(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 August 2019 (15.08.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/154986 A1

(51) International Patent Classification:
A61K 47/68 (2017.01) *C07K 14/54* (2006.01)
A61P 35/00 (2006.01) *C07K 16/18* (2006.01)

(21) International Application Number:
PCT/EP2019/053136

(22) International Filing Date:
08 February 2019 (08.02.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
18156141.6 09 February 2018 (09.02.2018) EP
18179313.4 22 June 2018 (22.06.2018) EP

(71) Applicant: **PHILOGEN S.P.A.** [IT/IT]; La Lizza 7, 53100 Siena (IT).

(72) Inventors: **VILLA, Alessandra**; c/o Philochem AG, Libernstrasse 3, 8112 Otelfingen (CH). **MATASCI, Mattia**; c/o Philochem AG, Libernstrasse 3, 8112 Otelfingen (CH). **ONGARO, Tiziano**; c/o Philochem AG, Libernstrasse 3, 8112 Otelfingen (CH).

(74) Agent: **MICHALSKI HÜTTERMANN & PARTNER PATENTANWÄLTE MBB** et al.; Speditionstraße 21, 40221 Düsseldorf (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: EDB TARGETING IL-12 COMPOSITIONS

Clone (SEQ ID NO)	...F35-L19... linker sequence (nucleotide)	...F35-L19... linker sequence (aminoacid)	Production yields
AKKAS (18/9)	AATGCTTCCGGAGGGGAGCTAAAGGTGGCGGTGGCAAGGCAGGGGAGGGAGTGAGGTGCAG	NAS GGGAKGGGGKAGGGS EVQ	4.6 mg/L
AP7 (19/15)	AATGCTTCCGCACCAGCACCAGCACCAGCACCAGCACCAGCACCAGCACCAGAGGTGCAG	NAS APAPAPAPAPAPAP EVQ	4.7 mg/L
DDS (20/10)	AATGCTTCCGGAGGTGGGGGTGATGGTGGGGGAGGTGACGGCGGAGGTGGGTCTGAGGTGCAG	NAS GGGGDGGGGDGGGGS EVQ	5.0 mg/L
AP6 (21/14)	AATGCTTCCGCACCAGCACCAGCACCAGCACCAGCACCAGAGGTGCAG	NAS APAPAPAPAPAP EVQ	3.5 mg/L
$(G4S)_2$ (22/11)	AATGCTTCCGGTGGAGGCGGGTCAGGCGGAGGGGGTTCTGGCGGTGGCGGATCGGAGGTGCAG	NAS GGGGSGGGGSGGGGS EVQ	3.5 mg/L
SES (23/12)	AATGCTTCCGGTGGGGGTGGGTCCGGAGGCGGAGGCGAAGGCGGAGGTGGGTCGGAGGTGCAG	NAS GGGGSGGGGEGGGGS EVQ	4.4 mg/L
Alpha3 (24/13)	AATGCTTCCGCAGAAGCAGCAGCAAAAGAAGCAGCAGCAAAAGAAGCAGCAGCAAAAGCAGAGGTGCAG	NAS AEAAAKEAAAKEAAAKA EVQ	4.5 mg/L
SAD (25/4)	AATGCTTCCGGGTCTGCAGACGGCGGATCATCAGCTGGGGGAAGTGACGCAGGAGAGGTGCAG	NAS GSADGGSSAGGSDAG EVQ	9.0 mg/L

Fig. 1A

(57) Abstract: The present invention relates to compositions comprising an IL-12 protein having a first and second subunit, an EDB-binding domain, and a linker between the IL-12 protein and the EDB- binding domain.

WO 2019/154986 A1

EDB Targeting IL-12 Compositions

FIELD OF THE INVENTION

The present application relates to compositions comprising a cytokine, an antigen binding domain, and an improved linker.

BACKGROUND

IL-12 is a heterodimeric cytokine comprising two disulfide-linked subunits, p35 and p40. IL-12 stimulates the production of IFNγ from T-cells and natural killer cells, and also induces differentiation of Th1 helper cells. IL-12 is a key mediator of innate and cell-mediated immunity, with the potential for anti-cancer and anti-metastatic activity.

Like many other cytokines, however, the administration of IL-12 is associated with severe toxicity (Car et al., 1999), even at doses as low as 1μg per kg per day, discouraging its development as an anticancer drug.

SUMMARY OF THE INVENTION

The present invention provides, among other things, improved compositions and methods that can be used to effectively treat various diseases and disorders associated with the expression of EDB fibronectin.

In particular, the present invention provides IL-12 linked EDB binding domains having preferred therapeutic properties over known recombinant IL-12 constructs. The compositions described herein surprisingly are superior to previously known IL-12 constructs designed to target EDB and solve the long-known problem of safely and effectively administering IL-12 for the targeted treatment of a disease or disorder, e.g., a cancer. The compositions and methods described herein provide for improved therapeutic potential of IL-12 by enhancing one or more of its biodistribution profile, its tolerability, its therapeutic window, and its efficacy in reaching the site of disease. The constructs described herein also surprisingly exhibit superior manufacturability.

There remains a need in the art for improvements in tissue penetration of immunocytokine treatments. There is also a need in the art for improved manufacturing of immunocytokine treatments as these are highly complex proteins which are difficult to produce.

It is hence an object of the present invention to provide improved versions immunocytokines, e.g., of protein therapeutics comprising an IL-12 and an EDB fibronectin binding domain. It is a further object of the present invention to provide immunocytokines which exhibit more efficient production. It is another object of the present invention to provide immunocytokines with improved *in vivo* performance, e.g., target binding or tissue penetration.

The present invention provides compositions comprising an antigen-binding domain and a cytokine having such superior properties. The invention and general advantages of its features, including suitable linkers, will be discussed in detail below.

According to one aspect of the invention, a composition is provided comprising

a. an IL-12 protein comprising a first IL-12 subunit and a second IL-12 protein subunit;

b. a peptide or protein comprising an EDB binding domain; and

c. a linker between the IL-12 protein and the peptide or protein comprising the EDB binding domain.

Preferably, the two subunits of IL-12 are joined to one another by a given linker, according to the following scheme (N->C orientation): p40-linker 1-p35.

Preferably IL-12 is human IL-12. According to some embodiments of the invention, the single chain diabody binds to a splice isoform of fibronectin. Preferably, said extra-domain B (ED-B) of fibronectin is the extra-domain B of human fibronectin (UniProt: P02751).

In some embodiments, the linker between the IL-12 protein and the peptide or protein comprising the EDB binding domain comprises GSADGGSSAGGSDAG (SEQ ID NO: 4).

In some embodiments, the peptide or protein comprising the EDB binding domain comprises an scFv. In some embodiments, the peptide or protein comprising the EDB binding domain is a diabody. In some embodiments, the peptide or protein comprising the EDB binding domain is a single chain diabody.

In some embodiments, the first subunit of the IL-12 protein is a p40 and the second subunit is a p35.

In some embodiments, the first subunit of the IL-12 protein is a p40 comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO:1 or a fragment thereof, wherein the IL-12 protein can activate an IL-12 receptor.

In some embodiments, the second subunit of the IL-12 protein is a p35 comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO:3 or a fragment thereof, wherein the IL-12 protein can activate an IL-12 receptor.

In some embodiments, the peptide or protein comprising an EDB binding domain is monospecific or bispecific.

In some embodiments, the peptide or protein comprising an EDB binding domain binds to the extra-domain B (ED-B) of fibronectin.

In some embodiments, the peptide or protein comprising an EDB binding domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to one or more of the amino acid sequences set forth in SEQ ID NOs: 28 to 33.

In some embodiments, the peptide or protein comprising an EDB binding domain comprises each of the amino acid sequences of SEQ ID NOs: 28 to 33.

In some embodiments, the peptide or protein comprising an EDB binding domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to one or more of the amino acid sequences set forth in SEQ ID NOs: 7 and 5.

In some embodiments, the peptide or protein comprising an EDB binding domain comprises each of the amino acid sequences set forth in SEQ ID NOs: 7 and 5.

In some embodiments, the peptide or protein comprising an EDB binding domain comprises at least one of

a) the sequence pair according to the above description, with the proviso that at least one of the domains has a sequence identity of ≥80 % relative to SEQ ID NO:7 or SEQ ID NO:5, respectively and/or
b) the sequence pair according to the above description, with the proviso that at least one of the domains has up to 10 amino acid substitutions relative to SEQ ID NO:7 or SEQ ID NO:5, respectively,

while maintaining its capability to bind to the extra-domain B (ED-B) of fibronectin.

In some embodiments, the peptide or protein comprises at least one amino acid substitution where the at least one amino acid substitution is a conservative amino acid substitution.

In some embodiments, the peptide or protein comprising an EDB binding domain

- has a target binding affinity of ≥50 % to the extra-domain B (ED-B) of fibronectin, compared to one of the peptides or proteins comprising an anti-EDB binding domain as described above and/or
- competes for binding to the extra-domain B (ED-B) of fibronectin with one of peptides or proteins comprising an EDB binding domain as described above.

In some embodiments, the peptide or protein comprising an EDB binding domain comprises two L19 VH domains and two L19 VL domains.

In some embodiments, the two L19 VH domains have the same amino acid sequence;

- the two L19 VH domains have a different amino acid sequence;
- the two L19 VL domains have the same amino acid sequence; or
- the two L19 VL domains have a different amino acid sequence.

In some embodiments, the peptide or protein comprising an EDB binding domain comprises one L19 VH domain and one L19 VL domain.

In some embodiments, the composition comprises:

- a p40 domain linked to a p35 domain by a first linker (also called “linker 1”);
- a first L19 VH domain linked to the p35 domain by a SAD linker;
- a first L19 VL domain linked to the first L19 VH domain by a third linker (also called “linker 3”);
- a second L19 VH domain linked to the first L19 VL domain by a fourth linker (also called “linker 4”);
- a second L19 VL domain linked to the second L19 VH domain by a fifth linker (also called “linker 5”).

In some embodiments, the third linker and fifth linker comprise the same amino acid sequence, and/or can be replaced against one another.

In some embodiments, the p40 domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 1 or a fragment thereof.

In some embodiments, the p35 domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 3 or a fragment thereof.

In some embodiments, the first linker ("linker 1") is a GS linker.

In some embodiments, the first linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 2.

In some embodiments, the first L19 VH domain, the second L19 VH domain, or both comprise an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to at least one amino acid sequence set forth in SEQ ID NOs: 28-30.

In some embodiments, the first L19 VL domain, the second L19 VL domain, or both comprise an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to at least one amino acid sequence set forth in SEQ ID NOs: 31-33.

In some embodiments, the first L19 VH domain, the second L19 VH domain, or both comprise an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 7.

In some embodiments, the first L19 VL domain, the second L19 VL domain, or both comprise an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 5.

In some embodiments, the SAD linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 4.

In some embodiments, the third linker ("linker 3") is a GS linker.

In some embodiments, the third linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 6.

In some embodiments, the fifth linker ("linker 5") is a GS linker.

In some embodiments, the fifth linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 6.

In some embodiments, the third linker ("linker 3") and fifth linker ("linker 5") comprise the same amino acid sequence, and/or can be replaced against one another.

In some embodiments, the fourth linker ("linker 4") is a GS linker.

In some embodiments, the fourth linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 8.

In some embodiments, the composition comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 16.

In some embodiments, the composition consists of an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 16.

According to another aspect of the invention, the use of the composition according to any one of the aforementioned claims (for the manufacture of a medicament) is provided in the treatment of a human or animal subject

- being diagnosed for,
- suffering from or
- being at risk of

developing a neoplastic disease, or for the prevention of such condition.

In some embodiments, the neoplastic disease is selected from the group consisting of malignant melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma, urothelial carcinoma, head and neck squamous cell carcinoma (HNSCC), microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer, hepatocellular cancer, gastric cancer, squamous cell carcinoma of the skin, cervical cancer, and diffuse large B-cell lymphoma (DLBCL).

According to another aspect of the invention, the use of the composition according to the above disclosure (for the manufacture of a medicament) for the inhibition of angiogenesis in a human or animal subject is provided.

According to another aspect of the invention, a pharmaceutical composition comprising at least the composition according to the above description, and optionally one or more pharmaceutically acceptable excipients, is provided.

According to another aspect of the invention, a combination comprising (i) the composition according to the above description or the pharmaceutical composition according to the above description and (ii) one or more therapeutically active compounds is provided.

According to another aspect of the invention, a method for treating or preventing a disorder or condition associated with expression or overexpression of ED-B fibronectin, comprising administering to a subject in need thereof an effective amount of the composition according to

the above description, the pharmaceutical composition according to the above description, or the combination according to the above description is provided.

According to another aspect of the invention, a therapeutic kit of parts is provided, comprising:

a) the composition according to the above description, the pharmaceutical composition according to the above description, or the combination according to the above description,
b) an apparatus for administering the composition, composition or combination, and
c) instructions for use.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1B: Results of protein expression experiments. See below for materials and methods.

Fig. 1A: A 15-mer linker having the amino acid sequence of SEQ ID NO:4 (nicknamed "SAD" herein) shows by far the best production yields of all variants (also called "clones" herein). The yield is almost 100% better than the 2nd best variant, DDS.

The two sequences in the line AKKAS are SEQ ID NOs: 9 and 18, the two sequences in the line AP7 are SEQ ID NOs:15 and 19, the two sequences in the line DDS are SEQ ID NOs: 10 and 20, the two sequences in the line AP6 are SEQ ID NOs: 14 and 21, the two sequences in the line G4S are SEQ ID NOs: 11 and 22, the two sequences in the line SES are SEQ ID NOs: 12 and 23, the two sequences in the line alpha3 are SEQ ID NOs: 13 and 24, and the two sequences in the line SAD are SEQ ID NOs: 4 and 25.

There are N-terminal and C-terminal residues (or 5'- or 3'-nucleotides) in Fig. 1 which are shown in grey. These do not belong to the disclosure of the present application for which a search is necessary. They simply show the framework in which the respective linkers can be embedded.

Fig. 1B: SDS-PAGE characterization displayed a molecular weight around 120 kDa for all variants.

Fig. 2: ELISA experiments. All variants bind to the domain 7B89 of human fibronectin, both at 10 µg/ml and 1 µg/ml concentration.

Fig. 3: Biacore experiments. All variants show similar binding behavior to the domain 7B89 of human fibronectin.

Fig. 4: Size exclusion chromatography (SEC). All variants showed a comparable aggregation profile with the major peak at 13 ml corresponding to the monomeric immunocytokine, and the smaller peak at 10 ml corresponding to aggregates.

Fig. 5: Immunofluorescence staining experiments. All variants specifically stained the vasculature of frozen syngeneic F9 teratocarcinoma specimens as compared to the negative control.

Fig. 6: *In vivo* tumor targeting. All variants and a positive control were radioiodinated with ^{125}I and injected (4-9 µg protein / animal) into immunocompetent mice bearing s.c. implanted F9 murine teratocarcinoma. The radioactivity counted 24 hours after the injection showed an accumulation in the tumor for all variants. However, the "SAD" variant showed a superior accumulation in the tumor as compared to the other seven clones (~2,9 % ID/g (= injected dose per gram of tissue) vs. the second best, which is $(G4S)_3$, and shows ~2,4 % ID/g).

Figs. 7 and 8: Exemplary immunocytokine formats using IL-12 and an anti-fibronectin antibody.

Fig. 9: SEC analysis of the different fusion proteins (A) huIL-12L19L19 "SAD" Batch-A (B) huIL-12L19L19 "SAD" Batch-B (C) huIL-12L19L19 "Old" Batch A (D) huIL-12L19L19 "Old" Batch B.

Fig. 10: Biacore experiment. The "SAD" variant showed to have an improved apparent affinity as compared to variant with the "Old" linker toward the fibronectin 7B89 domain (3.8 nM *vs* 6.7 nM). This surprising result is unexpected, since the variation of the linker may affect the stability of the protein but usually not the affinity to its target.

Fig. 11: *In vivo* tumor targeting experiment. The "SAD" and the "Old" variants were radioiodinated with ^{125}I and injected (10-11 μg protein / animal) into immunocompetent mice bearing s.c. implanted F9 murine teratocarcinoma. The "SAD" variant showed to have an improved tumor targeting ability as compared to the "Old" linker variant.

