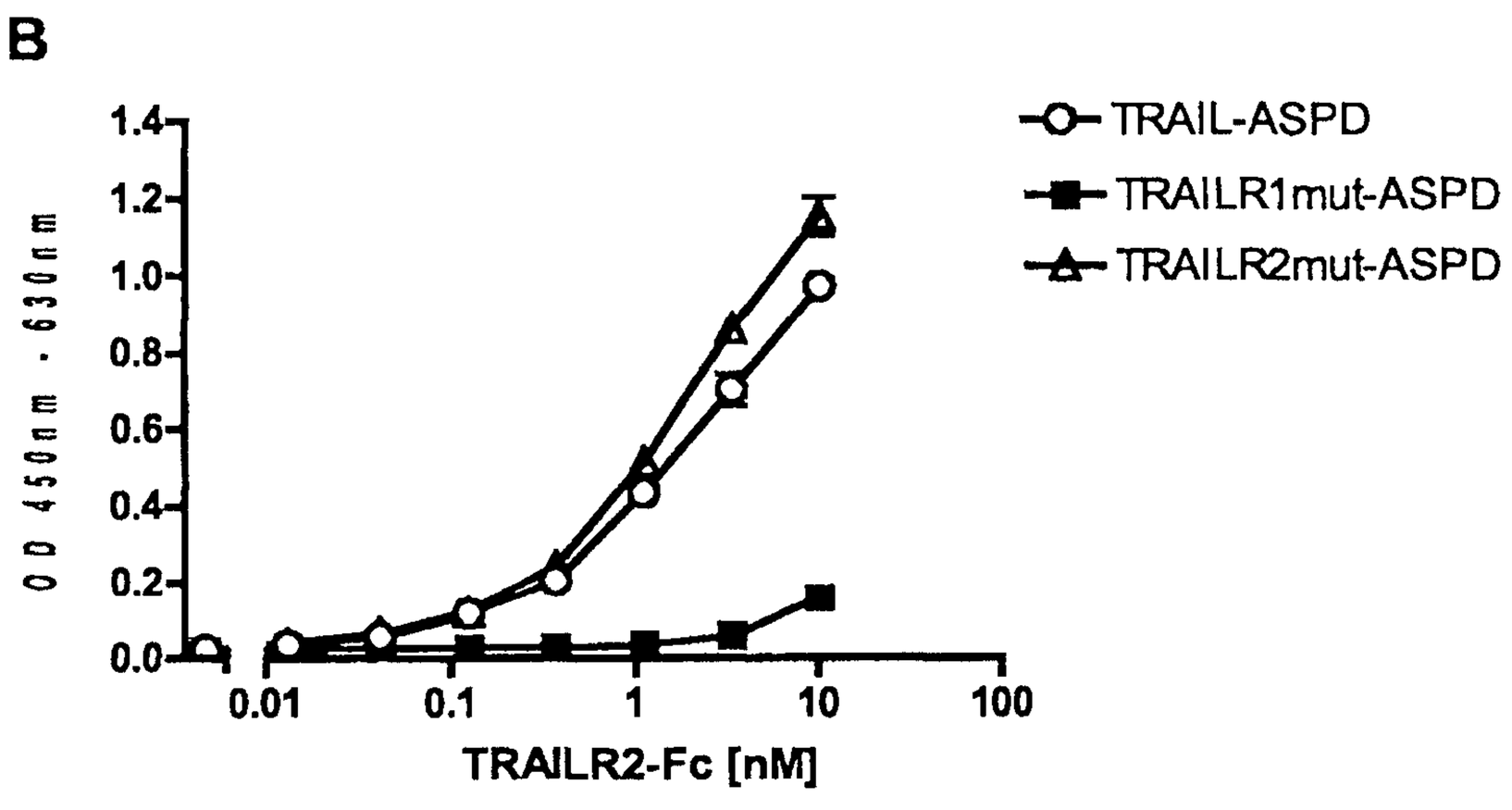
**TRAIL-Receptor selective ligands (TRAILR1mut and TRAILR2mut) immobilized on Streptactin plates, are differentially detected by TRAIL-Receptor 1-Fc or TRAIL-Receptor 2-Fc**



# Figure 15

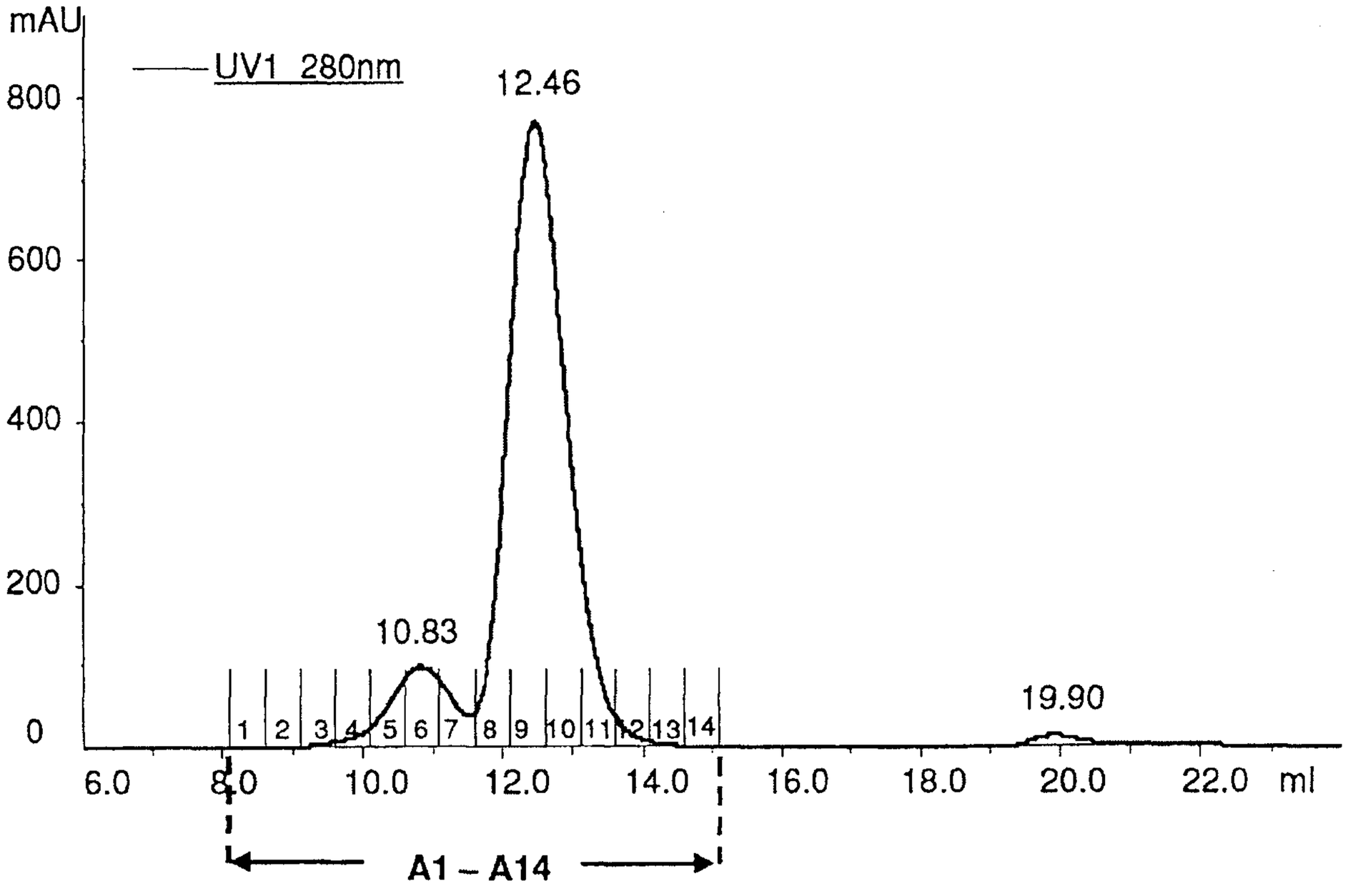
## Binding of TRAIL-Receptors to Receptor-selective "mutein" ligands