DETAILED DESCRIPTION

Definitions

An "antibody" refers to a molecule of the immunoglobulin family comprising a tetrameric structural unit. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" chain (about 25 kD) and one "heavy" chain (about 50-70 kD), connected through a disulfide bond. Recognized immunoglobulin genes include the κ, λ, α, γ, δ, ε, and μ constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either κ or λ. Heavy chains are classified as γ, μ, α, δ, or ε, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD, and IgE, respectively. Antibodies can be of any isotype/class (e.g., IgG, IgM, IgA, IgD, and IgE), or any subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, IgA2).

Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used structurally and functionally. The N-terminus of each chain defines a variable (V) region or domain of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these regions of light and heavy chains respectively. The pairing of a V_H and V_L together forms a single antigen-binding site. In addition to V regions, both heavy chains and light chains contain a constant (C) region or domain. A secreted form of an immunoglobulin C region is made up of three C domains, CH1, CH2, CH3, optionally CH4 (Cμ), and a hinge region. A membrane-bound form of an immunoglobulin C region also has membrane and intracellular domains. Each light chain has a V_L at the N-terminus followed by a constant domain (C) at its other end. The constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention, the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The N-terminus is a variable region

and at the C-terminus is a constant region; the CH3 and CL domains actually comprise the carboxy-terminal domains of the heavy and light chain, respectively. The VL is aligned with the VH and the CL is aligned with the first constant domain of the heavy chain. As used herein, an "antibody" encompasses conventional antibody structures and variations of antibodies. Thus, within the scope of this concept are full length antibodies, chimeric antibodies, humanized antibodies, human antibodies, and antibody fragments thereof.

Antibodies exist as intact immunoglobulin chains or as a number of well-characterized antibody fragments produced by digestion with various peptidases. The term "antibody fragment," as used herein, refers to one or more portions of an antibody that retain the ability to specifically interact with (*e.g.*, by binding, steric hindrance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'$_2$, a dimer of Fab' which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The F(ab)'$_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'$_2$ dimer into a Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region. Paul, *Fundamental Immunology* 3d ed. (1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. As used herein, an "antibody fragment" refers to one or more portions of an antibody, either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies, that retain binding specificity and functional activity. Examples of antibody fragments include Fv fragments, single chain antibodies (ScFv), Fab, Fab', Fd (Vh and CH1 domains), dAb (Vh and an isolated CDR); diabodies and single chain diabodies; and multimeric versions of these fragments (*e.g.*, F(ab')$_{2,}$) with the same binding specificity. Antibody fragments can also be incorporated into cytokine engrafted proteins to achieve the binding specificity and activity provided in the present disclosure.

A "Fab" domain as used herein comprises a heavy chain variable domain, a constant region CH1 domain, a light chain variable domain, and a light chain constant region CL domain. The interaction of the domains is stabilized by a disulfide bond between the CH1 and CL domains. In some embodiments, the heavy chain domains of the Fab are in the order, from N-terminus to C-terminus, VH-CH and the light chain domains of a Fab are in the order, from N-terminus to C-terminus, VL-CL. In some embodiments, the heavy chain domains of the Fab are in the

order, from N-terminus to C-terminus, CH-VH and the light chain domains of the Fab are in the order CL-VL. Although Fabs were historically identified by papain digestion of an intact immunoglobulin, in the context of this disclosure, a "Fab" is typically produced recombinantly by any method. Each Fab fragment is monovalent with respect to antigen binding, i.e., it has a single antigen- binding site.

"Complementarity-determining domains" or "complementarity-determining regions" ("CDRs") interchangeably refer to the hypervariable regions of V_L and V_H. CDRs are the target protein-binding site of antibody chains that harbors specificity for such target protein. There are three CDRs (CDR1-3, numbered sequentially from the N-terminus) in each human V_L or V_H, constituting about 15-20% of the variable domains. CDRs are structurally complementary to the epitope of the target protein and are thus directly responsible for the binding specificity. The remaining stretches of the V_L or V_H, the so-called framework regions (FR), exhibit less variation in amino acid sequence (Kuby, Immunology, 4th ed., Chapter 4. W.H. Freeman & Co., New York, 2000).

Positions of CDRs and framework regions can be determined using various well known definitions in the art, *e.g.*, Kabat, Chothia, international ImMunoGeneTics database (IMGT) and AbM (see, *e.g.*, Johnson *et al.,* Nucleic Acids Res., 29:205-206 (2001); Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987); Chothia *et al.,* Nature, 342:877-883 (1989); Chothia *et al.,* J. Mol. Biol., 227:799-817 (1992); Al-Lazikani *et al.,* J.Mol.Biol., 273:927-748 (1997)). Definitions of antigen combining sites are also described in the following: Ruiz *et al.,* Nucleic Acids Res., 28:219–221 (2000); and Lefranc, M.P., Nucleic Acids Res., 29:207-209 (2001); MacCallum *et al.,* J. Mol. Biol., 262:732-745 (1996); and Martin *et al.,* Proc. Natl. Acad. Sci. USA, 86:9268–9272 (1989); Martin *et al.,* Methods Enzymol., 203:121–153 (1991); and Rees *et al.,* In Sternberg M.J.E. (ed.), Protein Structure Prediction, Oxford University Press, Oxford, 141–172 (1996).

Under Kabat, CDR amino acid residues in the V_H are numbered 31-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the V_L are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). Under Chothia, CDR amino acids in the V_H are numbered 26-32 (HCDR1), 52-56 (HCDR2), and 95-102 (HCDR3); and the amino acid residues in V_L are numbered 26-32 (LCDR1), 50-52 (LCDR2), and 91-96 (LCDR3). By combining the CDR definitions of both Kabat and Chothia, the CDRs consist of amino acid

residues 26-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3) in human VH and amino acid residues 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3) in human VL.

An "antibody variable light chain" or an "antibody variable heavy chain" as used herein refers to a polypeptide comprising the V_L or V_H, respectively. The endogenous V_L is encoded by the gene segments V (variable) and J (junctional), and the endogenous V_H by V, D (diversity), and J. Each of V_L or V_H includes the CDRs as well as the framework regions (FR). The term "variable region" or "V-region" interchangeably refer to a heavy or light chain comprising FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4. A V-region can be naturally occurring, recombinant or synthetic. In this application, antibody light chains and/or antibody heavy chains may, from time to time, be collectively referred to as "antibody chains." As provided and further described herein, an "antibody variable light chain" or an "antibody variable heavy chain" and/or a "variable region" and/or an "antibody chain" optionally comprises a cytokine polypeptide sequence engrafted into a CDR.

The C-terminal portion of an immunoglobulin heavy chain as disclosed herein, comprising, e.g., CH2 and CH3 domains, is the "Fc" domain. An "Fc region" as used herein refers to the constant region of an antibody excluding the first constant region (CH1) immunoglobulin domain. Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc may include the J chain. For IgG, Fc comprises immunoglobulin domains Cγ2 and Cγ3 and the hinge between Cγ1 and Cγ. It is understood in the art that boundaries of the Fc region may vary, however, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, using the numbering is according to the EU index as in Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, Va.). "Fc region" may refer to this region in isolation or this region in the context of an antibody or antibody fragment. "Fc region" includes naturally occurring allelic variants of the Fc region, *e.g.,* in the CH2 and CH3 region, including, e.g., modifications that modulate effector function. Fc regions also include variants that don't result in alterations to biological function. For example, one or more amino acids are deleted from the N-terminus or C-terminus of the Fc region of an immunoglobulin without substantial loss of biological function. For example, in certain embodiments a C-terminal lysine is modified replaced or removed. In particular embodiments one or more C-terminal residues in the Fc region is altered or removed. In certain embodiments

one or more C-terminal residues in the Fc (e.g., a terminal lysine) is deleted. In certain other embodiments one or more C-terminal residues in the Fc is substituted with an alternate amino acid (e.g., a terminal lysine is replaced). Such variants are selected according to general rules known in the art so as to have minimal effect on activity (see, e.g., Bowie, *et al.,* Science 247:306-1310, 1990). The Fc domain is the portion of the immunoglobulin (Ig) recognized by cell receptors, such as the FcR, and to which the complement-activating protein, C1 q, binds. The lower hinge region, which is encoded in the 5' portion of the CH2 exon, provides flexibility within the antibody for binding to FcR receptors.

A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, and drug; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

A "humanized" antibody is an antibody that retains the reactivity (e.g., binding specificity, activity) of a non-human antibody while being less immunogenic in humans. This can be achieved, for instance, by retaining non-human CDR regions and replacing remaining parts of an antibody with human counterparts. *See*, *e.g.*, Morrison *et al.,* Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984); Morrison and Oi, Adv. Immunol., 44:65-92 (1988); Verhoeyen *et al.,* Science, 239:1534-1536 (1988); Padlan, Molec. Immun., 28:489-498 (1991); Padlan, Molec. Immun., 31(3):169-217 (1994).

A "human antibody" includes antibodies having variable regions in which both the framework and CDR regions are derived from sequences of human origin. Furthermore, if an antibody contains a constant region, the constant region also is derived from such human sequences, *e.g.*, human germline sequences, or mutated versions of human germline sequences or antibody containing consensus framework sequences derived from human framework sequences analysis, for example, as described in Knappik *et al.,* J. Mol. Biol. 296:57-86, 2000). Human antibodies may include amino acid residues not encoded by human sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo, or a conservative substitution to promote stability or manufacturing).

The term "isolated," when applied to a nucleic acid or protein, denotes that the nucleic acid or protein is essentially free of other cellular components with which it is associated in the natural state. It is preferably in a homogeneous state. It can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein that is the predominant species present in a preparation is substantially purified. In particular, an isolated gene is separated from open reading frames that flank the gene and encode a protein other than the gene of interest. The term "purified" denotes that a nucleic acid or protein gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or protein is at least 85% pure, more preferably at least 95% pure, and most preferably at least 99% pure.

The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.,* Nucleic Acid Res. 19:5081 (1991); Ohtsuka *et al.,* J. Biol. Chem. 260:2605-2608 (1985); and Rossolini *et al.,* Mol. Cell. Probes 8:91-98 (1994)).

The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. As used herein, the term "peptide or protein comprising an EDB binding domain" relates to a peptide or protein which, as a whole or by virtue of a portion/fragment thereof, binds to EDB, i.e., Extra domain-B containing fibronectin.

Generally, a peptide can for example be a monomeric molecule having a length of ≥ 3 amino acid residues and ≤ 50 amino acid residues (hence, an oligo- or polypeptide), while a protein can for example be a monomeric or bi- or polymeric molecule with one or more protein each chain having a length of ≥ 50 amino acid residues.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG, and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alter, add or delete a single amino acid or a small percentage of amino acids in the encoded sequence are a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles. The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see*, *e.g.*, Creighton, *Proteins* (1984)).

In this context, a "conservative amino acid substitution", as used herein, has a smaller effect on antibody function than a non-conservative substitution. Although there are many ways to classify amino acids, they are often sorted into six main groups on the basis of their structure and the general chemical characteristics of their R groups.

In some embodiments, a "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. For example, families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with

- basic side chains (e.g., lysine, arginine, histidine),
- acidic side chains (e.g., aspartic acid, glutamic acid),
- uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine),
- nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan),
- beta-branched side chains (e.g., threonine, valine, isoleucine) and
- aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Other conserved amino acid substitutions can also occur across amino acid side chain families, such as when substituting an asparagine for aspartic acid in order to modify the charge of a peptide. Conservative changes can further include substitution of chemically homologous non-natural amino acids (i.e. a synthetic non-natural hydrophobic amino acid in place of leucine, a synthetic non-natural aromatic amino acid in place of tryptophan).

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence (*e.g.*, a polypeptide), which does not comprise additions or deletions, for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same sequences. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, at least 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity over a specified region, or, when not specified, over the entire sequence of a reference sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. The disclosure provides polypeptides or polynucleotides that are substantially identical to the polypeptides or polynucleotides, respectively, exemplified herein. Optionally, the identity exists over a region that is at least about 15, 25 or 50 nucleotides in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides in length, or over the full length of the reference sequence. With respect to amino acid sequences, identity or substantial identity can exist over a region that is at least 5, 10, 15 or 20 amino acids in length, optionally at least about 25, 30, 35, 40, 50, 75 or 100 amino acids in length, optionally at least about 150, 200 or 250 amino acids in length, or over the full length of the reference sequence. With

respect to shorter amino acid sequences, *e.g.*, amino acid sequences of 20 or fewer amino acids, substantial identity exists when one or two amino acid residues are conservatively substituted, according to the conservative substitutions defined herein.

The terms "subject," "patient," and "individual" interchangeably refer to a mammal, for example, a human or a non-human primate mammal. The mammal can also be a laboratory mammal, *e.g.*, mouse, rat, rabbit, hamster. In some embodiments, the mammal can be an agricultural mammal (e.g., equine, ovine, bovine, porcine, camelid) or domestic mammal (e.g., canine, feline).

As used herein, the terms "treat," "treating," or "treatment" of any disease or disorder refer in some embodiments, to ameliorating the disease or disorder (*i.e.*, slowing or arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment, "treat," "treating," or "treatment" refers to alleviating or ameliorating at least one physical parameter including those which may not be discernible by the patient. In yet another embodiment, "treat," "treating," or "treatment" refers to modulating the disease or disorder, either physically, (*e.g.*, stabilization of a discernible symptom), physiologically, (*e.g.*, stabilization of a physical parameter), or both. In yet another embodiment, "treat," "treating," or "treatment" or "prophylaxis" refers to preventing or delaying the onset or development or progression of a disease or disorder.

The term "co-administer" refers to the simultaneous presence of two (or more) active agents in an individual. Active agents that are co-administered can be concurrently or sequentially delivered.

Various aspects of the invention are described in detail in the following sections. The use of sections is not meant to limit the invention. Each section can apply to any aspect of the invention. The invention is not limited to the particular component parts of the compositions described or process steps of the methods described as such compositions and methods may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting.

As used in the instant specification and the appended claims, the singular forms "a", "an", and "the" include singular and/or plural referents unless the context clearly dictates otherwise. In

this application, the use of "or" means "and/or" unless stated otherwise. It is moreover to be understood that, in case parameter ranges are given which are delimited by numeric values, the ranges are deemed to include these limitation values. The disclosures of all of the art cited herein are incorporated by reference in their entireties.

The term "GS Linker", as used herein, relates to a peptide linker that consist predominantly, or exclusively, of Glycin and Serin residues (also called "Gly-Ser linker"). In different embodiments, the GS linker is the linker sown herein in any of SEQ ID NOs: 2, 6 or 8.

A "conservative amino acid substitution", as used herein, has a smaller effect on antibody function than a non-conservative substitution. Although there are many ways to classify amino acids, they are often sorted into six main groups on the basis of their structure and the general chemical characteristics of their R groups.

As used herein, the term "target binding affinity" refers to the affinity of a binding molecule according to the invention, to its target, and is expressed numerically using "KD" values. In general, a higher KD value corresponds to a weaker binding. In some embodiments, the "KD" is measured by a radiolabeled antigen binding assay (MA) or surface plasmon resonance (SPR) assays, using, e.g., a BIAcore™-2000 or a BIAcore™-3000. In certain embodiments, an "on-rate" or "rate of association" or "association rate" or "kon" and an "off-rate" or "rate of dissociation" or "dissociation rate" or "koff" are also determined with the surface plasmon resonance (SPR) technique. In additional embodiments, the "KD", "kon", and "koff" are measured using the Octet® Systems.

As used herein, the term "competes for binding" is used in reference to one of the antibodies defined by the sequences as above, meaning that the actual antibody as an activity which binds to the same target, or target epitope or domain or subdomain, as does said sequence defined antibody, and is a variant of the latter. The efficiency (e.g., kinetics or thermodynamics) of binding may be the same as or greater than or less than the efficiency of the latter. For example, the equilibrium binding constant for binding to the substrate may be different for the two antibodies.

As used herein, the term "maintaining the capability to bind to a given target" means, for example, that the respective variant has a target binding affinity of ≥50 % compared to that of the non-modified peptide.

EDB Fibronectin

Fibronectin (UniProt: P02751) is a high-molecular weight (~440kDa) glycoprotein of the extracellular matrix that binds to membrane-spanning receptor proteins called integrins. Similar to integrins, fibronectin binds extracellular matrix components such as collagen, fibrin, and heparan sulfate proteoglycans (e.g. syndecans).

Fibronectin has been implicated in carcinoma development. In lung carcinoma, fibronectin expression is increased, especially in non-small cell lung carcinoma. The adhesion of lung carcinoma cells to fibronectin enhances tumorigenicity and confers resistance to apoptosis-inducing chemotherapeutic agents. Fibronectin may promote lung tumor growth/survival and resistance to therapy, and has been discussed to represent a novel target for the development of new anticancer drugs.

Fibronectin exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds. The fibronectin protein is produced from a single gene, but alternative splicing of its precursor mRNA, produced from a single copy fibronectin gene, occurs at three sites coding for the EDA, EDB and IIICS domains and results in the creation of several isoforms.

Fibronectin isoforms comprising the EDA or EDB domains are known as oncofetal forms due to their importance in embryonic development and their restricted presence in normal adult tissues. These isoforms are also recognized as important markers of angiogenesis, a crucial physiological process in development and required by tumor cells in cancer progression. ED-B fibronectin is expressed in tumor tissues, particularly in breast carcinomas, brain tumors, lymphoma cells, and prostate cancers. Due to its tissue specific expression profile, ED-B fibronectin is an attractive tumor antigen to utilize for treatment targeting.

IL-12

Interleukin-12 is a heterodimeric cytokine with multiple biological effects on the immune system. It is made up of two subunits, p35 and p40, both of which are required for the secretion of the active form of IL-12, p70. Interleukin-12 acts on dendritic cells (DC), leading to increased maturation and antigen presentation, which can allow for the initiation of a T cell response to tumor specific antigens. It also drives the secretion of IL-12 by DCs, creating a positive feedback mechanism to amplify the response. Once a response is initiated, IL-12 plays a fundamental role in directing the immune system towards a Th1 cytokine profile, inducing CD4+ T cells to secrete interferon-gamma (IFN-γ) and leading to a CD8+ cytotoxic T cell response.

IL-12 is also a strong pro-inflammatory cytokine that leads to the secretion of other cytokines including tumor necrosis factor-alpha (TNF-α) which, combined with IFN-γ, is a prerequisite for the development of CD4+ cytotoxic T lymphocytes (CTL). Furthermore, IL-12 can promote the activation of innate immune cells such as macrophages and eosinophils through its induction of IFN-γ and other cytokines. This activation then leads to IL-12 secretion by these cells and further amplification of both the innate and acquired responses. However, high levels of IL-12, and consequently IFN-γ, have also been associated with induction of antagonistic molecules such as IL-10 and the depletion of signaling molecules downstream of IL-12, such as STAT4.

Previous attempts at utilizing IL-12 as a therapeutic agent were unsuccessful as IL-12 showed at best modest anti-tumor effects which were often accompanied by unacceptably toxic side effects, including fever, fatigue, hematological changes, hyperglycemia, and/or organ dysfunction.

"p35" as used herein means a polypeptide that comprises an amino acid sequence having at least eighty percent (80%) identity to the amino acid sequence indicated below:
RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEA
CLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLL
MDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVT
IDRVMSYLNAS (SEQ ID NO: 3).

"p40" as used herein means a polypeptide that comprises an amino acid sequence having at least eighty percent (80%) identity to the amino acid sequence indicated below:
IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGD
AGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLT
TISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSVECQEDSACPAAEESLPI
EVMVDAVHKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFS
LTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCS (SEQ ID NO:1).

"IL-12" as used herein means a polypeptide that (i) comprises both:

(a) p35, or a fragment thereof, wherein p35 comprises an amino acid sequence having at least eighty percent (80%) identity to the amino acid sequence indicated below:
RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEA
CLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLL
MDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVT
IDRVMSYLNAS (SEQ ID NO:3)
and

(b) p40, or a fragment thereof, wherein p40 comprises an amino acid sequence having at least eighty percent (80%) identity to the amino acid sequence indicated below:
IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGD
AGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLT
TISTDLTFSVKSSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSVECQEDSACPAAEESLPI
EVMVDAVHKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFS
LTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCS (SEQ ID NO:1)

and (ii) can activate an IL-12 receptor.

Linkers

In certain embodiments one or more peptide linkers are independently selected from a $(Gly_n\text{-}Ser)_m$ sequence, a $(Gly_n\text{-}Ala)_m$ sequence, or any combination of a $(Gly_n\text{-}Ser)_m/(Gly_n\text{-}Ala)_m$ sequence, wherein each n is independently an integer from 1 to 5 and each m is independently

an integer from 0 to 10. In some embodiments a peptide linker is $(Gly_4\text{-}Ser)_m$ wherein m is an integer from 0 to 10. In some embodiments a peptide linker is $(Gly_4\text{-}Ala)_m$ wherein m is an integer from 0 to 10. Examples of linkers include, but are not limited to, certain embodiments one or more linkers include G_4S repeats, e.g., the Gly-Ser linker GGGGS (SEQ ID NO:34), or $(GGGGS)_m$ wherein m is a positive integer equal to or greater than 1. For example, m=1, m=2, m=3. m=4, m=5 and m=6, m=7, m=8, m=9 and m=10. In some embodiments, the linker includes multiple repeats of GGGGS (SEQ ID NO:34), including, but is not limited to $(GGGGS)_3$ or $(GGGGS)_4$. In some embodiments, Ser can be replaced with Ala e.g., linkers G/A such as (GGGGA) (SEQ ID NO:35), or $(GGGGA)_m$ wherein m is a positive integer equal to or greater than 1. In some embodiments, the linker includes multiple repeats of GGGGA (SEQ ID NO:35). In other embodiments, a linker includes combinations and multiples of GGGGS (SEQ ID NO: 34), and GGGGA (SEQ ID NO: 35).

In some embodiments a linker comprises the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO:2). In some embodiments a linker comprises the amino acid sequence GSADGGSSAGGSDAG (SEQ ID NO:4). In some embodiments a linker comprises the amino acid sequence GSSGG (SEQ ID NO:6). In some embodiments a linker comprises the amino acid sequence SSSSGSSSSGSSSSG (SEQ ID NO:8). In some embodiments a linker comprises the amino acid sequence GGGAKGGGGKAGGGS (SEQ ID NO:9). In some embodiments a linker comprises the amino acid sequence GGGGDGGGGDGGGGS (SEQ ID NO:10). In some embodiments a linker comprises the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO:11). In some embodiments a linker comprises the amino acid sequence GGGGSGGGGEGGGGS (SEQ ID NO:12). In some embodiments a linker comprises the amino acid sequence AEAAAKEAAAKEAAAKA (SEQ ID NO:13). In some embodiments a linker comprises the amino acid sequence APAPAPAPAPAP (SEQ ID NO:14). In some embodiments a linker comprises the amino acid sequence APAPAPAPAPAPAP (SEQ ID NO:15).

Anti-EDB linked IL-12

The present invention provides, among other things, methods and compositions for treating diseases or disorders associated with the expression of EDB-fibronectin, including cancers. Described herein are new compositions and methods which utilize fibronectin as a target to accomplish cancer-directed delivery of IL-12. This approach promises to fully exploit the

therapeutic potential of IL-12, while reducing systemic toxicity and increasing the therapeutic window of IL-12.

Although other constructs containing IL-12 and an EDB fibronectin targeting domain have been previously described, the presently disclosed compositions are surprisingly superior.

WO2006/119897, the content of which is incorporated herein by reference, discloses three different molecular formats of IL-12 combined with an EDB fibronectin targeting antibody named "L19".

One format was sc(IL-12)-scFv(L19), as illustrated in Fig. 7A. This format, consisting of an IL-12 heterodimer in which the two subunits are joined via a peptide linker (hence, "single chain" IL-12, or sc(IL-12)), and said IL-12 is then joined, via a second peptide linker, to the L19 antibody which is also in the single chain Fv format (hence, scFv(L19)). This format showed modest tumor-targeting ability, consistent with the prior art findings.

Another format was a homodimer of sc(IL-12)-SIP(L19), as illustrated in Fig. 7B. The SIP format ("small immune protein") has been developed by the applicants in WO2003/076469 and is also nicknamed "miniantibody". SIP is a homodimer consisting of two subunits comprising a scFv joined to the CH4 domain. The two CH4 domains are joined to one another by a disulfide bridge. Despite the prior art indication that tumor-targeting properties of L19 could be improved using the SIP format, increased tumor uptake of this conjugate was not observed.

Another format was a heterodimer of IL-12 p40 and p35 subunits joined to one another by a disulfide bridge, and each subunit fused to scFv(L19), forming a scFv(L19)-p35/p40-scFv(L19) heterodimer, as illustrated in Fig. 7C. With this heterodimeric format a marked improvement in tumor uptake of the composition was achieved.

In WO2013/014149, content of which is incorporated herein by reference, the applicant has disclosed two new alternative molecular formats of IL-12 joined to the anti-EDA fibronectin tumor targeting antibody named "F8".

Therein, another format of IL-12 immunoconjugates, comprises a "single chain diabody". It essentially consists of two scFv antibodies with a short - five amino acid - linker (therefore forming a "diabody") joined to one another by a longer – fifteen amino acid - peptide linker.

It was shown that a molecular format featuring IL-12 fused to a monospecific F8 single chain diabody (see Fig. 8B) proved to be superior in terms of tumor targeting to either (i) a scFv(F8)-p35/p40-scFv(F8) heterodimer (Fig. 8A) which, in its L19 variant, proved to be the best format disclosed in WO2006/119897 or (ii) two IL-12 molecules joined to a diabody (Fig. 8C).

L19

"L19 antibody" as used herein means any antibody that binds to EDB Fibronectin or any portion thereof and comprises an amino acid sequence having at least seventy-five percent (75%) identity to one or more of the following amino acid sequences:

L19 VH (SEQ ID NO: 7)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYAD
SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTLVTVSS

L19 VL (SEQ ID NO: 5)
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDR
FSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIK

CDR1 VH (SEQ ID NO: 28)
SFSMS

CDR2 VH (SEQ ID NO: 29)
SISGSSGTTYYADSVKG

CDR3 VH (SEQ ID NO: 30)
PFPYFDY

CDR1 VL (SEQ ID NO: 31)
RASQSVSSSFLA

CDR2 VL (SEQ ID NO: 32)
YASSRAT

CDR3 VL (SEQ ID NO: 33)
QQTGRIPPT

L19 Diabody (SEQ ID NO: 36)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYAD
SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTLVTVSSGSSGGEIV
LTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSG
SGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIK

Pharmaceutical compositions

A further aspect of the present invention relates to a pharmaceutical composition comprising at least one conjugate of the invention and optionally a pharmaceutically acceptable excipient.

Pharmaceutical compositions of the present invention typically comprise a therapeutically effective amount of a conjugate according to the invention and optionally auxiliary substances such as pharmaceutically acceptable excipient(s). Said pharmaceutical compositions are prepared in a manner well known in the pharmaceutical art. A carrier or excipient may be a liquid material which can serve as a vehicle or medium for the active ingredient. Suitable carriers or excipients are well known in the art and include, for example, stabilisers, antioxidants, pH-regulating substances, controlled-release excipients.

The pharmaceutical preparation of the invention may be adapted, for example, for parenteral use and may be administered to the patient in the form of solutions or the like. Compositions comprising the composition of the present invention may be administered to a patient. Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to the patient. Such benefit may be at least amelioration of at least one symptom. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc., is within the responsibility of general practitioners and other medical doctors.

Treatments may be repeated at daily, twice-weekly, weekly, or monthly intervals at the discretion of the physician.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may comprise a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally comprise a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

It is further to be understood that embodiments disclosed herein are not meant to be understood as individual embodiments which would not relate to one another. Features discussed with a particular embodiment are meant to be disclosed also in connection with other embodiments shown herein. If, in one case, a specific feature is not disclosed with one embodiment, but with another, the skilled person would understand that does not necessarily mean that said feature is not meant to be disclosed in combination with said other embodiment. The skilled person would understand that it is the gist of this application to disclose said feature also for the other embodiment, but that just for purposes of clarity and to keep the specification in a manageable volume this has not been done.

Furthermore, the contents of the references referred to herein are incorporated by reference. This refers, particularly, for prior art documents that disclose standard or routine methods. In that case, the incorporation by reference has mainly the purpose to provide sufficient enabling disclosure, and avoid lengthy repetitions.

Generally, the composition of the invention is capable binding to specific target structures in a cell, tissue, organ or patient, which target structures are defined by the specificity of the peptide or protein comprising the EDB binding domain.

Once at the target, IL-12 stimulates the production of IFNγ from T-cells and natural killer cells, and also induces differentiation of Th1 helper cells. IL-12 is a key mediator of innate and cell-mediated immunity. If the peptide or protein comprising the EDB binding domain in the construct is specific for a target structure, e.g., a receptor or an extracellular matrix protein, which characterizes a neoplasticity, e.g., a tumor, a hematologic disease, or cells being in the process of transforming into cancer, the binding of the composition evokes an IL-12 mediated potent anti-cancer and anti-metastatic activity.

The applicants have surprisingly discovered that when a linker comprising an amino acid motif comprising GSADGGSSAGGSDAG is used to link IL-12 to peptide or protein comprising an EDB binding domain (i.e., a diabody as disclosed in WO2013/014149), a better tumor targeting performance as well as a superior production yield can be achieved. At the same time, the binding behaviour of this variant is superior to the binding behaviour of the shorter GSADGG linker, nicknamed herein "Old", and disclosed in WO2013/014149.

The applicants have first evaluated and characterized eight clones of human IL-12 joined to the L19 antibody in single chain diabody format (huIL-12L19L19) with different polypeptide linkers between the cytokine and the L19 single chain diabody.

Five clones nicknamed: (i) "AKKAS" (ii) "DDS" (iii) "$(G4S)_3$" (iv) "SAD" and (v) "SES" contain linkers for conjugation of immunocytokines to recombinant antibodies and have been chosen due to their different electric charge characteristic (neutral, positively charged, negatively charged).

Three additional clones nicknamed (vi) Alpha3 (vii) AP6 and (viii) AP7 were developed. With regards to these three clones, the principles reported in Chen et al (2013) were considered and put into practice. This review suggests that rigid linkers might have a better stability and might maintain the correct distance between the cytokine and the antibody, thus increasing the therapeutic efficacy.

None of the linkers (i) – (viii) were previously tested in this specific immunocytokine.

It was surprisingly found that the "SAD" linker greatly enhances (i) the tumor targeting performance and (ii) the production yield of IL-12 joined to a peptide or protein comprising the EDB binding domain, without (iii) compromising the binding behaviour to ED-B as compared to the other clones. This is quite surprising as despite the consideration of the principles reported by Chen, only the SAD linker resulted in a composition having a number of superior properties when compared to both the "Old" clone (described in more detail below), and the other new variants described herein.

Finally, a ninth clone nicknamed "Old" comprising a linker disclosed in WO2013/014149 was compared to the "SAD" linker. It was surprisingly found that the "SAD" linker despite sharing the first part of the sequence with the "Old" linker has a superior binding affinity to ED-B.

Furthermore, after size-exclusion chromatography, the "SAD" linker shows a higher monomeric portion as compared to the "Old" linker, meaning that assembly of the entire conjugate is more efficient. The higher monomeric portion given by the "SAD" linker would be expected to increase the overall manufacturing yield.

As used herein, the term "single chain diabody" relates to a construct of two single chain Fv (scFv) antibodies with a short linker, preferably 3 – 10 amino acids long, more preferably 5 amino acid long (also known as "diabodies"), joined to one another by a longer linker, preferably 5 – 20 amino acids long, more preferably 15 amino acid long, according to the following scheme (N->C orientation): L19VH-linker3-L19VL-linker4-L19VH-linker3-L19VL.

According to some embodiments of the invention, the first subunit of the heterodimeric IL-12 protein is p40 and the second subunit is p35,

As discussed above, Fibronectin isoforms comprising the EDA or EDB domains are known as oncofetal forms due to their developmental importance and their re-expression in tumors, contrasting with restricted presence in normal adult tissues.

These isoforms are also recognized as important markers of angiogenesis, a crucial physiological process in development and required by tumor cells in cancer progression.

Hence, the extra-domain B (ED-B) of fibronectin is an attractive target for anti-cancer therapy, including the use of immunocytokines as discussed herein.

According to some embodiments of the invention, the single-chain diabody may comprise an antigen-binding site having the complementarity determining regions (CDRs), or the VH and/or VL domains of an antibody capable of specifically binding to an antigen of interest, for example, one or more CDRs or VH and/or VL domains of an antibody capable of specifically binding to an antigen of the extra-domain B of fibronectin.