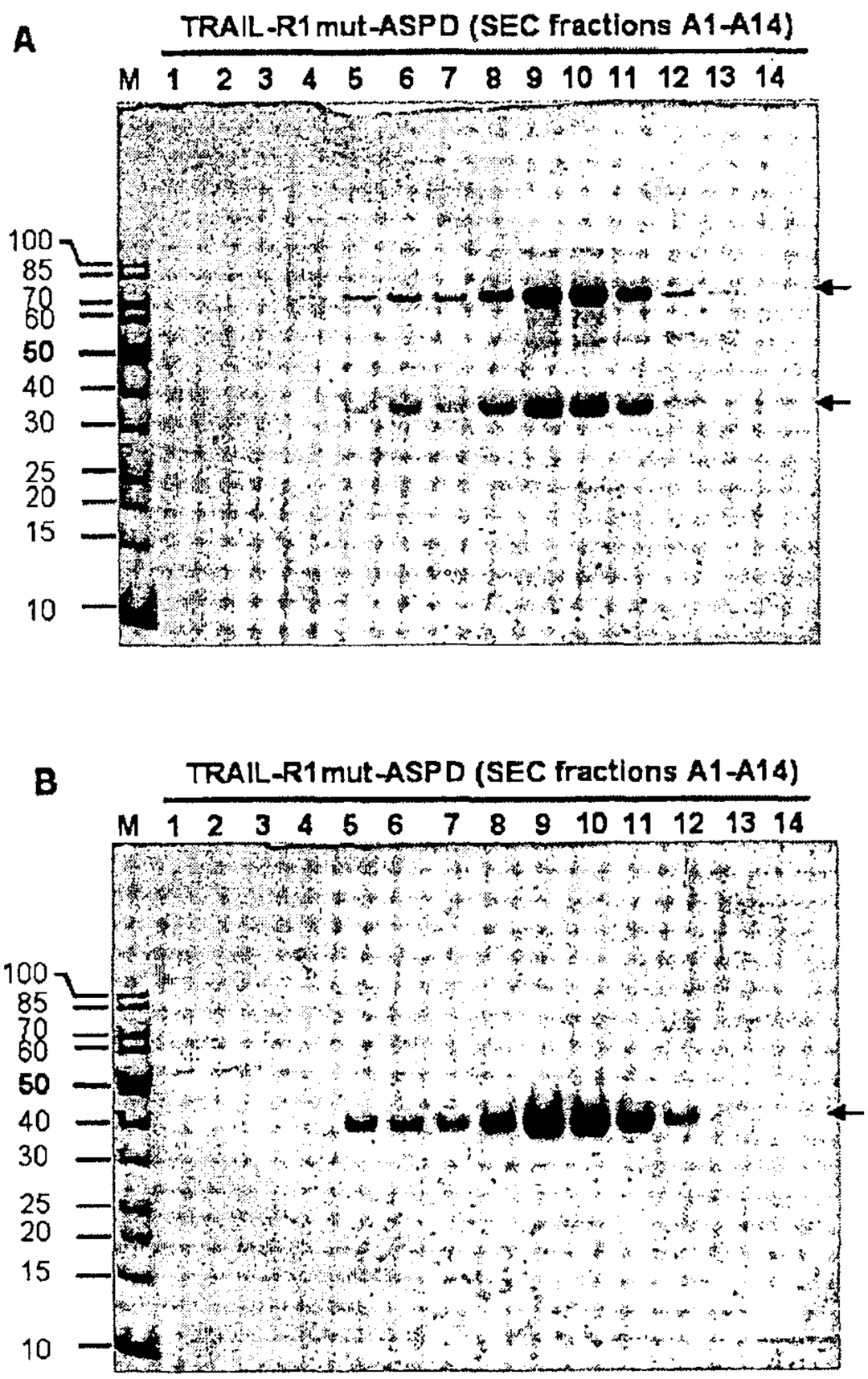
# Figure 16

## Size exclusion chromatography of affinity purified TRAILR1mut-ASPD



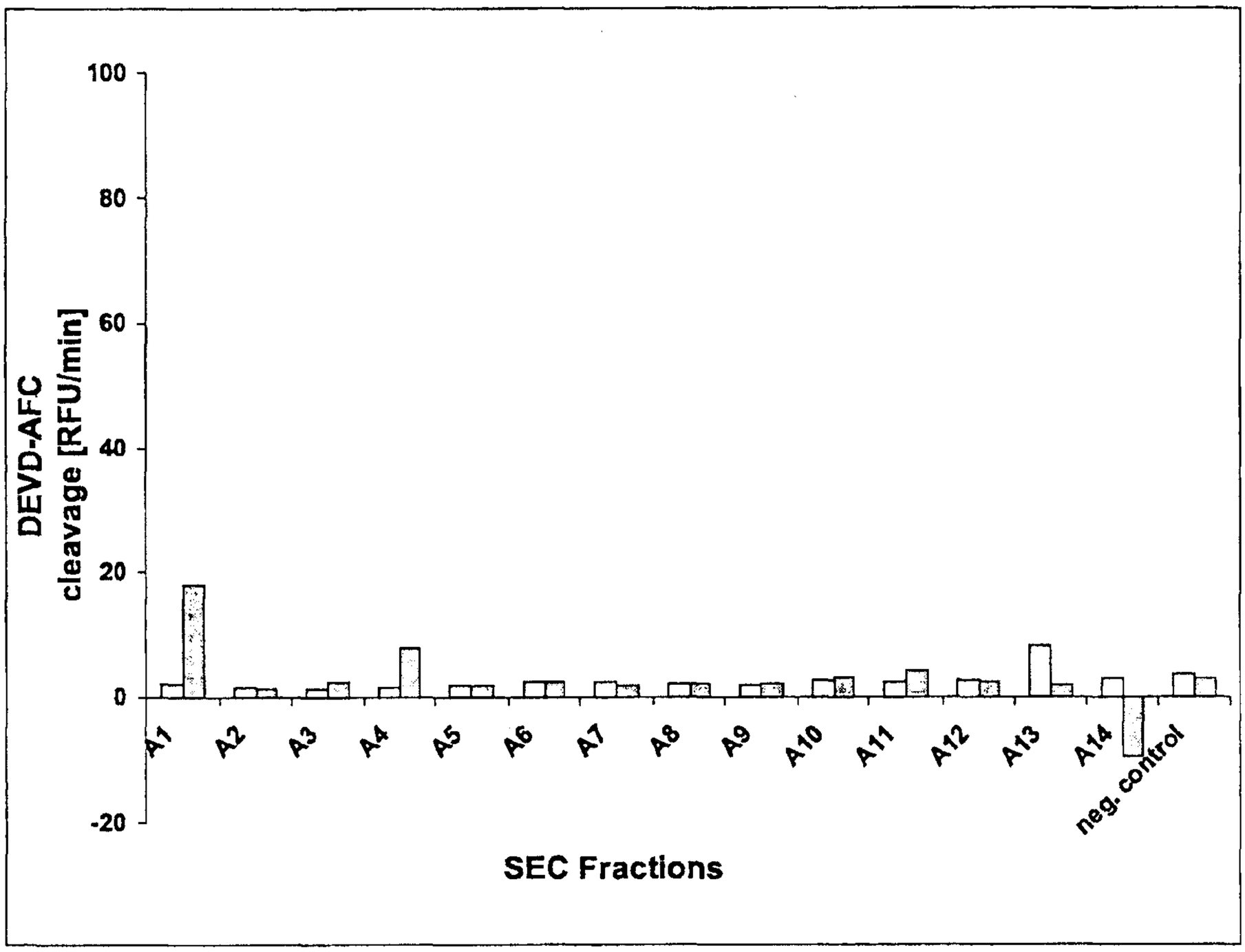
## Figure 17

**Silver stained SDS-PAGE of SEC fractions A1-A14 from affinity purified TRAILR1mut-ASPD**



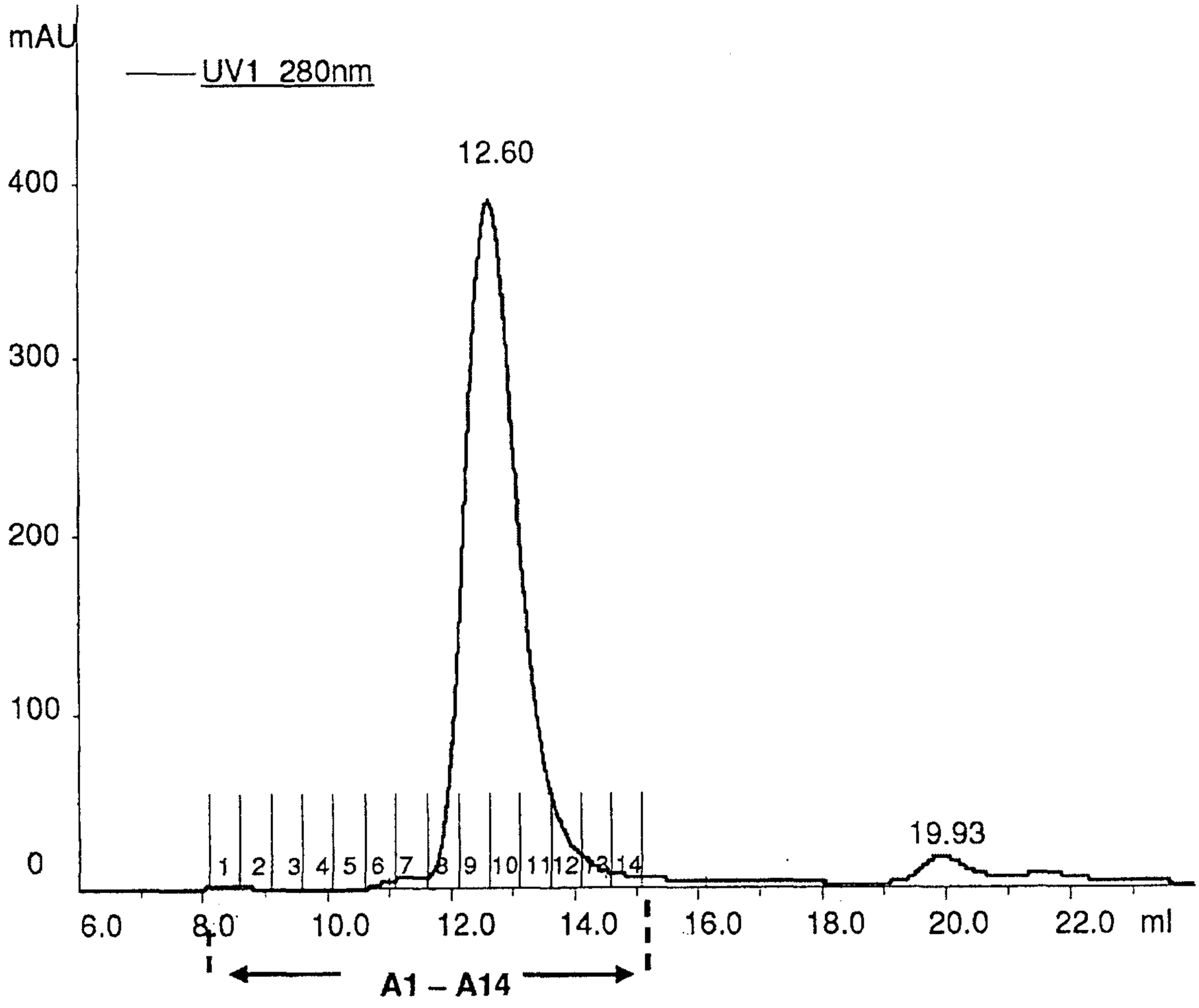
## Figure 18

**Caspase activity of SEC fractions A1-A14 from affinity purified TRAILR1mut-ASPD on Jurkat cells**



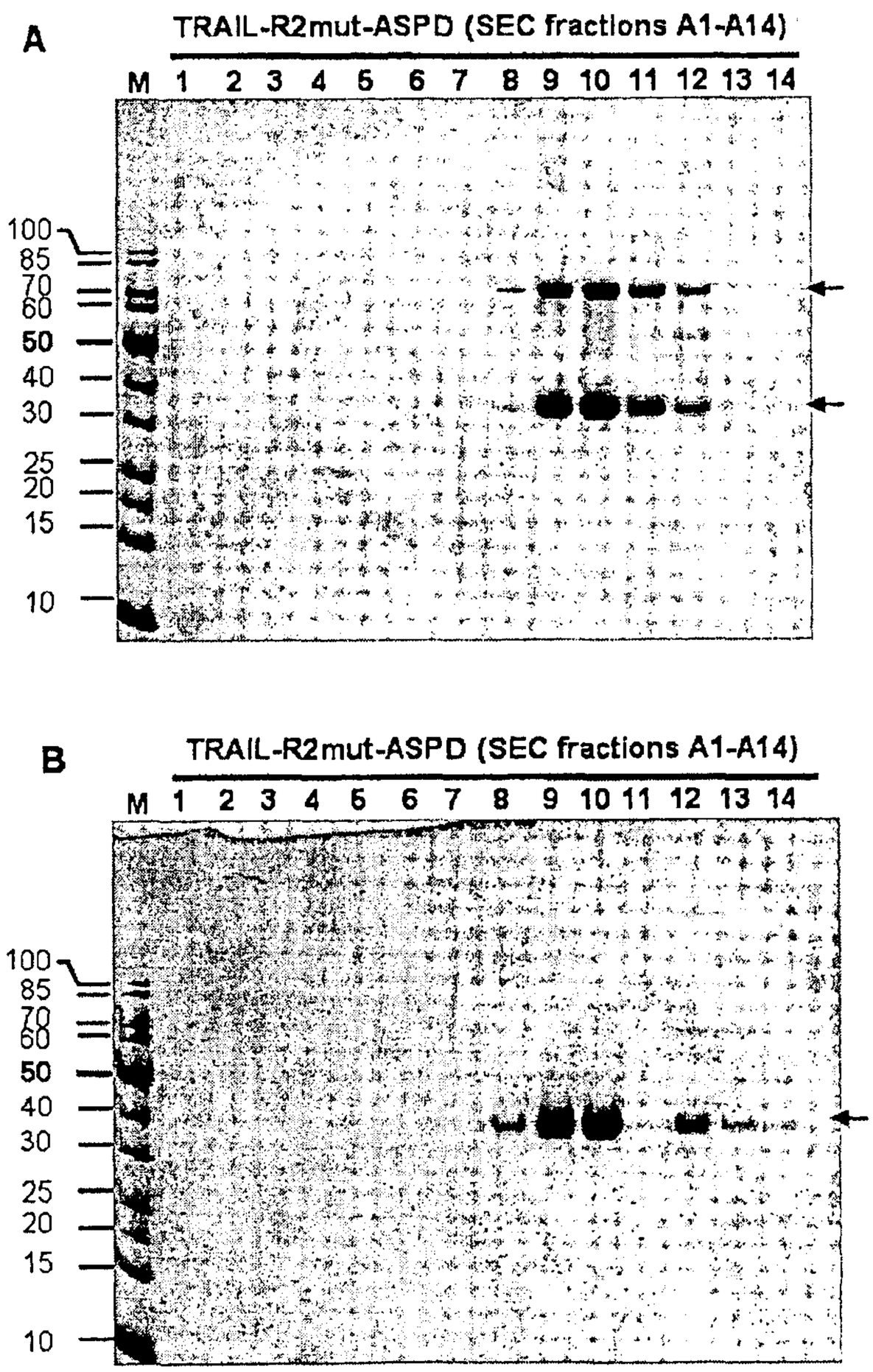
# Figure 19

## Size exclusion chromatography of affinity purified TRAILR2mut-ASPD



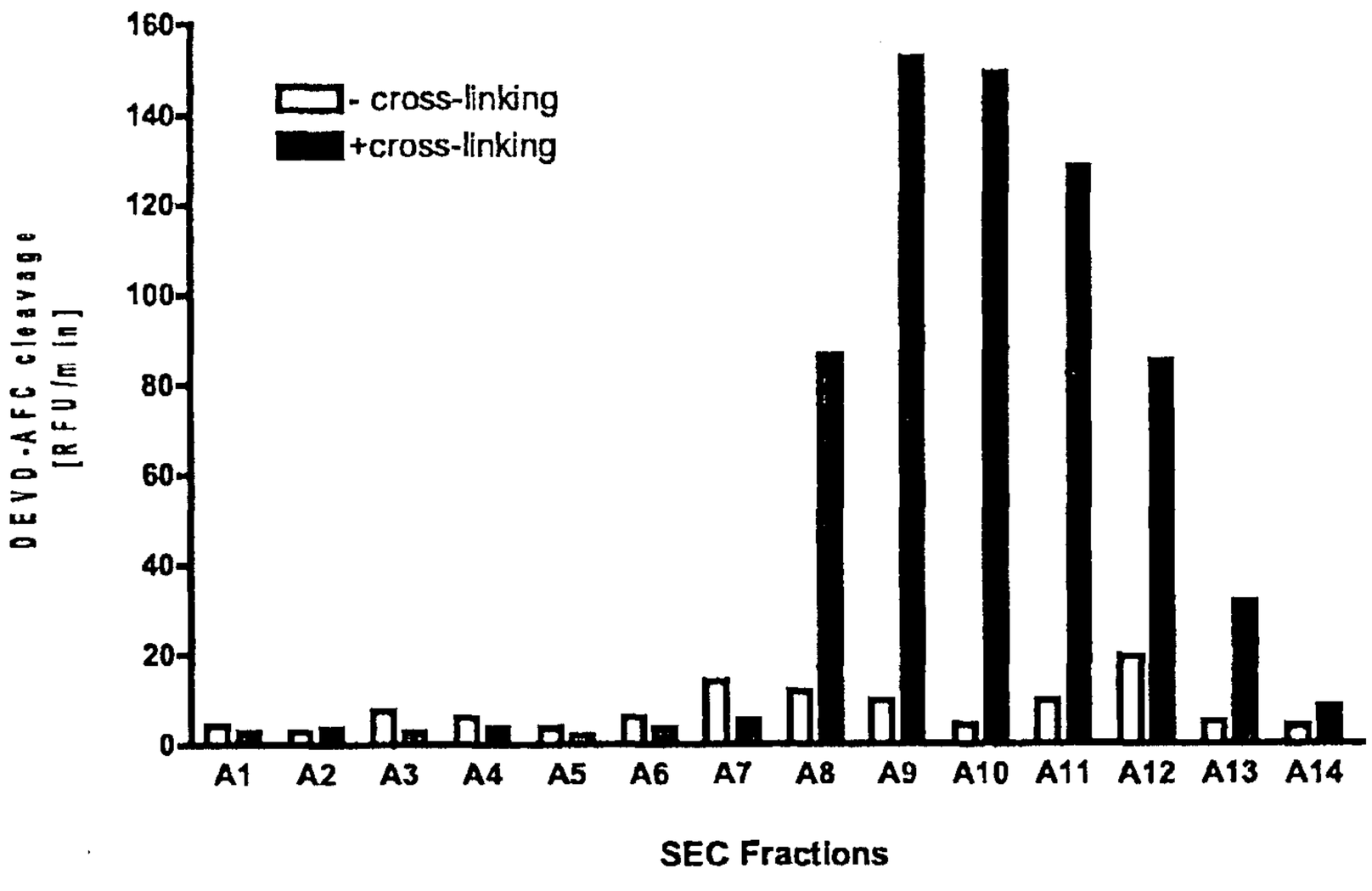
## Figure 20

**Silver stained SDS-PAGE of SEC fractions A1-A14 from affinity purified