An antigen binding site may be provided by means of arrangement of complementarity determining regions (CDRs). The structure for carrying a CDR or a set of CDRs will generally be an antibody heavy or light chain sequence or substantial portion thereof in which the CDR or set of CDRs is located at a location corresponding to the CDR or set of CDRs of naturally occurring VH and VL antibody variable domains encoded by rearranged immunoglobulin genes. The structures and locations of immunoglobulin variable domains may be determined by reference to Kabat *et al.* (1987) (Sequences of Proteins of Immunological Interest. 4th Edition. US Department of Health and Human Services.), and updates thereof, now available on the Internet (at immuno.bme.nwu.edu or find "Kabat" using any search engine).

By CDR region or CDR, it is intended to indicate the hypervariable regions of the heavy and light chains of the immunoglobulin as defined by Kabat *et al.* (1987) Sequences of Proteins of Immunological Interest, 4th Edition, US Department of Health and Human Services (Kabat *et al.*, (1991a), Sequences of Proteins of Immunological Interest, 5th Edition, US Department of Health and Human Services, Public Service, NIH, Washington, and later editions). An antibody typically contains 3 heavy chain CDRs and 3 light chain CDRs. The term CDR or CDRs is used here in order to indicate, according to the case, one of these regions or several, or even the whole, of these regions which contain the majority of the amino acid residues responsible for the binding by affinity of the antibody for the antigen or the epitope which it recognizes.

Thus the single-chain diabody may comprise an antigen-binding site having one, two, three, four, five or six CDR's, or the VH and/or VL domains of antibody L19.

According to some embodiments of the invention, the single-chain diabody may comprise an antigen binding site having the complementarity determining regions (CDRs) of antibody L19 set forth in SEQ ID NOs: 28-33. The antigen binding site may comprise VH and/or VL domains of antibody L19 set forth in SEQ ID NOs: 7 and 5, respectively.

An antigen-binding site may comprise one, two, three, four, five or six CDRs of antibody L19. Amino acid sequences of the CDRs of L19 are:

SEQ ID NO: 28 (VH CDR1);
SEQ ID NO: 29 (VH CDR2);
SEQ ID NO: 30 (VH CDR3);
SEQ ID NO: 31 (VL CDR1);
SEQ ID NO: 32 (VL CDR2), and/or
SEQ ID NO: 33 (VL CDR3).

SEQ ID NOs: 28-30 are the amino acid sequences of the VH CDR regions (1-3, respectively) of the human monoclonal antibody L19. SEQ ID NOs: 31-33 are the amino acid of the VL CDR regions (1-3, respectively) of the human monoclonal antibody L19. The amino acid sequence of the VH and VL domains of antibody L19 correspond to SEQ ID NOs: 7 and 5, respectively.

According to some embodiments of the invention, the single chain diabody comprises at least one of

a) a set comprising the 3 heavy chain CDRs defined herein as SEQ ID NOs: 28 – 30, and the 3 light chain CDRs defined herein as SEQ ID NOs: 31 – 33
b) a set of 3 heavy chain CDRs in the VH defined herein as SEQ ID NO: 7 and a set of 3 light chain CDRs in the VL defined herein as SEQ ID NO: 5
c) a heavy chain CDR/light chain CDR combination of a) or b), with the proviso that at least one of the CDRs has up to 3 amino acid substitutions relative to the respective

CDR as specified in a) or b), while maintaining its capability to bind to the extra-domain B (ED-B) of fibronectin,

d) a heavy chain CDR/light chain CDR combination of a) or b), with the proviso that at least one of the CDRs has a sequence identity of ≥66 % relative to the respective CDR as specified in a) or b), while maintaining its capability to bind to the extra-domain B (ED-B) of fibronectin,

wherein the CDRs are embedded in a suitable protein framework so as to be capable to bind to the extra-domain B (ED-B) of fibronectin.

In some embodiments, at least one of the CDRs has a sequence identity of ≥67, preferably ≥68, more preferably any one of ≥69, ≥70, ≥71, ≥72, ≥73, ≥74, ≥75, ≥76, ≥77, ≥78, ≥79, ≥80, ≥81, ≥82, ≥83, ≥84, ≥85, ≥86, ≥87, ≥88, ≥89, ≥90, ≥91, ≥92, ≥93, ≥94, ≥95, ≥96, ≥97, ≥98 or most preferably ≥99 % sequence identity relative to the respective CDRs.

In another embodiment, at least one of the CDRs has been modified by affinity maturation or other modifications, resulting in a sequence modification compared to the sequences disclosed above.

In some embodiments, at least one of the CDRs has up to 2, and preferably 1 amino acid substitutions relative to the respective CDR as specified in a) or b).

According to some embodiments of the invention, the single chain diabody comprises at least one of

a) a VH and VL domains of antibody L19 set forth in SEQ ID NOs: 7 and 5

b) the heavy chain/light chain variable domain sequence pair of a), with the proviso that at least one of the domains has a sequence identity of ≥80 % relative to SEQ ID NO: 7 or SEQ ID NO: 5, respectively and/or

c) the heavy chain/light chain variable domain sequence pair of a), with the proviso that at least one of the domains has up to 10 amino acid substitutions relative to SEQ ID NO: 7, or SEQ ID NO: 5, respectively,

while maintaining its capability to bind to the extra-domain B (ED-B) of fibronectin.

In some embodiments, at least one of the domains has a sequence identity of ≥81, preferably ≥82, more preferably ≥83, ≥84, ≥85, ≥86, ≥87, ≥88, ≥89, ≥90, ≥91, ≥92, ≥93, ≥94, ≥95, ≥96, ≥97, ≥98 or most preferably ≥99 % relative to SEQ ID NO: 7, or SEQ ID NO: 5, respectively.

In some embodiments, at least one of the domains has up to 9, preferably up to 8, more preferably up to 7, 6, 5, 4, 3 or 2 and most preferably up to 1 amino acid substitutions relative to SEQ ID NO: 7, or SEQ ID NO: 5, respectively.

According to some embodiments of the invention, at least one amino acid substitution in the single chain diabody is a conservative amino acid substitution.

According to one further embodiment, the composition has the full-length structure "[p40]-[linker1]-[p35]-[SADlinker]-[L19VH]-[linker3]-[L19VL]-[linker4]-[L19VH]-[linker3]-[L19VL]".

According to one further embodiment, the composition has a full-length sequence according to SEQ ID NO: 16.

Disorders

According to a further aspect of the invention, the use of the composition according to the above description (for the manufacture of a medicament) in the treatment of a human or animal subject

- being diagnosed for,
- suffering from or
- being at risk of

developing a neoplastic disease, or for the prevention of such condition, is provided.

According to a further aspect of the invention, the use of the composition according to any of the aforementioned claims (for the manufacture of a medicament) in the inhibition of angiogenesis in a human or animal subject.

Thus, a conjugate as herein described may be used in a method of treating a neoplastic disease or inhibiting angiogenesis by targeting IL-12 to the neovasculature *in vivo*.

The term "neoplastic disease" encompasses malignant transformations and cancers, including tumors and hematological diseases.

Also contemplated is a method of treating cancer or inhibiting angiogenesis by targeting an agent, in particular a therapeutic agent e.g. IL-12, to the neovasculature in a patient, the method comprising administering a therapeutically effective amount of a conjugate as herein described to the patient. Conditions treatable using the composition as described herein include cancer, other tumors and neoplastic conditions. The composition may be used to inhibit angiogenesis and thereby treat rheumatoid arthritis, diabetic retinopathy, age-related muscular degeneration, angiomas, tumors and cancer. Treatment may include prophylactic treatment. The composition may also be administered in diagnostic methods, e.g. targeting and diagnosis of angiogenesis, which may be associated with any of the above conditions. Other diseases and conditions may also be diagnosed and treated, according to the nature of the protein therapeutic or diagnostic agent contained in the composition, and the specificity of the targeting portion.

Cancers suitable for treatment as described herein include any type of solid or non-solid cancer or malignant lymphoma and especially liver cancer, lymphoma, leukemia (e.g. acute myeloid leukemia), sarcomas, skin cancer, bladder cancer, breast cancer, uterine cancer, ovarian cancer, prostate cancer, lung cancer, colorectal cancer, cervical cancer, head and neck cancer, oesophageal cancer, pancreatic cancer, renal cancer, stomach cancer and cerebral cancer. Cancers may be familial or sporadic. Cancers may be metastatic or non-metastatic.

Preferably, the cancer is a cancer selected from the group of kidney cancer, breast cancer, liver cancer, lung cancer, lymphoma, sarcoma (e.g. gastrointestinal stromal tumor), skin cancer (e.g. melanoma), colorectal cancer, and neuroendocrine tumors.

In some embodiments, the neoplastic disease is characterized by expression or overexpression of ED-B fibronectin.

Compositions of the invention may be administered to a patient in need of treatment via any suitable route, usually by injection into the bloodstream and/or directly into the site to be treated, e.g. tumor or tumor vasculature. The precise dose and its frequency of administration will depend upon a number of factors, the route of treatment, the size and location of the area to be treated (e.g. tumor).

With respect to responsiveness, a subject responds to treatment if a parameter of a cancer (e.g., a hematological cancer, e.g., cancer cell growth, proliferation and/or survival) in the subject is retarded or reduced by a detectable amount, e.g., about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more as determined by any appropriate measure, *e.g.*, by mass, cell count or volume. In one example, a subject responds to treatment if the subject experiences a life expectancy extended by about 5%, 10%, 20%, 30%, 40%, 50% or more beyond the life expectancy predicted if no treatment is administered. In another example, a subject responds to treatment, if the subject has an increased disease-free survival, overall survival or increased time to progression. Several methods can be used to determine if a patient responds to a treatment including, for example, criteria provided by NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®).

Combination Therapy

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. Other treatments may include the administration of suitable doses of pain relief drugs such as non-steroidal anti-inflammatory drugs (e.g. aspirin, ibuprofen or ketoprofen) or opiates such as morphine, or antiemetics.

According to a further aspect of the invention, a combination of the composition or the pharmaceutical composition according to the above description and one or more therapeutically active compounds is provided.

According to a further aspect of the invention, a method for treating or preventing a disorder or condition associated with expression or overexpression of ED-B fibronectin, comprising administering to a subject in need thereof an effective amount of the composition, the pharmaceutical composition or the combination according to the above description is provided.

Kits

According to a further aspect of the invention, a kit of parts comprising:

a) the composition, the pharmaceutical composition or the combination according to the above description,
b) an apparatus for administering the composition, composition or combination, and
c) instructions for use

is provided.

In some embodiments, such kit of parts comprises a pre-filled syringe provided with a suitable patient leaflet. In another embodiment, such kit of parts comprises an infusion bottle with suitable user instructions.

The components of a kit are preferably sterile and in sealed vials or other containers.

A kit may further comprise instructions for use of the components in a method described herein. The components of the kit may be comprised or packaged in a container, for example a bag, box, jar, tin or blister pack.

EXAMPLES

While the invention has been illustrated and described in detail in the drawings and foregoing description, such illustration and description are to be considered illustrative or exemplary and not restrictive; the invention is not limited to the disclosed embodiments. Other variations to the disclosed embodiments can be understood and effected by those skilled in the art in practicing the claimed invention, from a study of the drawings, the disclosure, and the appended claims. In the claims, the word “comprising” does not exclude other elements or

steps, and the indefinite article "a" or "an" does not exclude a plurality. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage. Any reference signs in the claims should not be construed as limiting the scope.

All amino acid sequences disclosed herein are shown from N-terminus to C-terminus; all nucleic acid sequences disclosed herein are shown 5'->3'.

EXAMPLE 1

The applicants have surprisingly discovered that when certain linkers are used to join IL-12 to a single-chain diabody (i.e. the superior format disclosed in WO2013/014149), a better tumor targeting performance as well as a superior production yield can be achieved.

The applicants have evaluated and characterized eight clones of human IL-12 joined to the L19 antibody in single chain diabody format (huIL-12L19L19) with different polypeptide linker between the cytokine and the L19 single chain diabody.

Five clones nicknamed: (i) "AKKAS" (ii) "DDS" (iii) "$(G4S)_3$" (iv) "SAD" and (v) "SES" contain linkers for conjugation of immunocytokines to recombinant antibodies and have been chosen due to their different electric charge characteristic (neutral, positively charged, negatively charged).

Three additional clones nicknamed (vi) Alpha3 (vii) AP6 and (viii) AP7 were developed. With regards to these three clones, the teachings reported in Chen et al (2013) were considered and put into practice. This review suggests that rigid linkers might have a better stability and might maintain the correct distance between the cytokine and the antibody, thus increasing the therapeutic efficacy.

None of the linkers (i) – (viii) were previously tested in this specific immunocytokine.

It was surprisingly found that the "SAD" linker greatly enhances the tumor targeting performance and the production yield of IL-12 joined to a single chain diabody, while being equally capable to bind to ED-B as compared to the other clones.

Materials & Methods

The variants tested in the examples have the following common structure:

Domain (N->C)	p40	Linker1	p35	Linker2	L19VH	Linker3	L19VL	Linker4	L19VH	Linker3/5	L19VL
SEQ ID NO	1	2	3	4, 9-15	7	6	5	8	7	6	5

The different variants (also called "clones" herein) differ from one another in the sequence of linker 2, as detailed in Table 2:

Table 2

Linker 2	SEQ ID NO	Sequence
AKKAS	9	GGGAKGGGGKAGGGS
DDS	10	GGGGDGGGGDGGGGS
$(G4S)_3$	11	GGGGSGGGGSGGGGS
SAD	4	GSADGGSSAGGSDAG
SES	12	GGGGSGGGGEGGGGS
Alpha3	13	AEAAAKEAAAKEAAAKA
AP6	14	APAPAPAPAPAP
AP7	15	APAPAPAPAPAPAP

Cloning of the eight fusion proteins with different linkers

The huIL-12L19L19 coding sequence has been generated by assembling different PCR fragments: the L19 antibody and the IL12 payload. The L19 gene was PCR amplified from a previously generated fusion protein L19-IL2 template using suitable primers. A second L19 gene was PCR amplified with suitable primers.

At the same time, part of the gene of the p35 domain of IL-12 was PCR amplified from a previously generated IL12-based immunocytokine using suitable primers. The two intermediate fragments were PCR-assembled (to generate a P35-L19 fragment), double digested with BamHI/BspEI and cloned into the double digested vector containing a p35. The

newly generated p35-L19 vector was subsequently double digested with BspEI/NotI-HF and ligated with a second L19 diabody fragment gene. The fragment p35-L19L19 was digested by BamHI/NotI-HF and cloned into the previously double digested mammalian cell expression vector pcDNA3.1 (+) carrying the p40 subunit gene, resulting in the full length IL12-L19L19.

Different linkers between the IL12 and the single-chain diabody L19 fragments were inserted by means of PCR assembly of fragments "A" (encoding for a part of p35 with the linker), and fragment "B" (encoding for the linker and a part of the antibody). The different fragments "A" and fragments "B" were amplified from IL12-L19L19 as template using suitable primers.

The cloning strategy designed for clone AP7 led to the generation of a mutant clone (named AP6). All PCR products were double digested with BamHI-HF and BspEI restriction enzymes and ligated into a P35-L19L19 pcDNA3.1 plasmid. The resulting plasmids were amplified, double digested with NotI-HF and BamHI-HF restriction enzymes and the insert was sub-cloned into a pcDNA3.1 plasmid containing IL12. Resulting DNA plasmids were amplified and used for cell transfection.

Expression purification of the eight fusion proteins with different linkers

For the production of the various human IL-12 fusions, CHO-S cells in suspension were used. The huIL-12L19L19 variants were expressed using transient gene expression. For 1 ml of production 4×10^6 CHO-S cells in suspension were centrifuged and resuspended in 1 mL of a medium suitable for CHO-S. 0.625 µg of plasmid DNAs followed by 2.5 µg polyethylene imine (PEI; 1 mg/mL solution in water at pH 7.0) per million cells were then added to the cells and gently mixed. The transfected cultures were incubated in a shaker incubator at 31°C for 6 days.

Finally, the fusion proteins produced by transient gene expression, were purified from the cell culture medium by protein A affinity chromatography and then dialyzed against PBS.

SDS-PAGE

The correct molecular weight of the fusion proteins was analyzed under reducing and non-reducing conditions by SDS-PAGE 10% and SDS-PAGE 12%.