TRAILR2mut-ASPD**



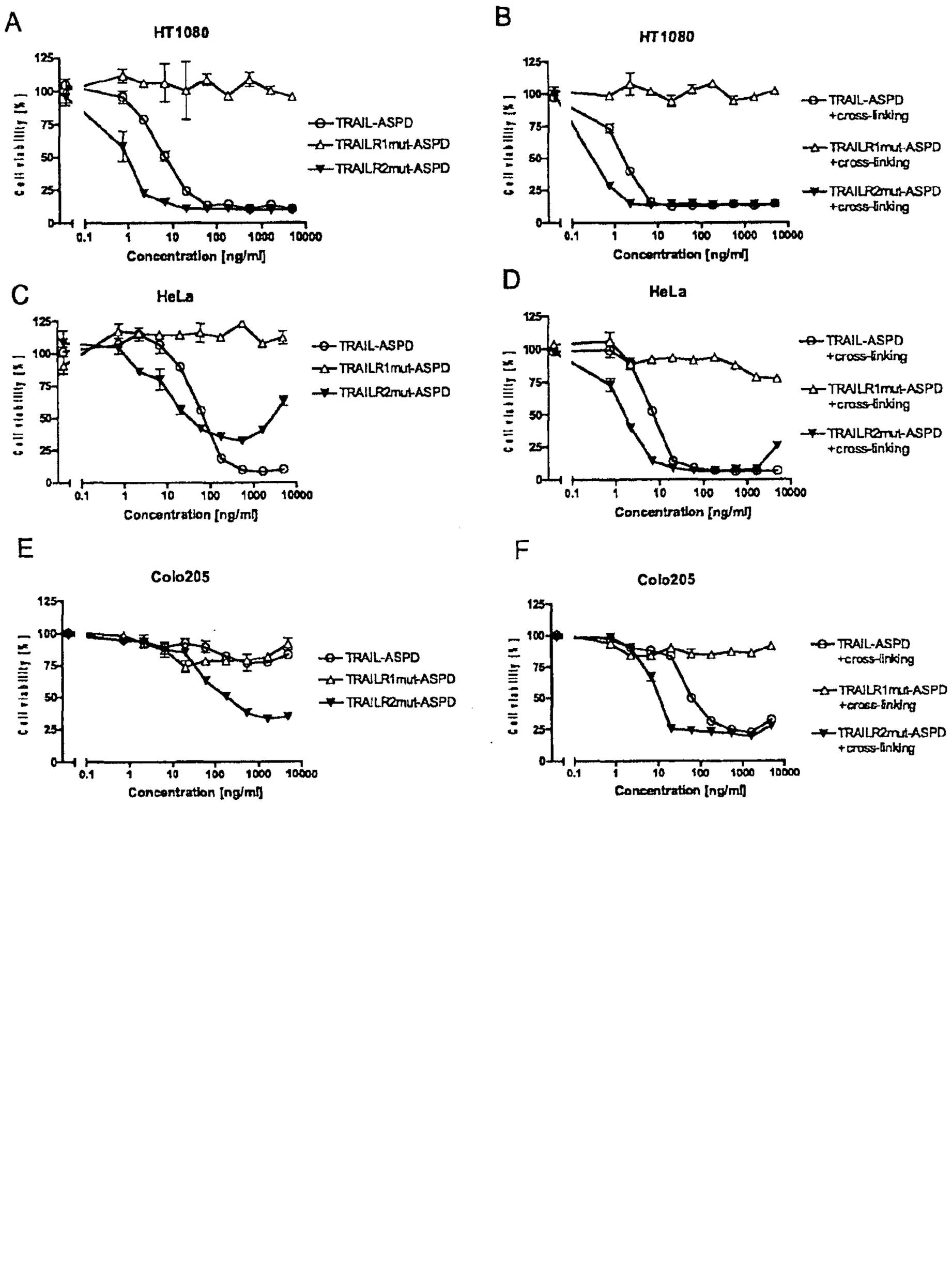
## Figure 21

**Jurkat Kill Assay Jurkat of SEC fractions A1-A14 from affinity purified TRAILR2mut-ASPD**



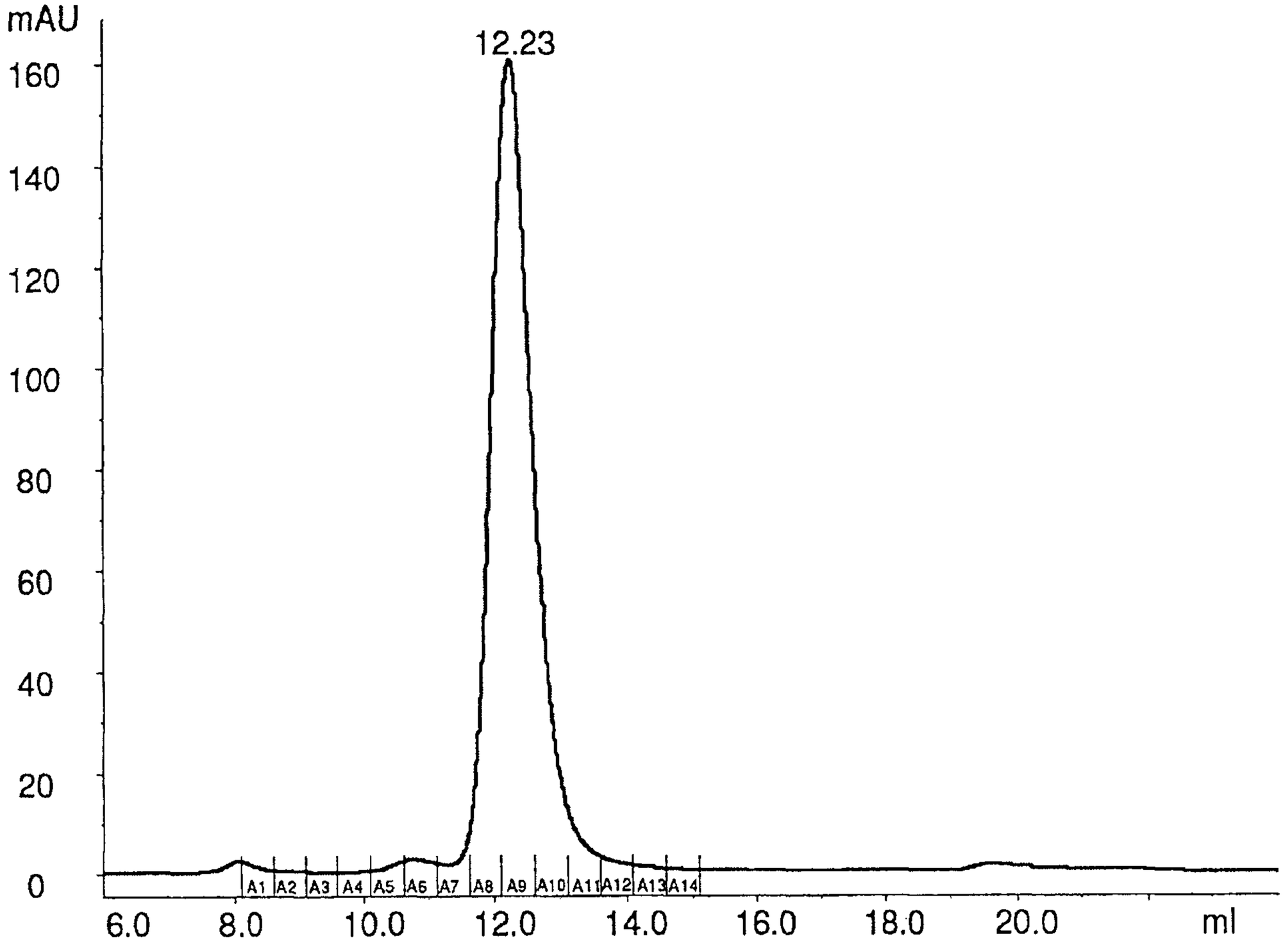
## Figure 22

**Cytotoxic activity of TRAIL-ASPD, TRAILR1mut-ASPD and TRAILR2mut-ASPD on human cancer cells.**



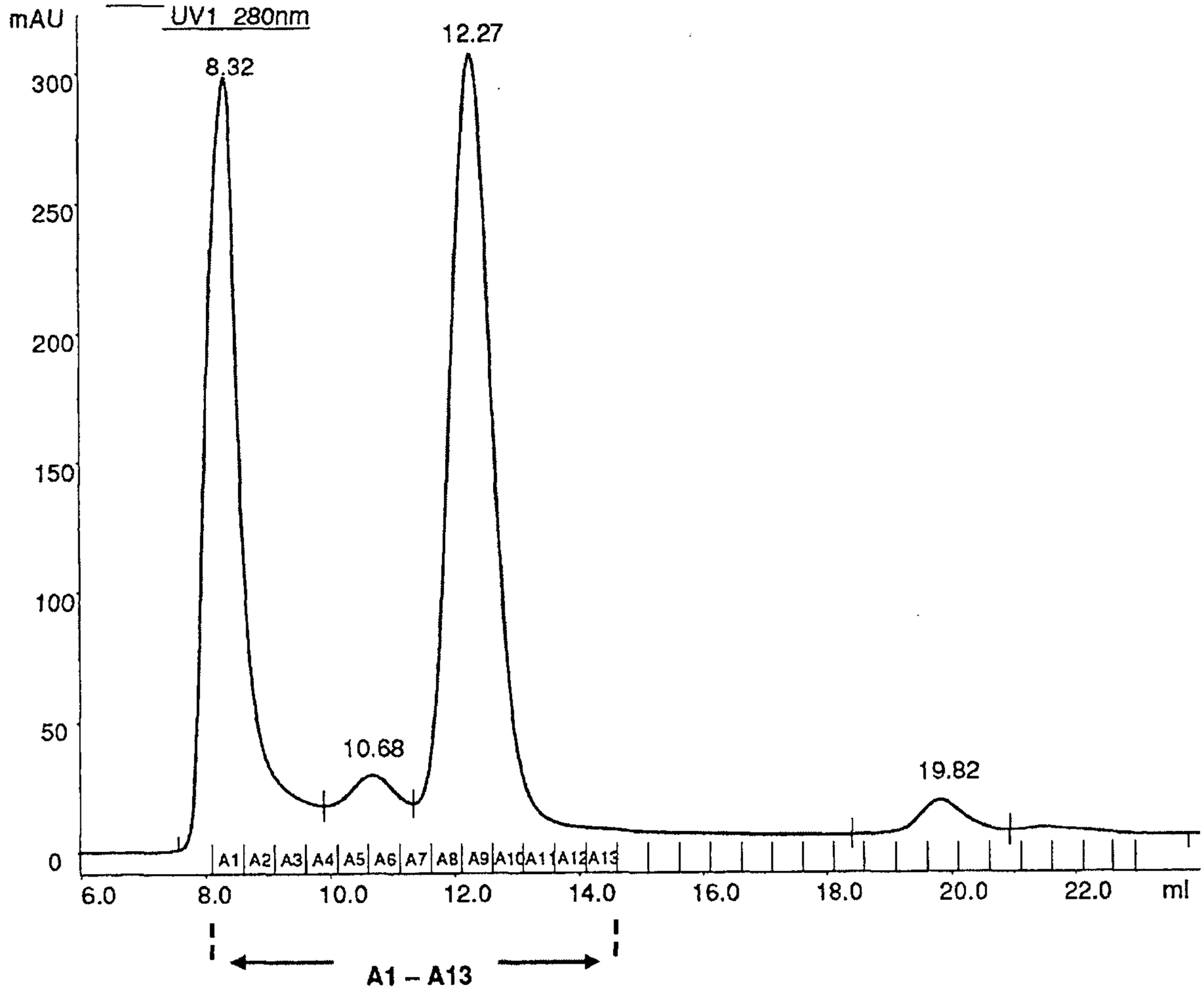
# Figure 23

## Receptor selective TRAIL-SPD proteins are highly souble



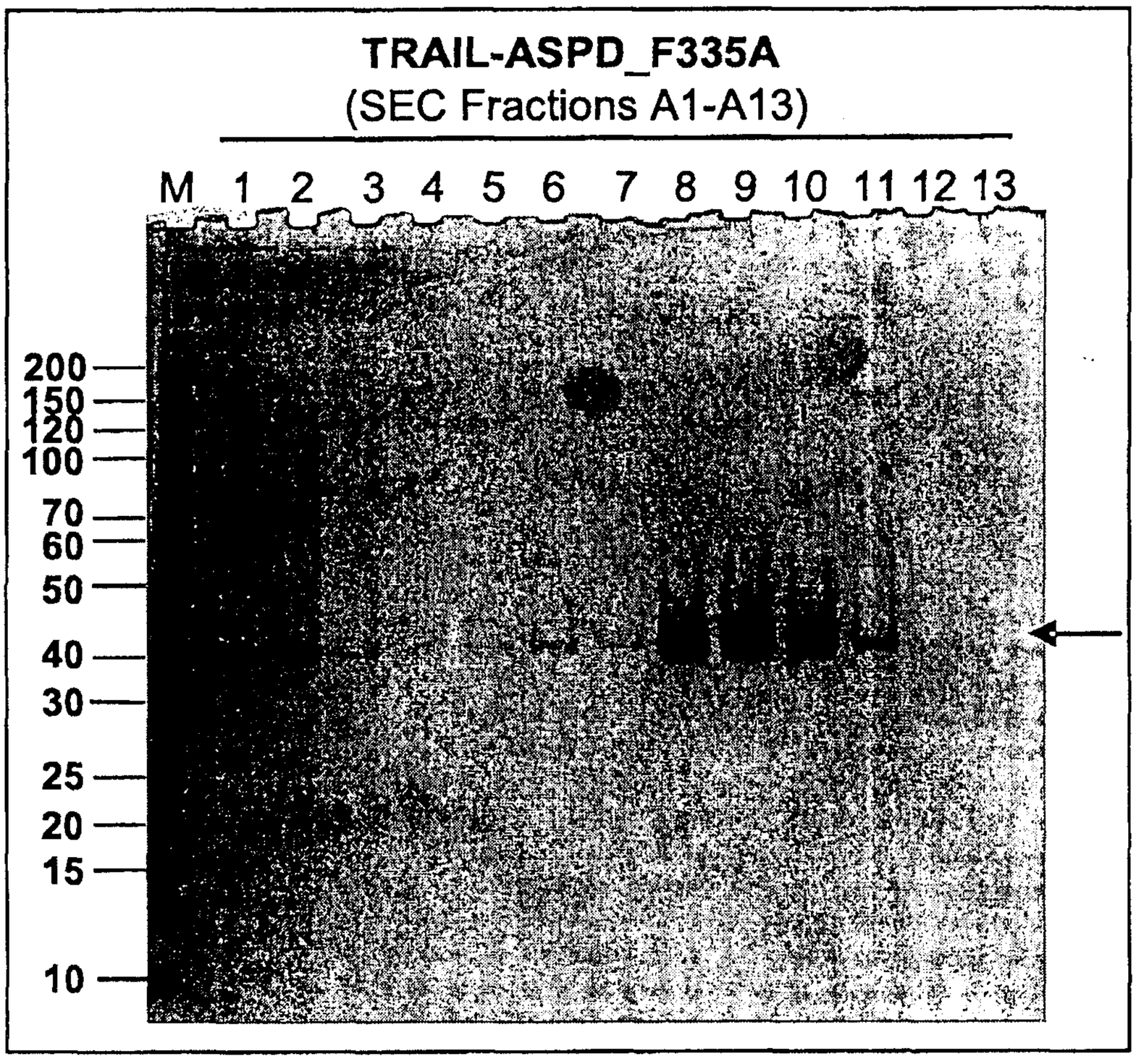
# Figure 24

## SEC of affinity purified TRAIL-ASPD_F335A



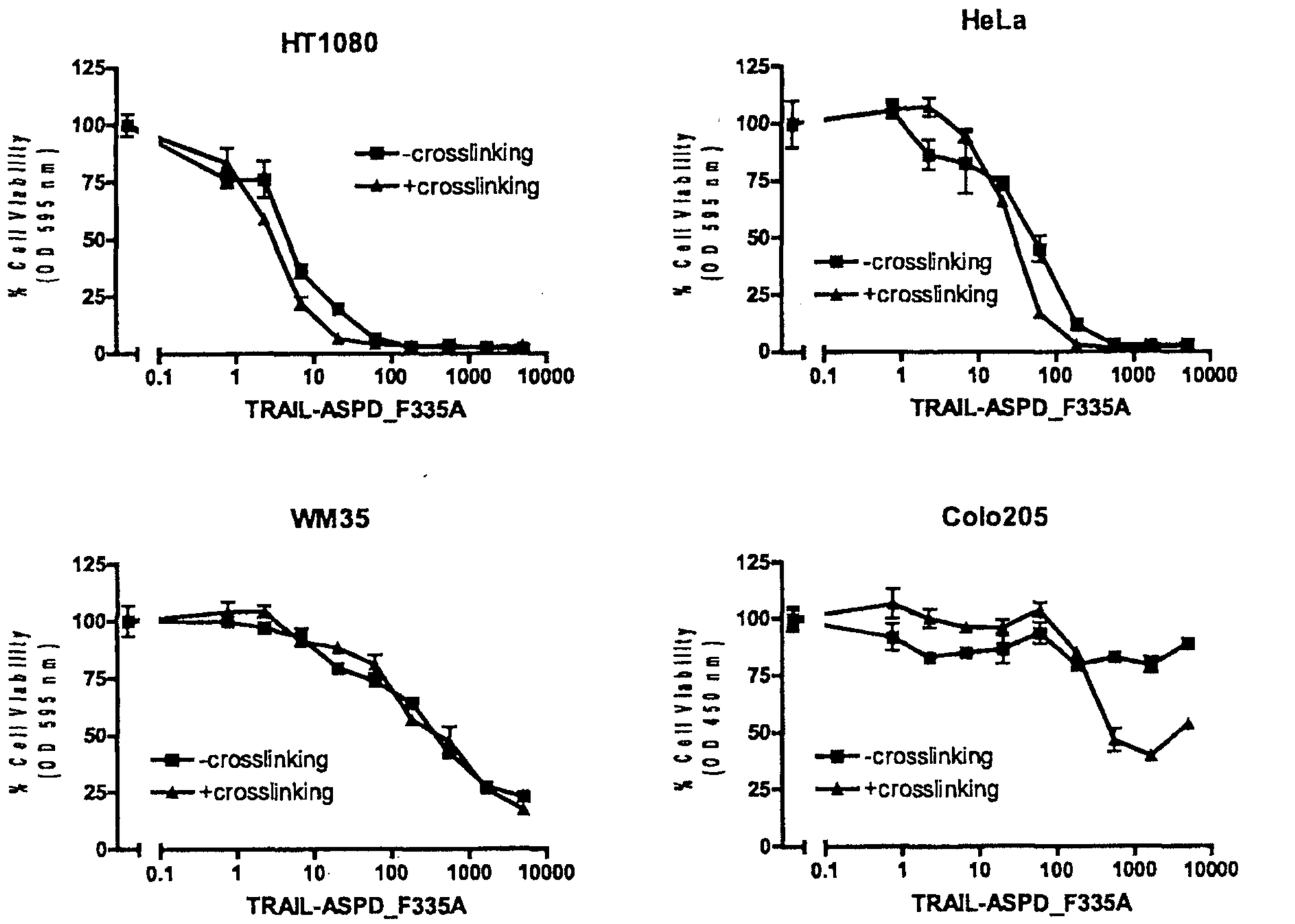
# Figure 25

## Silver stained SDS-PAGE of SEC fractions A1-A13



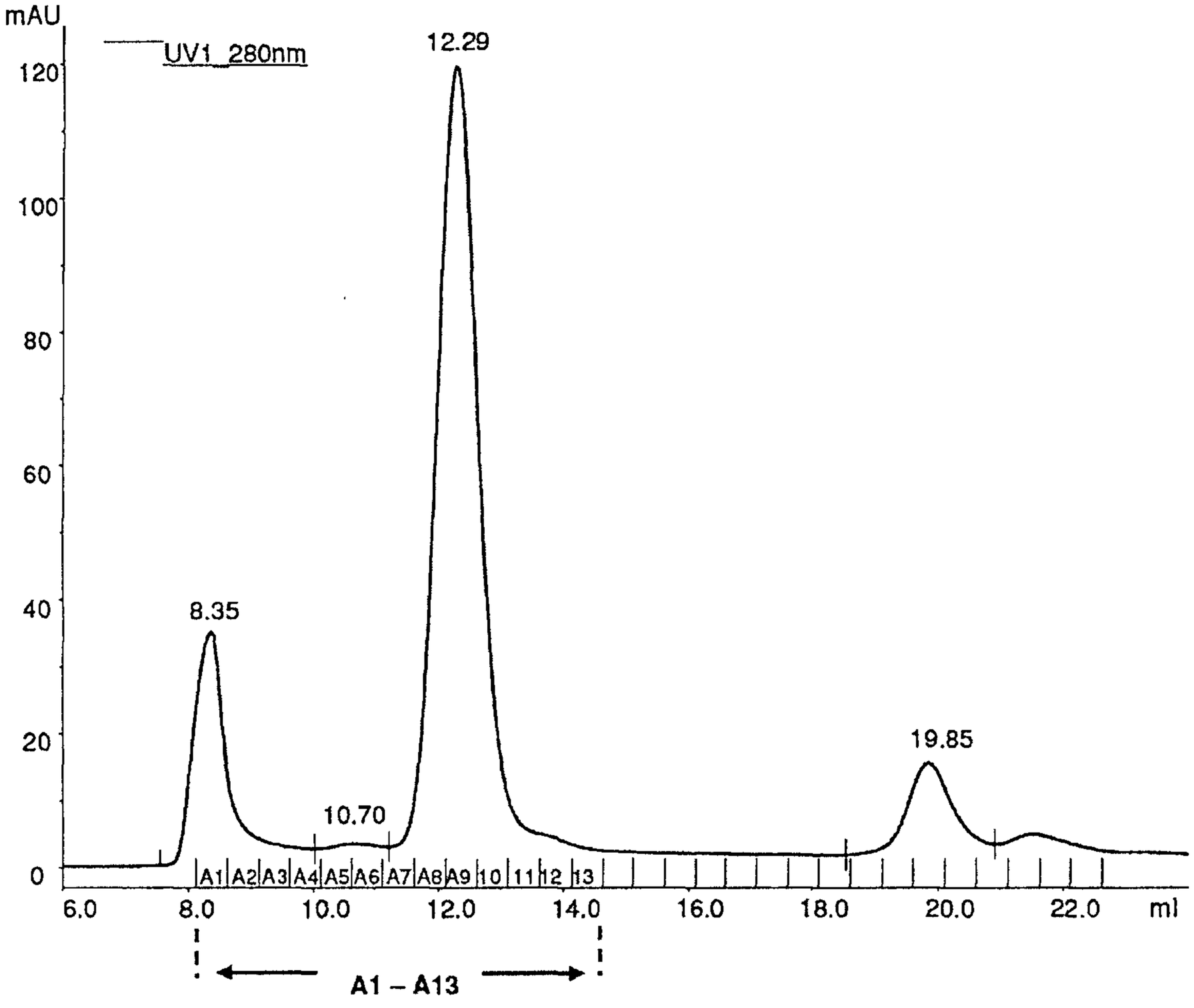