ELISA

To check the correct binding of the various IL-12 fusions, Elisa plates were coated overnight with 50 ug/ml fibronectin domain 7B89 (see WO2001/062800 A1, the content of which is incorporated herein by reference). The immunocytokines were tested at 10 ug/ml and 1 ug/ml. As secondary reagent, Protein A horseradish peroxidase was used. The assay was developed with BM- Blue POD soluble substrate. The colorimetric reaction was stopped by the addition of 333 mM H_2SO_4 and the absorbance was measured at wavelengths 450 nm and 650 nm using a microtiter plate reader.

Size exclusion chromatography and Biacore

Size-exclusion chromatography was performed on an ÄKTA FPLC system using the Superdex 200 increase column. Surface plasmon resonance experiments affinity measurements were performed by Biacore X100 instrument with purified huIL-12L19L19 clones on a fibronectin 7B89 domain coated CM5 chip. Samples were injected as serial-dilution, concentration range from 1 μM to 250 nM.

Immunofluorescence

To confirm the ability of the various huIL-12 fusions to bind cancer cells, immunofluorescence was performed onto frozen syngeneic F9 teratocarcinoma specimen cryostat sections (8 um). The tumor slices were fixed by ice-cold aceton (5 min). After fixation, coverslips were washed and blocked with 20% fetal bovine serum in PBS for 45 min. HuIL-12L19L19 clones at concentration 5 μg/ml were added in 2% BSA/PBS solution 1h at room temperature. Coverslips were then washed twice with PBS and secondary antibody mouse anti-human interleukin-12, final dilution 1: 1000 was added in 2% BSA/PBS solution at room temperature for 1h. Coverslips were then washed again twice with PBS and tertiary antibody Goat Anti Mouse, final dilution 1:500, was added. DAPI was used to counterstain nuclei.

Radiolabelling and *in vivo* tumor targeting

To confirm the ability of the various IL-12 fusions to bind *in vivo* tumor, their targeting ability was evaluated by biodistribution analysis. 100 µg of each IL-12L19L19 clone were radioiodinated with ^{125}I and Chloramine T hydrate and purified on a PD10 column. Radiolabeled proteins were injected into the lateral tail vein of immunocompetent mice bearing s.c. implanted F9 murine teratocarcinoma. Injected dose per mouse varied between 4 and 9 µg. Mice were sacrificed 24 h after injection. Organs were weighed and radioactivity was counted using a Packard Cobra gamma counter. Radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g ± standard error).

RESULTS

Cloning, expression and SDS-PAGE

The eight variants of huIL-12L19L19 fusion proteins were successfully cloned each one with a different polypeptide linker between the cytokine and the L19 single-chain diabody. The SDS-PAGE characterization displayed a molecular weight around 120 kDa for all variants, which confirms the expected protein size (about 109 kDa not glycosylated). The expression yields (by transient gene expression in CHO-S cells) ranged for all variants between 3.5 and 5 mg/L. Surprisingly the clone nicknamed "SAD" showed a yield of 9 mg/L which is remarkably higher than the yield of the other 7 clones. The results are shown in Fig. 1.

ELISA

In ELISA, the eight clones Alpha3, AP6, AP7, DDS, SES, AKKAS, $(G4S)_3$ and SAD all confirmed the binding (both at 10 µg/ml and 1 µg/ml concentration) towards the domain 7B89 of human fibronectin. The results are shown in Fig. 2.

BiaCore

A more precise affinity constant determination was performed by Biacore analysis on a domain 7B89 of human fibronectin coated chip (Fig. 3). Samples were injected as serial-dilution, concentration equal to 1000 nM, 750 nM, 500 nM and 250 nM (Fig. 3). The apparent KD was estimated by Biacore X100 Evaluation Software.

Size Exclusion Chromatography

The eight clones Alpha3, AP6, AP7, DDS, SES, AKKAS, $(G4S)_3$ and SAD were characterized on size exclusion chromatography (SEC-200increase), where all clones showed a comparable profile with the major peak corresponding to the monomeric immunocytokine (Fig. 4).

Immunofluorescence

An immunofluorescence experiment was performed with the clones Alpha3, AP6, AP7, DDS, SES, AKKAS, $(G4S)_3$ and SAD onto frozen syngeneic F9 teratocarcinoma specimen cryostat section (8 um). All the clones showed a specific binding on the vasculature as compared to the negative control (Fig. 5).

***In vivo* tumor targeting**

In vivo targeting was evaluated by biodistribution analysis. The eight clones Alpha3, AP6, AP7, DDS, SES, AKKAS, $(G4S)_3$ and SAD as well as the positive control (the L19 single chain diabody joined to murine IL-12) were radioiodinated with ^{125}I and injected (4-9 μg protein/animal) into immunocompetent mice bearing s.c. implanted F9 murine teratocarcinoma. The radioactivity counted 24 hours after the injection, showed an accumulation in the tumor for all the clones, however the "SAD" clone showed a superior accumulation in the tumor as compared to the other seven clones. (Fig. 6).

EXAMPLE 2

In a further set of comparative experiments, it was surprisingly found that the "SAD" linker is also superior to the old (and shorter) GSADGG linker (SEQ ID NO: 26) disclosed in WO2013/014149 in terms of binding capacity, monomeric profile and tumor targeting ability.

Material & Methods

The variants tested in this example have the following common structure:

Domain (N->C)	p40	Linker1	p35	Linker2	L19VH	Linker3	L19VL	Linker4	L19VH	Linker3/5	L19VL
SEQ ID NO	1	2	3	4, 26	7	6	5	8	7	6	5

The different variants (also called "clones" herein) differ from one another in the sequence of linker 2:

Linker 2	SEQ ID NO	Sequence
SAD	4	GSADGGSSAGGSDAG
Old	26	GSADGG

Cloning of fusion proteins

Fusion proteins comprising huIL-12 fused via a 6 or 15 amino acids linker, to the L19 antibody in single chain diabody format (namely huIL-12L19L19 "Old", and huIL-12L19L19 "SAD" variants respectively) were cloned along the lines described above.

Expression of fusion proteins

Fusion proteins comprising huIL-12 fused via a 6 or 15 amino acids linker, to the L19 antibody in single chain diabody format (namely huIL-12L19L19 "Old", and huIL-12L19L19 "SAD" variants respectively) were produced by transient gene expression in suspension adapted CHO cell cultures. Following transfection cells were maintained in ProCHO-4 medium (supplemented with 4 mM ultraglutamine), for 6 days at 31°C under shaking conditions, after which the culture supernatant was harvest by centrifugation and further processed to purify the fusion protein.

Purification of fusion proteins using protein A resin

Transfected CHO cell suspension cultures were centrifuged for 30 minutes at 5000 rpm at 4 °C. The supernatant was further clarified by filtration using 0.45 um filters. Protein A resin was added to the filtered supernatant and the mixture incubated under shaking conditions for ca 1h. The resin was than collected into a liquid chromatography column, and washed with "buffer A" (100 mM NaCl, 0.5 mM EDTA, 0.1% Tween 20 in PBS pH 7.4) followed by a

second wash with "buffer B" (500 mM NaCl 0.5 mM EDTA in PBS pH 7.4). The fusion proteins comprising huIL-12 were eluted by gravity flow using 100 mM TEA. Aliquots were collected and fractions containing the fusion protein, as confirmed by UV spectrometry, were pooled and dialyzed overnight against PBS.

Size exclusion chromatography of fusion proteins

Size exclusion chromatography of fusion proteins was performed using a Superdex 200 increase 10/300 GL column with PBS as running buffer on a ÄKTA-FPLC system. 100 µl protein solutions were injected into a loop and automatically injected onto the column. UV absorbance at 280 nm was assessed over time. SEC profiles of the fusion proteins were analyzed using the peak integration function of the UNICORN software to quantify the percentage of the monomeric fraction with respect either to the total % area or to the peak % area. To exclude peak artifacts due to sample loading or to salts present in the sample buffers, only the interval between retention volume 5-17.5 mL was considered for quantification.

BIACore

Surface plasmon resonance experiments affinity measurements were performed by BiacoreX100 instrument with the purified "Old" and "SAD" clones on a fibronectin 7B89 domain freshly coated CM5 chip. Samples were injected as serial-dilution, concentration equal to 250nM, 125 nM and 62.5 mM (Fig. 10). The apparent KD was estimated by Biacore X100 Evaluation Software.

Radiolabelling and *In vivo* tumor targeting

Purified protein samples huIL-12L19L19 "SAD" (with linker GSADGGSSAGGSDAG, SEQ ID NO 4) and huIL-12L19L19 "Old" (with linker GSADGG, SEQ ID NO 26) (100 μg) were radioiodinated with ^{125}I and Chloramine T hydrate and purified on a PD10 column. Proteins were radioiodinated after Protein A affinity chromatography. Proteins were injected into the lateral tail vein of immunocompetent (129/Sv) mice bearing subcutaneously implanted F9 murine teratocarcinoma. Injected dose per mouse varied between 10 and 11 μg. Mice were sacrificed 24 hours after injection. Organ samples were weighed and radioactivity was counted

using a Packard Cobra gamma counter. The protein uptake in the different organs was calculated and expressed as the percentage of the injected dose per gram of tissue (%ID/g ± standard error). The protein uptake into the tumor was adjusted by the tumor growth according to Tarli et al. (1999).

RESULTS

Expression and purification of fusion proteins and Size Exclusion Chromatography

The two huIL-12L19L19 "SAD", and huIL-12L19L19 "Old" variants were produced by transient gene expression in CHO cells. Experiments were performed in duplicate, where two sets of production experiments were performed on different days giving rise to batches A and B respectively. Following single step purification by Protein-A affinity chromatography, and dialysis versus PBS, homogeneity of protein samples was assessed by size exclusion chromatography (Fig. 9). Both protein variants showed a certain degree of protein aggregation as highlighted by the presence of high molecular weight variants eluting at early retention volume. The huIL-12L19L19 "SAD" in both cases showed a better profile as confirmed by quantification of the monomeric portion of the proteins using the peak integration function of the UNICORN software. Indeed, the huIL-12L19L19 "SAD" variant showed lower tendency to aggregation when compared to huIL-12L19L19 "Old", this considering either the monomeric peak area as a percentage of the total area under the curve above the baseline (mean values: 54.57% vs 46.69%, respectively), or the monomeric peak area as a percentage of the sum of all integrated peaks (mean values: 58.83% vs 52.74%, respectively) (Table 1).

Protein	Linker length (amino acids)	Batch	Monomeric Peak Retention Volume (mL)	Monomeric Peak Area / Total Area (*) (%)	Mean Area / Total Area (*) (%)	Monomeric Peak Area / Peak Area (°) (%)	Mean Area / Peak Area (°) (%)
huIL-12L19L19 "SAD"	15	A	11.70	54.06	54.57	58.51	58.83
huIL-12L19L19 "SAD"	15	B	11.72	55.08		59.14	
huIL-12L19L19 "Old"	6	A	11.87	46.73	46.69	48.99	52.74

huIL-12L19L19 "Old"	6	B	11.86	46.65		56.48	

Table 1: Quantification of the Monomeric fraction of the different fusion proteins assessed by the peak integration function of the UNICORN software. (*) Peak area as a percent of the total area under the curve above the baseline. (°) Peak area as a percent of the sum of all integrated peaks.

Biacore

The apparent KD was estimated by Biacore X100 Evaluation Software to be 6.7 nM for the "Old" clone (huIL-12L19L19 "Old" with the linker GSADGG) and 3.8 nM for the "SAD" clone (huIL-12L19L19 "SAD" with the linker GSADGGSSAGGSDAG) (Fig. 10).

***In vivo* tumor targeting**

The radioactivity counted 24 hours after the injection, showed that the "SAD" clone has an unexpectedly superior tumor uptake as compared to the "Old" clone (Fig.11).

EXAMPLE 3

The efficacy of the huIL-12L19L19 "SAD" variant is assessed in human patients having malignant melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma, urothelial carcinoma, head and neck squamous cell carcinoma (HNSCC), microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer, hepatocellular cancer, gastric cancer, squamous cell carcinoma of the skin, cervical cancer, and diffuse large B-cell lymphoma (DLBCL). At least one cohort of patients demonstrates disease progression on an immune checkpoint blockade therapy-based regimen administered as immediate prior treatment.

Patients receive huIL-12L19L19 "SAD" variant by intravenous administration once-weekly for 8 weeks. Patients receive doses of 4 μg /kg; 8 μg /kg; 12 μg /kg; 16 μg /kg; or 20 μg /kg.

Patients are followed for 6 months from the start of treatment, or until withdrawal of consent or progressive disease.

Pharmacokinetic analysis of IL12-L19L19L19-L12 is assessed using sandwich capture of the fusion molecule and the IL12 moiety. Human anti-fusion protein antibodies (HAFA) will be tested by Surface Plasmon Resonance analysis and by sandwich capture. Anti-tumor activity, e.g., efficacy, will be assessed at week 8, week 16 and week 24 using RECIST (version 1.1) for solid tumors or by LUGANO criteria for malignant lymphoma evaluation criteria.

Further embodiments

According to a first set of embodiments the following is provided:

1. A conjugate comprising
   a) a heterodimeric IL-12 protein having a first and second subunit,
   b) a single chain diabody, and
   c) a linker between the IL-12 protein and the single chain diabody, which linker comprises an amino acid motif comprising SAD
2. The conjugate according to point 1, wherein the SAD linker comprises the amino acid motif GSADGGSSAGGSDAG (SEQ ID NO: 4)
3. The conjugate according to any of points 1 - 2, wherein the first subunit of the heterodimeric IL-12 protein is p40 and the second subunit is p35,
4. The conjugate according to any of points 1 - 3, wherein the single chain diabody is monospecific or bispecific.
5. The conjugate according to any of points 1 - 4, wherein the single chain diabody binds to the extra-domain B (ED-B) of fibronectin
6. The conjugate according to any of points 1 - 5, wherein the single chain diabody comprises two L19 VH domains and two L19 VL domains
7. The conjugate according to any of points 1 - 6, which has the full-length structure [p40]-[linker1]-[p35]-[SAD linker]-[L19VH]-[linker3]-[L19VL]-[linker4]-[L19VH]-[linker3]-[L19VL]
8. The conjugate according to any of points 1 - 7, which has a full length sequence according to SEQ ID NO: 16

9. Use of the conjugate according to any of the aforementioned points (for the manufacture of a medicament) in the treatment of a human or animal subject

- being diagnosed for,
- suffering from or
- being at risk of

developing a neoplastic disease, or for the prevention of such condition.

10. Use of the conjugate according to any of the aforementioned points (for the manufacture of a medicament) in the inhibition of angiogenesis in a human or animal subject.

11. A pharmaceutical composition comprising at least the conjugate according to any of points 1 – 8, and optionally one or more pharmaceutically acceptable excipient.

12. A combination comprising (i) the conjugate according to any one of points 1 – 8 or the pharmaceutical composition according to point 11 and (ii) one or more therapeutically active compounds.

13. A method for treating or preventing a disorder or condition associated with expression or overexpression of ED-B fibronectin, comprising administering to a subject in need thereof an effective amount of the conjugate according to any one of points 1 – 8, the pharmaceutical composition according to point 11, or the combination according to point 12.

14. A therapeutic kit of parts comprising:

a) the conjugate according to any one of points 1 – 8, the pharmaceutical composition according to point 11 or the combination according to point 12,

b) an apparatus for administering the conjugate, composition or combination, and

c) instructions for use.

According to a second set of embodiments the following is provided:

1. A conjugate comprising

a) a heterodimeric IL-12 protein having a first and second subunit,

b) a single chain diabody, and

c) a linker between the IL-12 protein and the single chain diabody, which linker comprises an amino acid motif comprising GSADGGSSAGGSDAG (SEQ ID NO: 4)

2. The conjugate according to any of points 1, wherein the first subunit of the heterodimeric IL-12 protein is p40 and the second subunit is p35,

3. The conjugate according to any of points 1 - 2, wherein the single chain diabody is monospecific or bispecific.

4. The conjugate according to any of points 1 - 3, wherein the single chain diabody binds to the extra-domain B (ED-B) of fibronectin

5. The conjugate according to any of points 1 - 4, wherein the single chain diabody comprises two L19 VH domains and two L19 VL domains

6. The conjugate according to any of points 1 - 5, which has the full-length structure [p40]-[linker1]-[p35]-[SAD linker]-[L19VH]-[linker3]-[L19VL]-[linker4]-[L19VH]-[linker3]-[L19VL]

7. The conjugate according to any of points 1 - 6, which has a full length sequence according to SEQ ID NO: 16

8. Use of the conjugate according to any of the aforementioned points (for the manufacture of a medicament) in the treatment of a human or animal subject

- being diagnosed for,
- suffering from or
- being at risk of

developing a neoplastic disease, or for the prevention of such condition.