# Figure 26

## Cytotoxic effect of TRAIL-ASPD_F335A on human cancer cells



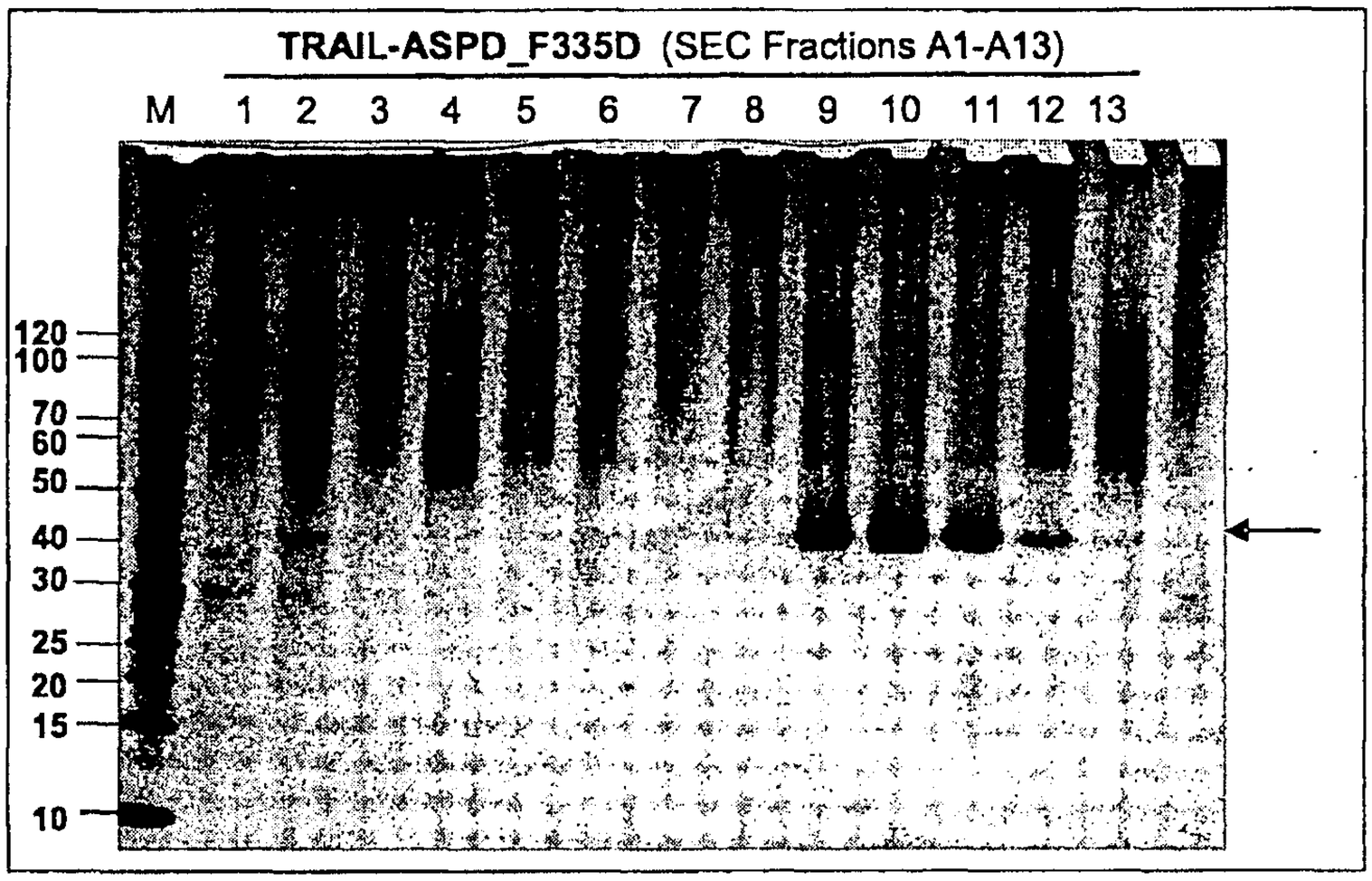
# Figure 27

## SEC of affinity purified TRAIL-ASPD_F335D



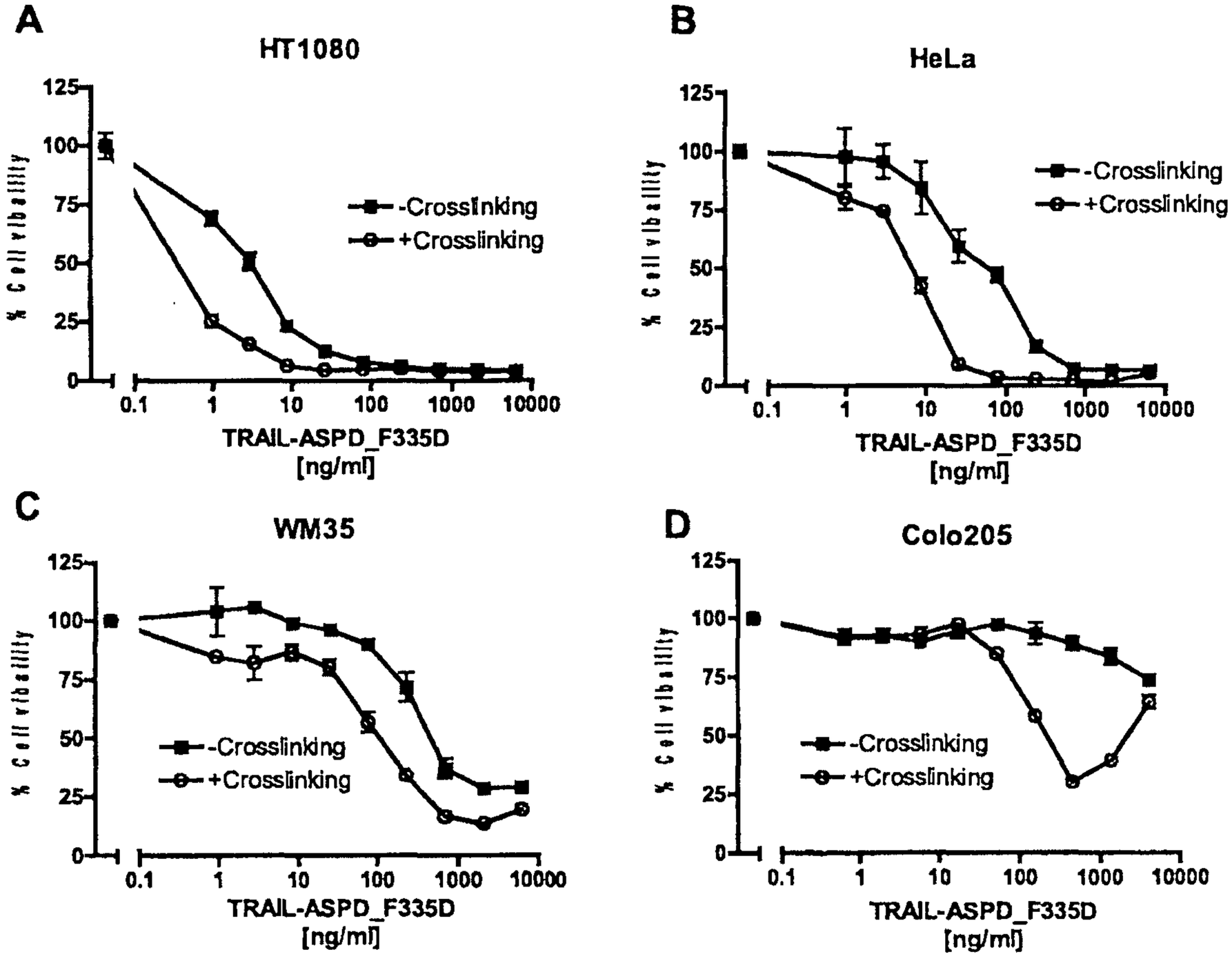
# Figure 28

## Silver stained SDS-PAGE of SEC from affinity purified TRAIL-ASPD_F335D



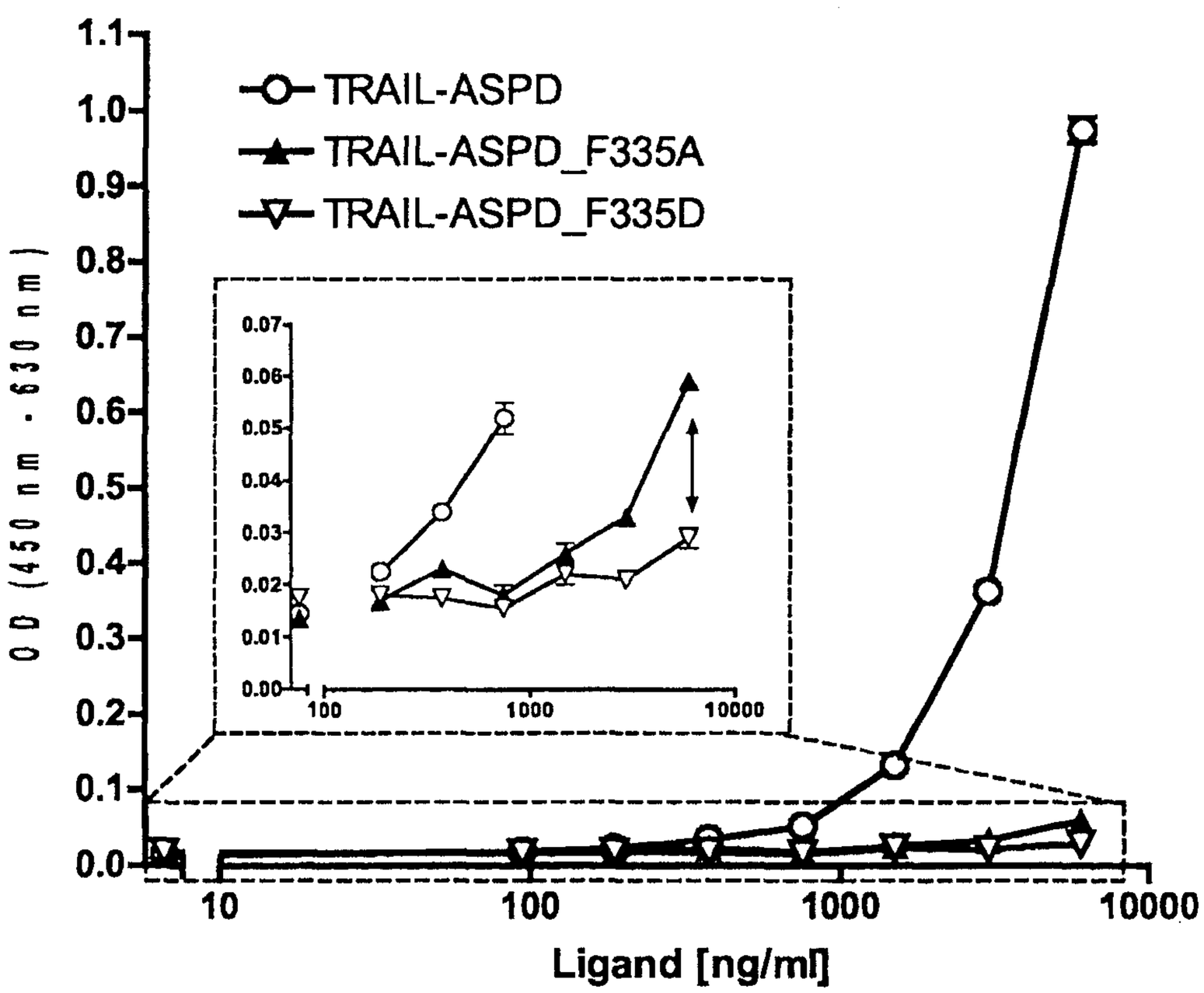