9. Use of the conjugate according to any of the aforementioned points (for the manufacture of a medicament) in the inhibition of angiogenesis in a human or animal subject.

10. A pharmaceutical composition comprising at least the conjugate according to any of points 1 – 7, and optionally one or more pharmaceutically acceptable excipient.

11. A combination comprising (i) the conjugate according to any one of points 1 – 7 or the pharmaceutical composition according to point 10 and (ii) one or more therapeutically active compounds.

12. A method for treating or preventing a disorder or condition associated with expression or overexpression of ED-B fibronectin, comprising administering to a subject in need thereof an effective amount of the conjugate according to any one of points 1 – 7, the pharmaceutical composition according to point 10, or the combination according to point 11.

13. A therapeutic kit of parts comprising:

a) the conjugate according to any one of points 1 – 7, the pharmaceutical composition according to point 10 or the combination according to point 11,

b) an apparatus for administering the conjugate, composition or combination, and

c) instructions for use.

According to a third set of embodiments the following is provided:

1. A conjugate comprising

a) a heterodimeric IL-12 protein having a first and second subunit,

b) a single chain diabody, and

c) a linker between the IL-12 protein and the single chain diabody, which linker comprises an amino acid motif comprising GSADGGSSAGGSDAG (SEQ ID NO: 4)

2. The conjugate according to point 1, wherein the first subunit of the heterodimeric IL-12 protein is p40 and the second subunit is p35.

3. The conjugate according to any one of points 1 - 2, wherein the single chain diabody is monospecific or bispecific.

4. The conjugate according to any one of points 1 - 3, wherein the single chain diabody binds to the extra-domain B (ED-B) of fibronectin

5. The conjugate according to any one of the points 1-4, wherein the single chain diabody comprises an antigen-binding site having the complementarity determining regions (CDRs) of antibody L19 set forth in SEQ ID NOs: 28 to 33.

6. The conjugate according to points 1-5, wherein the single chain diabody comprises the VH and VL domains of antibody L19 set forth in SEQ ID NOs: 7 and 5.

7. The conjugate according to any of points 1 - 6, wherein the single chain diabody comprises at least one of

a) the heavy chain/light chain variable domain sequence pair of point 6, with the proviso that at least one of the domains has a sequence identity of ≥80 % relative to SEQ ID NO: 7 or SEQ ID NO: 5, respectively and/or

b) the heavy chain/light chain variable domain sequence pair of point 6, with the proviso that at least one of the domains has up to 10 amino acid substitutions relative to SEQ ID NO: 7 or SEQ ID NO: 5, respectively,

while maintaining its capability to bind to the extra-domain B (ED-B) of fibronectin.

8. The conjugate according to any one of points 1 - 7, wherein at least one amino acid substitution in the single chain diabody is a conservative amino acid substitution

9. The conjugate according to any one of points 1 – 8, wherein the single chain diabody

• has a target binding affinity of ≥50 % to the extra-domain B (ED-B) of fibronectin, compared to one of the antibodies of point 5, or point 6, and/or

• competes for binding to bind to the extra-domain B (ED-B) of fibronectin with one of the antibodies of point 5, or point 6.

10. The conjugate according to any one of points 1 - 9, wherein the single chain diabody comprises two L19 VH domains and two L19 VL domains

11. The conjugate according to any one of points 1 - 10, which has the full-length structure [p40]-[linker1]-[p35]-[SAD linker]-[L19VH]-[linker3]-[L19VL]-[linker4]-[L19VH]-[linker3]-[L19VL]

12. The conjugate according to any one of points 1 - 11, which has a full length sequence according to SEQ ID NO: 16

13. Use of the conjugate according to any one of the aforementioned points (for the manufacture of a medicament) in the treatment of a human or animal subject

- being diagnosed for,
- suffering from or
- being at risk of

developing a neoplastic disease, or for the prevention of such condition.

14. Use of the conjugate according to any one of the aforementioned points (for the manufacture of a medicament) for the inhibition of angiogenesis in a human or animal subject.

15. A pharmaceutical composition comprising at least the conjugate according to any one of points 1 – 12, and optionally one or more pharmaceutically acceptable excipients.

16. A combination comprising (i) the conjugate according to any one of points 1 – 12 or the pharmaceutical composition according to point 15 and (ii) one or more therapeutically active compounds.

17. A method for treating or preventing a disorder or condition associated with expression or overexpression of ED-B fibronectin, comprising administering to a subject in need thereof an effective amount of the conjugate according to any one of points 1 – 12, the pharmaceutical composition according to point 15, or the combination according to point 16.

18. A therapeutic kit of parts comprising:

a) the conjugate according to any one of points 1 – 12, the pharmaceutical composition according to point 15 or the combination according to point 16,
b) an apparatus for administering the conjugate, composition or combination, and
c) instructions for use.

According to one further embodiment of the invention, the SAD linker comprises the amino acid motif GSADGGSSAGGSDAG (SEQ ID NO: 4). As used herein, the term “single chain diabody” relates to a construct of two single chain Fv (scFv) antibodies with a short linker, preferably 5 amino acid long, conjugated to one another by a longer linker, preferably 15 amino

acid long, according to the following scheme (N->C orientation): L19VH-linker$_3$-L19VL-linker4-L19VH-linker3-L19VL.

According to one embodiment of the invention, the linker$_3$ is GSSGG (SEQ ID NO: 6) and the linker$_4$ is SSSSGSSSSGSSSSG (SEQ ID NO: 8). According to one embodiment of the invention, the first subunit of the heterodimeric IL-12 protein is p40 and the second subunit is p35. Preferably, the two subunits are conjugated to one another by a given linker, according to the following scheme (N->C orientation): p40-linker$_1$-p35.

Preferably IL-12 is human IL-12. In a preferred embodiment, the linker1 is GGGGSGGGGSGGGGS (SEQ ID NO: 2). According to one embodiment of the invention, the single chain diabody is monospecific or bispecific. According to one embodiment of the invention, the single chain diabody binds to a splice isoform of fibronectin. According to one embodiment of the invention, the single chain diabody binds to the extra-domain B (ED-B) of fibronectin.

REFERENCES (the disclosures of which are herein incorporated by reference in their entireties)

Car et al., Toxicologic Pathology (1999), 27(1), 58-63
Chen et al., Adv Drug Deliv Rev. (2013), 65(10), 1357-1369
WO2013/014149
WO2006/119897
Tarli et al., Blood (1999), 94(1), 192-198.

SEQUENCES

The following sequences form part of the disclosure of the present application. A WIPO ST 25 compatible electronic sequence listing is provided with this application, too. For the avoidance of doubt, if discrepancies exist between the sequences in the following table and the electronic sequence listing, the sequences in this table shall be deemed to be the correct ones.

SEQ ID NO	qualifier	Sequence
1	P40	IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKT LTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNK TFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAE RVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIR DIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSK REKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCS
2	Linker 1	GGGGSGGGGSGGGGS
3	P35	RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDI TKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSS IYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVP QKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS
4	Linker 2 (“SAD”)	GSADGGSSAGGSDAG
5	L19VL	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYA SSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTK VEIK
6	Linker 3/Linker 5	GSSGG
7	L19VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSIS GSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFD YWGQGTLVTVSS
8	Linker 4	SSSSGSSSSGSSSSG
9	Linker 2 (“AKKAS”)	GGGAKGGGGKAGGGS
10	Linker 2 (“DDS”)	GGGGDGGGGDGGGGS
11	Linker 2 (“G4S$_3$”)	GGGGSGGGGSGGGGS
12	Linker 2 (“SES”)	GGGGSGGGGEGGGGS
13	Linker 2 (“Alpha 3”)	AEAAAKEAAAKEAAAKA
14	Linker 2 (“AP6”)	APAPAPAPAPAP
15	Linker 2 (“AP7”)	APAPAPAPAPAPAP
16	Full length SAD variant	IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKT LTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNK TFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAE RVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIR DIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSK REKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGGGSG GGGSGGGGSRNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCT SEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTS FMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQA LNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNASGS ADGGSSAGGSDAGEVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQ APGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDT AVYYCAKPFPYFDYWGQGTLVTVSSGSSGGEIVLTQSPGTLSLSPGERATLS CRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGEVQ LLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS GTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWG QGTLVTVSSGSSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQ

		QKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQ QTGRIPPTFGQGTKVEIK
17	Full length mulL-12-L19-L19	MWELEKDVYVVEVDWTPDAPGETVNLTCDTPEEDDITWTSDQRHGVIGSGKT LTITVKEFLDAGQYTCHKGGETLSHSHLLLHKKENGIWSTEILKNFKNKTFL KCEAPNYSGRFTCSWLVQRNMDLKFNIKSSSSSPDSRAVTCGMASLSAEKVT LDQRDYEKYSVSCQEDVTCPTAEETLPIELALEARQQNKYENYSTSFFIRDI IKPDPPKNLQMRPLKNSQVEVSWEYPDSWSTPHSYFSLKFFVRIQRKKEKMK ETEEGCNQKGAFLVERTSTEVQCKGGNVCVQAQDRYYNSSCSKWACVPCRVR SGGGGSGGGGSGGGGSRVIPVSGPARCLSQSRNLLKTTDDMVKTAREKLKHY SCTAEDIDHEDITRDQTSTLKTCLPLELHKNESCLATRETSSTTRGSCLPPQ KTSLMMTLCLGSIYEDLKMYQTEFQAINAALQNHNHQQIILDKGMLVAIDEL MQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRVVTINRVMGYLSS AGSADGEVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLE WVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK PFPYFDYWGQGTLVTVSSGSSGGEIVLTQSPGTLSLSPGERATLSCRASQSV SSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTLTISRLEP EDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGEVQLLESGGG LVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYAD SVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTLVTV SSGSSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAP RLLIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPP TFGQGTKVEIK
18	AKKAS linker nucleotide sequence	ggagggggag ctaaaggtgg cggtggcaag gcagggggag ggagt
19	AP7 linker nucleotide sequence	gcaccagcac cagcaccagc accagcacca gcaccagcac ca
20	DDS linker nucleotide sequence	ggaggtgggg gtgatggtgg gggaggtgac ggcggaggtg ggtct
21	AP6 linker nucleotide sequence	gcaccagcac cagcaccagc accagcacca gcacca
22	(G4S)3 linker nucleotide sequence	ggtggaggcg ggtcaggcgg agggggttct ggcggtggcg gatcg
23	SES linker nucleotide sequence	ggtgggggtg ggtccggagg cggaggcgaa ggcggaggtg ggtcg
24	Alpha3 linker nucleotide sequence	gcagaagcag cagcaaaaga agcagcagca aaagaagcag cagcaaaagc a
25	SAD linker nucleotide sequence	gggtctgcag acggcggatc atcagctggg ggaagtgacg cagga
26	Linker 2 (“Old”)	GSADGG
27	Full length “Old” variant	IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKT LTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNK TFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAE RVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIR DIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSK REKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGGGSG GGGSGGGGSRNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCT SEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTS FMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQA LNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNASGS ADGGEVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWV SSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPF PYFDYWGQGTLVTVSSGSSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSS SFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPED FAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGEVQLLESGGGLV QPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSSGTTYYADSV KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWGQGTLVTVSS GSSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRL LIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTF GQGTKVEIK

28	L19 VH CDR1	SFSMS
29	L19 VH CDR2	SISGSSGTTYYADSVKG
30	L19 VH CDR3	PFPYFDY
31	L19 VL CDR1	RASQSVSSSFLA
32	L19 VL CDR2	YASSRAT
33	L19 VL CDR3	QQTGRIPPT
34	GGGGS linker	GGGGS
35	GGGGA linker	GGGGA
36	L19 Diabody	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSIS GSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFD YWGQGTLVTVSSGSSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLA WYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVY YCQQTGRIPPTFGQGTKVEIK

What is claimed is:

1. A composition comprising

a. an IL-12 protein comprising a first IL-12 subunit and a second IL-12 protein subunit;

b. a peptide or protein comprising an EDB binding domain; and

c. a linker between the IL-12 protein and the peptide or protein comprising the EDB binding domain.

2. The composition according to claim 1, wherein the linker between the IL-12 protein and the peptide or protein comprising the EDB binding domain comprises GSADGGSSAGGSDAG (SEQ ID NO: 4).

3. The composition according to claims 1 or 2, wherein the peptide or protein comprising the EDB binding domain comprises an scFv.

4. The composition according to claims 1 or 2, wherein the peptide or protein comprising the EDB binding domain is a diabody.

5. The composition according to claims 1 or 2, wherein the peptide or protein comprising the EDB binding domain is a single chain diabody.

6. The composition according to any one of claims 1 - 5, wherein the first subunit of the IL-12 protein is a p40 and the second subunit is a p35.

7. The composition according to claims 1 - 6, wherein the first subunit of the IL-12 protein is a p40 comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO:1 or a fragment thereof, wherein the IL-12 protein can activate an IL-12 receptor.

8. The composition according to claims 1 -7, wherein the second subunit of the IL-12 protein is a p35 comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid

sequence set forth in SEQ ID NO:3 or a fragment thereof, wherein the IL-12 protein can activate an IL-12 receptor.

9. The composition according to any one of claims 1 - 8, wherein the peptide or protein comprising an EDB binding domain is monospecific or bispecific.

10. The composition according to any one of claims 1 - 9, wherein the peptide or protein comprising an EDB binding domain binds to the extra-domain B (ED-B) of fibronectin

11. The composition according to any one of claims 1 - 10, wherein the peptide or protein comprising an EDB binding domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to one or more of the amino acid sequences set forth in SEQ ID NOs: 28 to 33.

12. The composition according to any one of claims 1 - 10, wherein the peptide or protein comprising an EDB binding domain comprises each of the amino acid sequences of SEQ ID NOs: 28 to 33.

13. The composition according to any one of claims 1 - 12, wherein the peptide or protein comprising an EDB binding domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to one or more of the amino acid sequences set forth in SEQ ID NOs: 7 and 5.

14. The composition according to any one of claims 1 - 13, wherein the peptide or protein comprising an EDB binding domain comprises each of the amino acid sequences set forth in SEQ ID NOs: 7 and 5.

15. The composition according to any one of claims 1 - 14, wherein the peptide or protein comprising an EDB binding domain comprises at least one of

a) the sequence pair of claim 14, with the proviso that at least one of the domains has a sequence identity of ≥80 % relative to SEQ ID NO: 7 or SEQ ID NO: 5, respectively and/or

b) the sequence pair of claim 14, with the proviso that at least one of the domains has up to 10 amino acid substitutions relative to SEQ ID NO: 7 or SEQ ID NO: 5, respectively,

while maintaining its capability to bind to the extra-domain B (ED-B) of fibronectin.

16. The composition according to claim 15, wherein at least one amino acid substitution is a conservative amino acid substitution.

17. The composition according to any one of claims 1 – 16, wherein the peptide or protein comprising an EDB binding domain

- has a target binding affinity of ≥50 % to the extra-domain B (ED-B) of fibronectin, compared to one of the peptides or proteins comprising an EDB binding domain of claims 11 - 16, and/or
- competes for binding to the extra-domain B (ED-B) of fibronectin with one of peptides or proteins comprising an EDB binding domain of claims 11 -16.

18. The composition according to any one of claims 1 - 17, wherein the peptide or protein comprising an EDB binding domain comprises two L19 VH domains and two L19 VL domains.

19. The composition according to claim 18, wherein:

- the two L19 VH domains have the same amino acid sequence;
- the two L19 VH domains have a different amino acid sequence;
- the two L19 VL domains have the same amino acid sequence; or
- the two L19 VL domains have a different amino acid sequence.

20. The composition according to any one of claims 1 - 17, wherein the peptide or protein comprising an EDB binding domain comprises one L19 VH domain and one L19 VL domain.

21. The composition according to any one of claims 1 - 17, wherein the composition comprises:

- a p40 domain linked to a p35 domain by a first linker;
- a first L19 VH domain linked to the p35 domain by a SAD linker;
- a first L19 VL domain linked to the first L19 VH domain by a third linker;
- a second L19 VH domain linked to the first L19 VL domain by a fourth linker;
- a second L19 VL domain linked to the second L19 VH domain by a fifth linker.

22. The composition according to claim 21, wherein the p40 domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO:1 or a fragment thereof.

23. The composition according to any one of claims 21 - 22, wherein the p35 domain comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO:3 or a fragment thereof.

24. The composition according to any one of claims 21 - 23, wherein the first linker is a GS linker.

25. The composition according to any one of claims 21 - 24, wherein the first linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 2.

26 The composition according to any one of claims 21 - 25, wherein the first L19 VH domain, the second L19 VH domain, or both comprise an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to at least one amino acid sequence set forth in SEQ ID NOs: 28-30.

27. The composition according to any one of claims 21 - 26, wherein the first L19 VL domain, the second L19 VL domain, or both comprise an amino acid sequence having at least

75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to at least one amino acid sequence set forth in SEQ ID NOs: 31-33.

28. The composition according to any one of claims 21 - 27, wherein the first L19 VH domain, the second L19 VH domain, or both comprise an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 7.

29. The composition according to any one of claims 21 - 27, wherein the first L19 VL domain, the second L19 VL domain, or both comprise an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 5.

30 The composition according to any one of claims 21 - 29, wherein the SAD linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 4.

31. The composition according to any one of claims 21 - 30, wherein the third linker is a GS linker.

32. The composition according to any one of claims 21 - 31, wherein the third linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 6.

33. The composition according to any one of claims 21 - 32, wherein the fifth linker is a GS linker.

34. The composition according to any one of claims 21 - 33, wherein the third linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 6.

35. The composition according to any one of claims 21 - 34, wherein the third linker and fifth linker comprise the same amino acid sequence.

36. The composition according to any one of claims 21 - 35, wherein the fourth linker is a GS linker.

37. The composition according to any one of claims 21 - 36, wherein the fourth linker comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 8.

38. The composition according to any one of claims 1 - 37, wherein the composition comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 16.

39. The composition according to any one of claims 1 – 38, wherein the composition consists of an amino acid sequence having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the amino acid sequence set forth in SEQ ID NO: 16.

40. Use of the composition according to any one of claims 1 – 39 (for the manufacture of a medicament) in the treatment of a human or animal subject

- being diagnosed for,
- suffering from or
- being at risk of

developing a neoplastic disease, or for the prevention of such condition.

41. The use of claim 40, wherein the neoplastic disease is selected from the group consisting of malignant melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma, urothelial carcinoma, head and neck squamous cell carcinoma (HNSCC), microsatellite

instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer, hepatocellular cancer, gastric cancer, squamous cell carcinoma of the skin, cervical cancer, and diffuse large B-cell lymphoma (DLBCL).

42. Use of the composition according to any one claims 1 - 39 (for the manufacture of a medicament) for the inhibition of angiogenesis in a human or animal subject.

43. A pharmaceutical composition comprising the composition according to any one of claims 1 – 39, and optionally one or more pharmaceutically acceptable excipients.

44. A combination comprising (i) the composition according to any one of claims 1 – 39 or the pharmaceutical composition according to claim 43 and (ii) one or more therapeutically active compounds.

45. A method for treating or preventing a disorder or condition associated with expression or overexpression of ED-B fibronectin, comprising administering to a subject in need thereof an effective amount of the composition according to any one of claims 1 – 39, the pharmaceutical composition according to claim 43, or the combination according to claim 44.

46. A therapeutic kit of parts comprising:

a) the composition according to any one of claims 1 – 39, the pharmaceutical composition according to claim 43 or the combination according to claim 44,
b) an apparatus for administering the composition, composition or combination, and
c) instructions for use.

Clone (SEQ ID NO)	...P35-L19... linker sequence (nucleotide)	...P35-L19... linker sequence (aminoacid)	Production yields
AKKAS (18/9)	AATGCTTCCGGAGGGGGAGCTAAAGGTGGCGGTGGCAAGGCAGGGGGAGGGAGTGAGGTGCAG	NAS **GGGAKGGGGKAGGGS** EVQ	**4.6 mg/L**
AP7 (19/15)	AATGCTTCCGCACCAGCACCAGCACCAGCACCACCAGCACCACCAGCACCAGAGGTGCAG	NAS **APAPAPAPAPAPAP** EVQ	**4.7 mg/L**
DDS (20/10)	AATGCTTCCGGAGGTGGGGGTGATGGTGGGGGAGGTGACGGCGGAGGTGGGTCTGAGGTGCAG	NAS **GGGGDGGGGDGGGGS** EVQ	**5.0 mg/L**
AP6 (21/14)	AATGCTTCCGCACCAGCACCAGCACCAGCACCAGCACCAGCACCAGAGGTGCAG	NAS **APAPAPAPAPAP** EVQ	**3.5 mg/L**
$(G4S)_3$ (22/11)	AATGCTTCCGGTGGAGGCGGGTCAGGCGGAGGGGGGTTCTGGCGGTGGCGGATCGGGAGGTGCAG	NAS **GGGGSGGGGSGGGGS** EVQ	**3.5 mg/L**
SES (23/12)	AATGCTTCCGGTGGGGGTGGGTCCGGAGGCGGAGGCGAAGGCGGAGGTGGGTCGGAGGTGCAG	NAS **GGGGSGGGGEGGGGS** EVQ	**4.4 mg/L**
Alpha3 (24/13)	AATGCTTCCGCAGAAGCAGCAGCAAAAGAAAGCAGCAGCAAAAGAAGCAGCAAAAGCAGAGGTGCAG	NAS **AEAAAKEAAAKEAAAKA** EVQ	**4.5 mg/L**
SAD (25/4)	AATGCTTCCGGGTCTGCAGACGGGCGGATCATCAGCTGGGGGAAGTGACGCAGGAGAGGTGCAG	NAS **GSADGGSSAGGSDAG** EVQ	**9.0 mg/L**

Fig. 1A

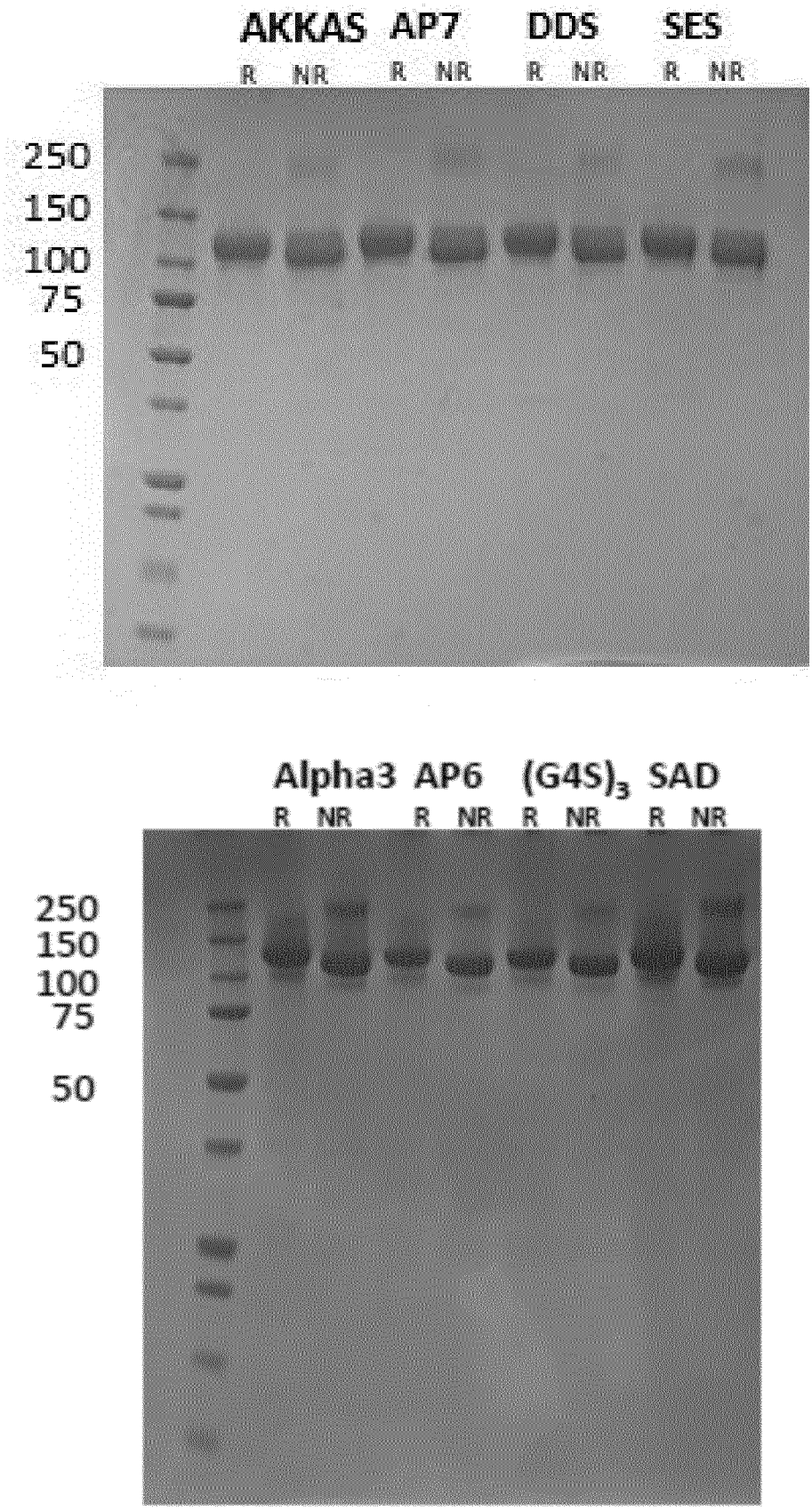
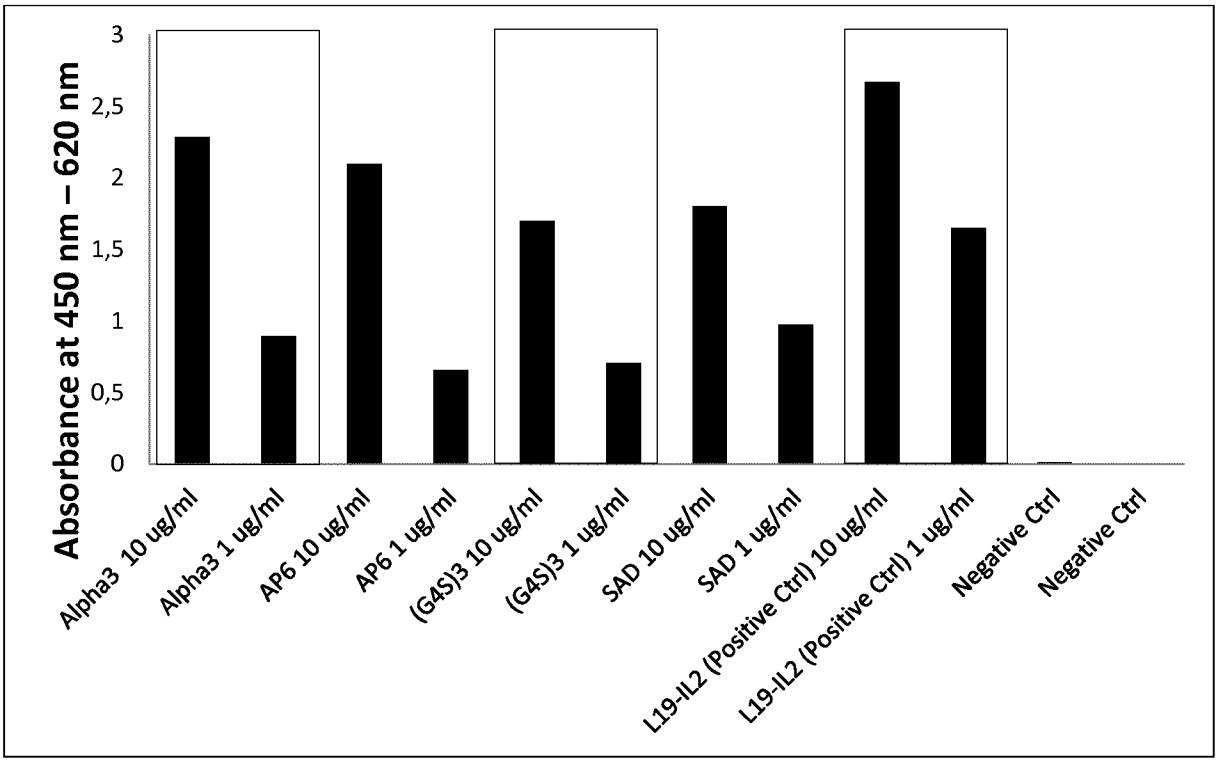

AKKAS AP7 DDS SES
R NR R NR R NR R NR
250
150
100
75
50
Alpha3 AP6 (G4S)3 SAD
R NR R NR R NR R NR
250
150
100
75
50

Fig. 1B


Absorbance at 450 nm – 620 nm
3
2,5
2
1,5
1
0,5
0
Alpha3 10 ug/ml
Alpha3 1 ug/ml
AP6 10 ug/ml
AP6 1 ug/ml
(G4S)3 10 ug/ml
(G4S)3 1 ug/ml
SAD 10 ug/ml
SAD 1 ug/ml
L19-IL2 (Positive Ctrl) 10 ug/ml
L19-IL2 (Positive Ctrl) 1 ug/ml
Negative Ctrl
Negative Ctrl

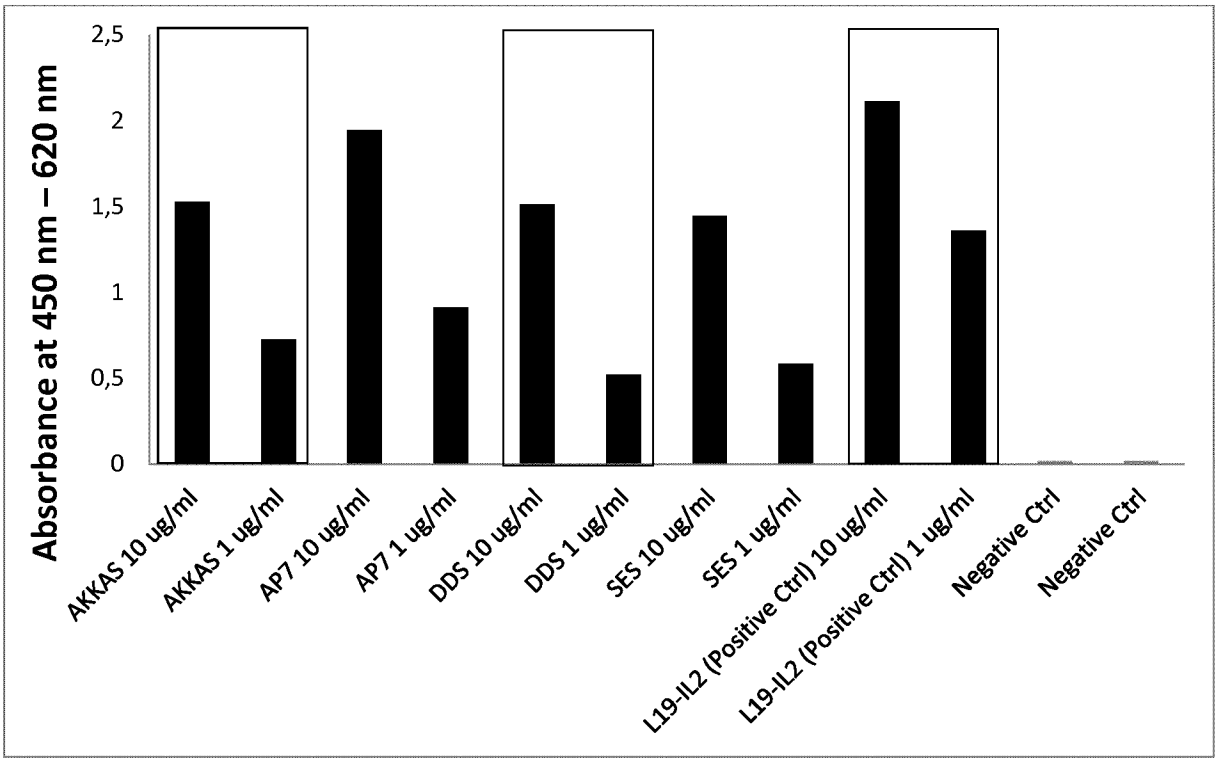

Absorbance at 450 nm – 620 nm
2,5
2
1,5
1
0,5
0
AKKAS 10 ug/ml
AKKAS 1 ug/ml
AP7 10 ug/ml
AP7 1 ug/ml
DDS 10 ug/ml
DDS 1 ug/ml
SES 10 ug/ml
SES 1 ug/ml
L19-IL2 (Positive Ctrl) 10 ug/ml
L19-IL2 (Positive Ctrl) 1 ug/ml
Negative Ctrl
Negative Ctrl