# Figure 29

## Cytotoxic effect TRAIL-SPD_F335D on human cancer cells



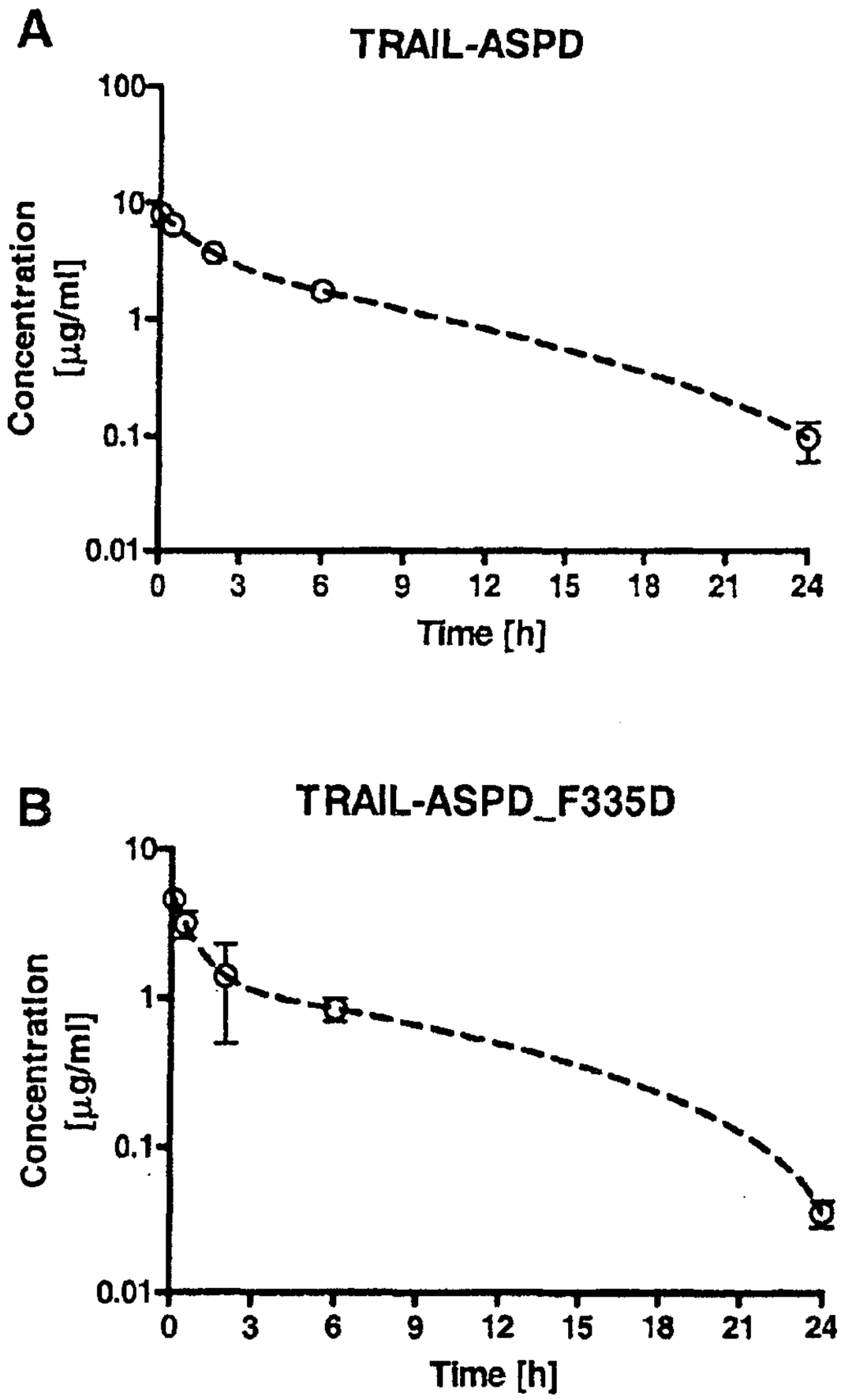
# Figure 30

## Binding of TRAIL-ASPD fusion protein to carbohydrates



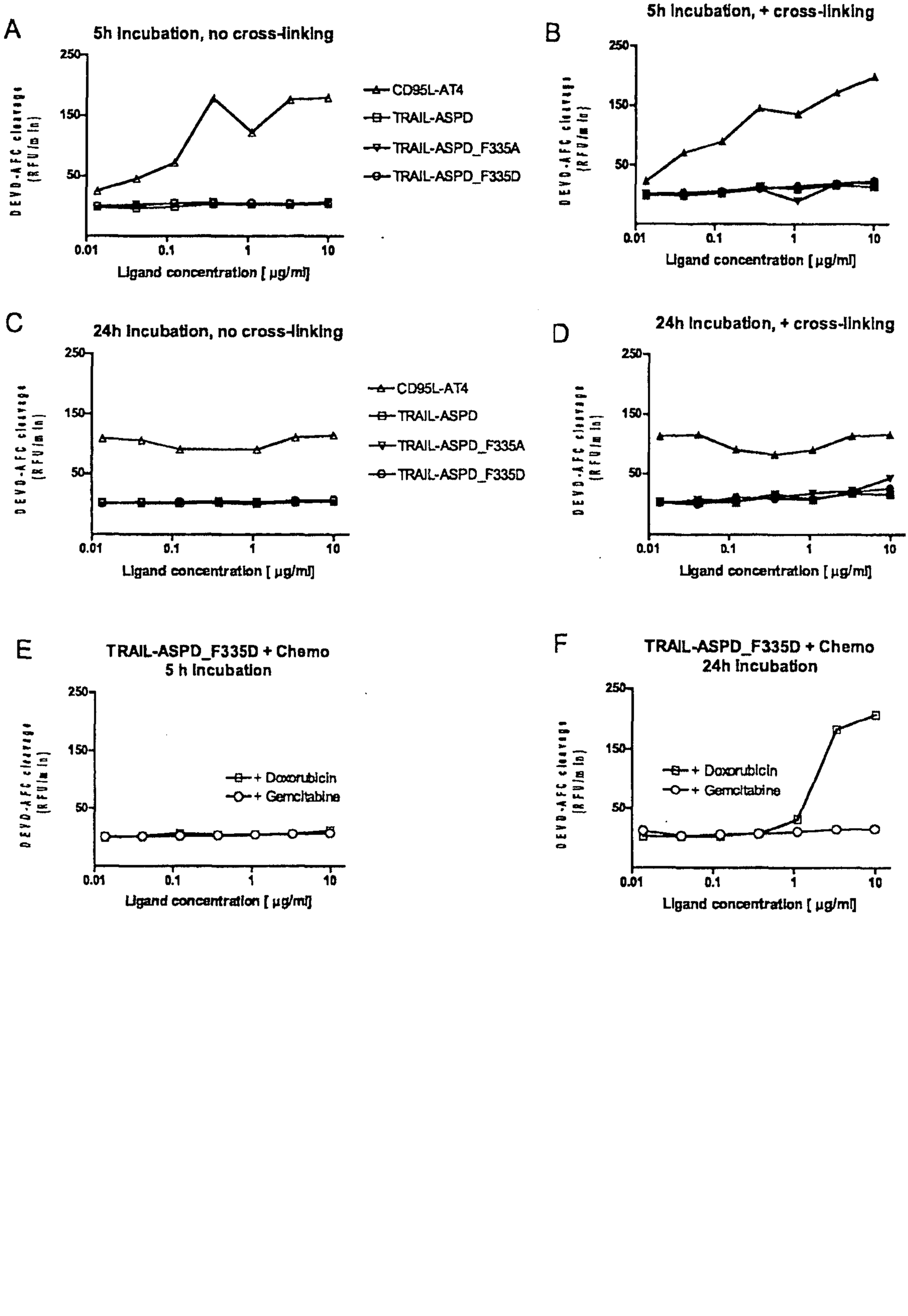
# Figure 31

## Pharmacokinetics of TRAIL-ASPD (A) or TRAIL-ASPD_F335 D (B) Fusion Proteins



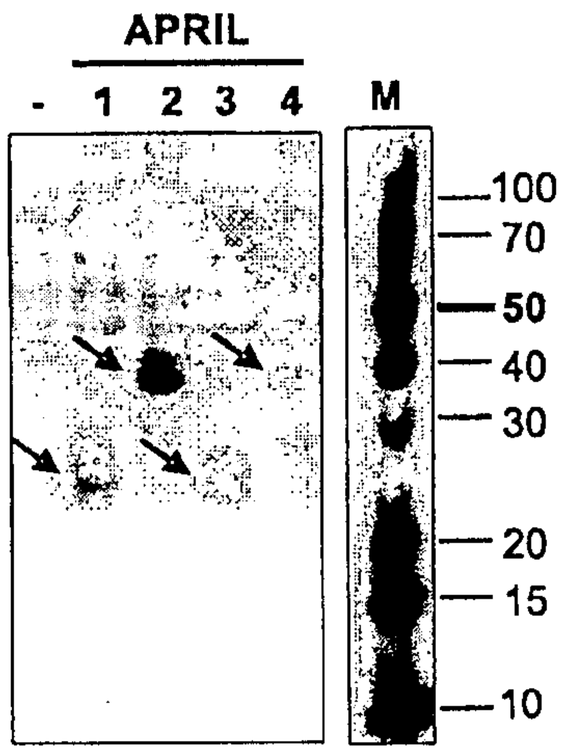
# Figure 32

## Caspase activity in primary human hepatocytes



## Figure 33

**Western Blot of supernatants from HEK293 cells transiently transfected with trimerized APRIL constructs**




| Lane | Construct |
|---|---|
| 1 | APRIL-A69 |
| 2 | APRIL-ASPD |
| 3 | APRIL-ACCSPD |
| 4 | APRIL-ACol11 |

# Figure 34

## TACI-Fc binds to APRIL-ASPD