Fig. 2

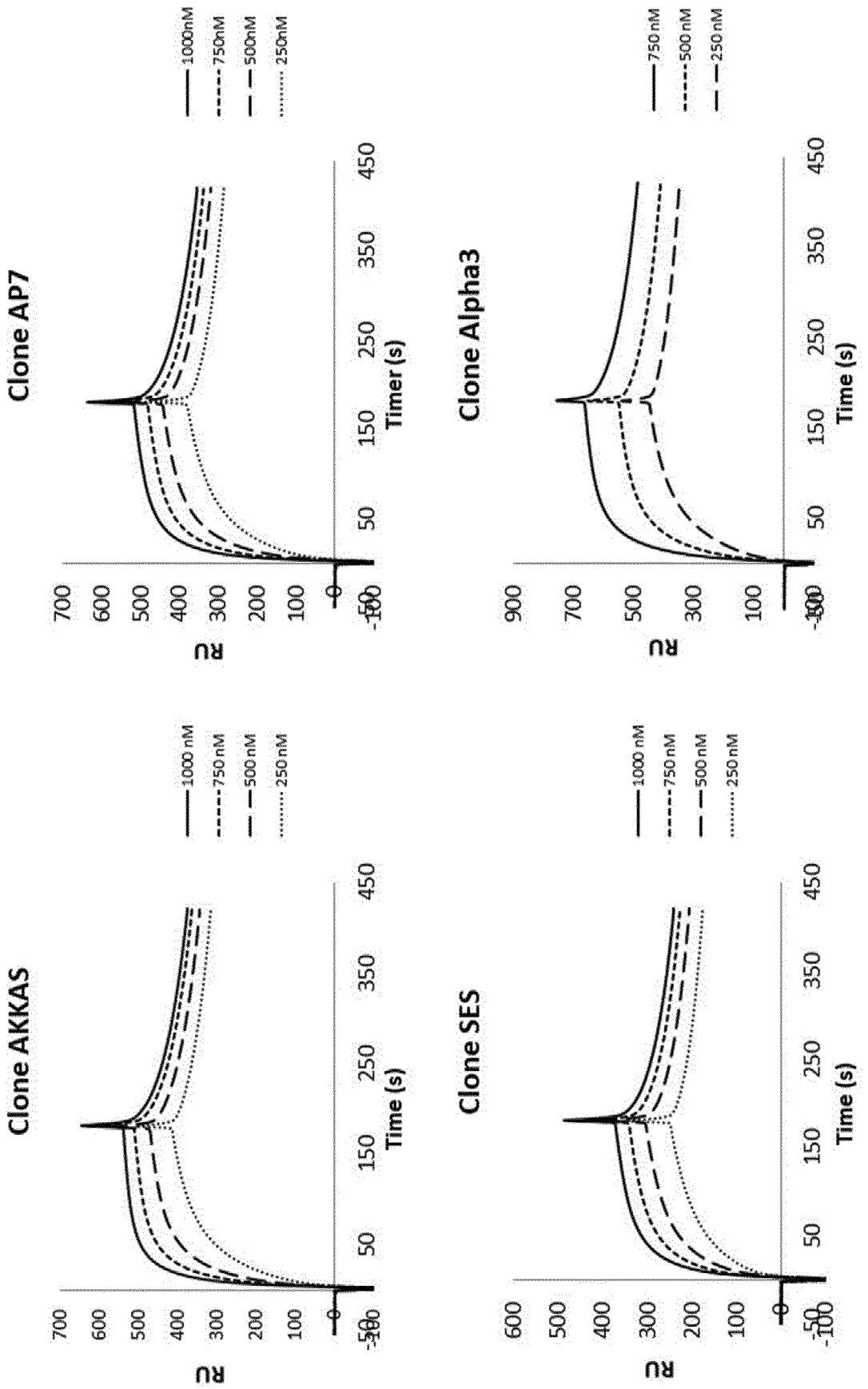

Clone AP7
1000nM
750nM
500nM
250nM
RU
Timer (s)
Clone Alpha3
750 nM
500 nM
250 nM
RU
Time (s)
Clone AKKAS
1000 nM
750 nM
500 nM
250 nM
RU
Time (s)
Clone SES
1000 nM
750 nM
500 nM
250 nM
RU
Time (s)

Fig. 3

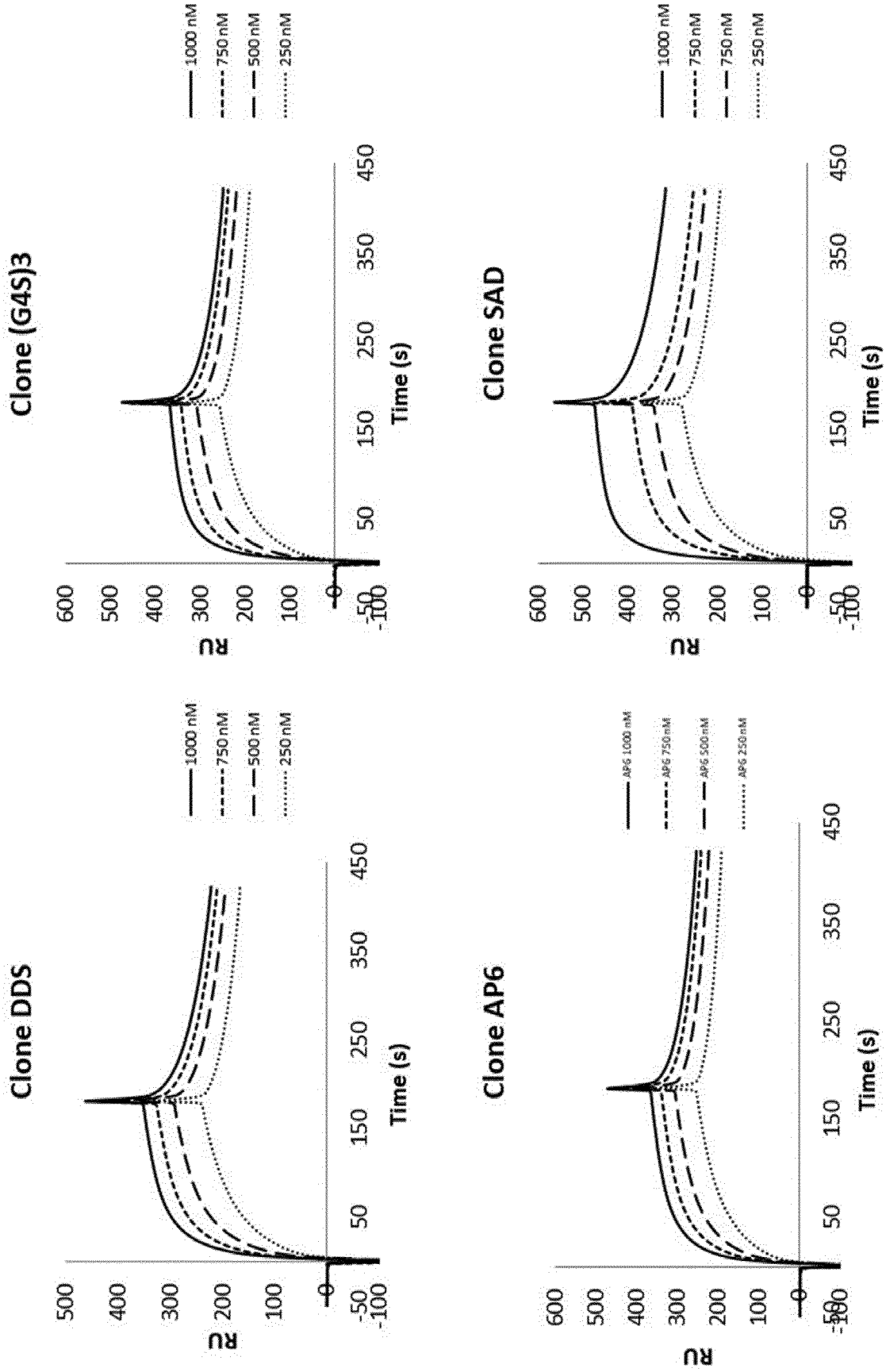

Clone (G4S)3
Clone SAD
Clone DDS
Clone AP6
1000 nM
750 nM
500 nM
250 nM
AP6 1000 nM
AP6 750 nM
AP6 500 nM
AP6 250 nM
RU
Time (s)

Fig. 3 ctd‘

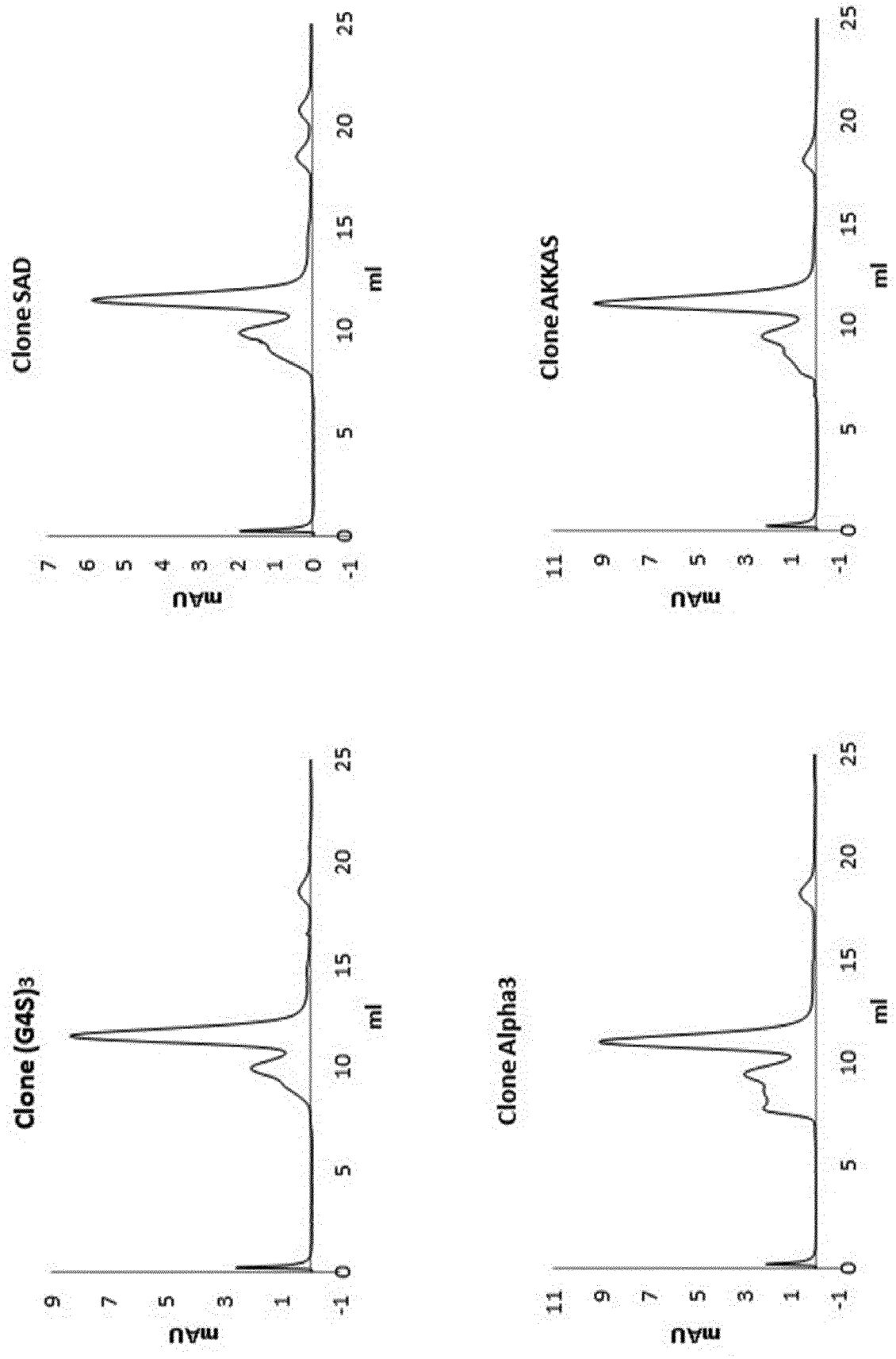

Clone SAD
mAU
ml
Clone AKKAS
mAU
ml
Clone (G4S)3
mAU
ml
Clone Alpha3
mAU
ml

Fig. 4

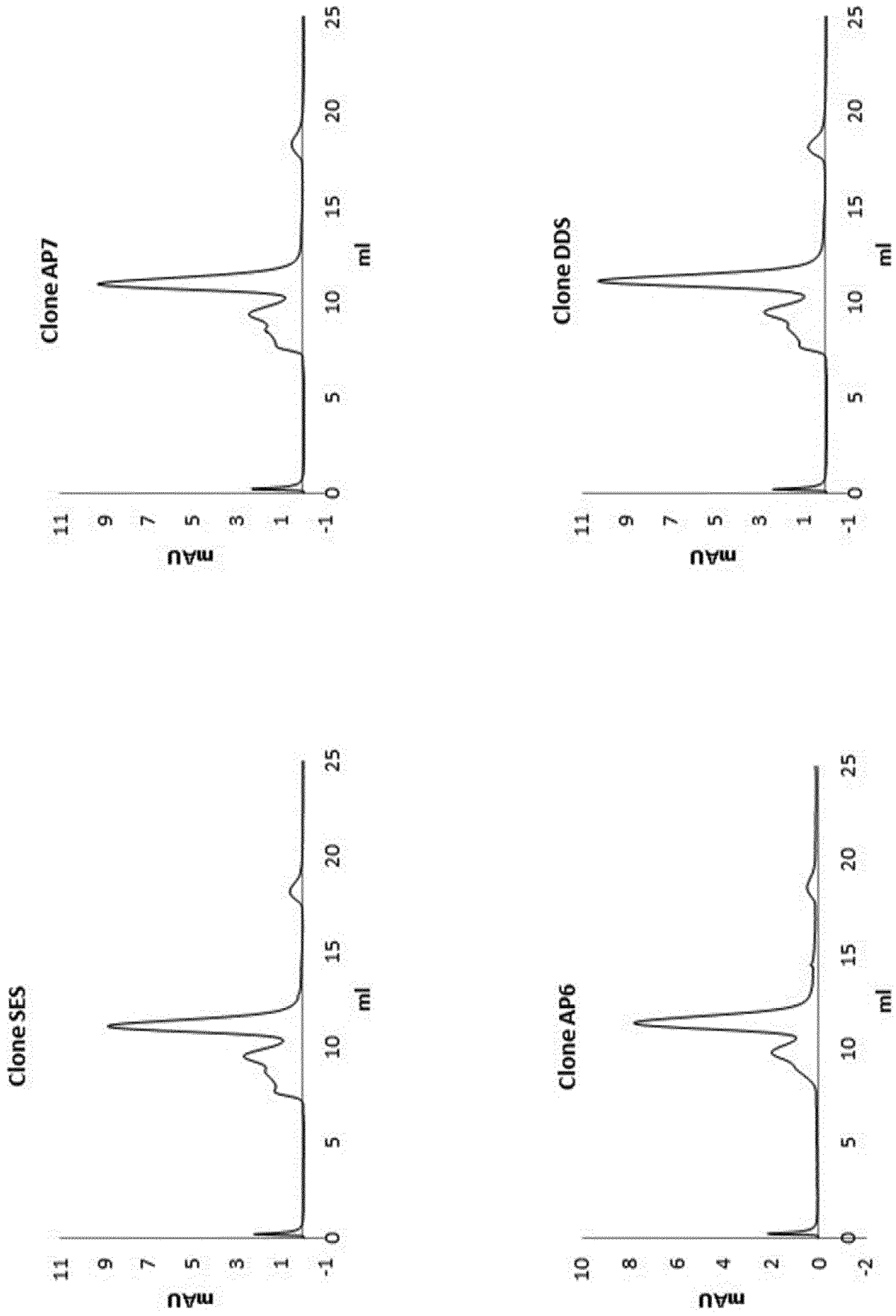

Clone AP7
mAU
ml
Clone DDS
mAU
ml
Clone SES
mAU
ml
Clone AP6
mAU
ml

Fig. 4 ctd‘

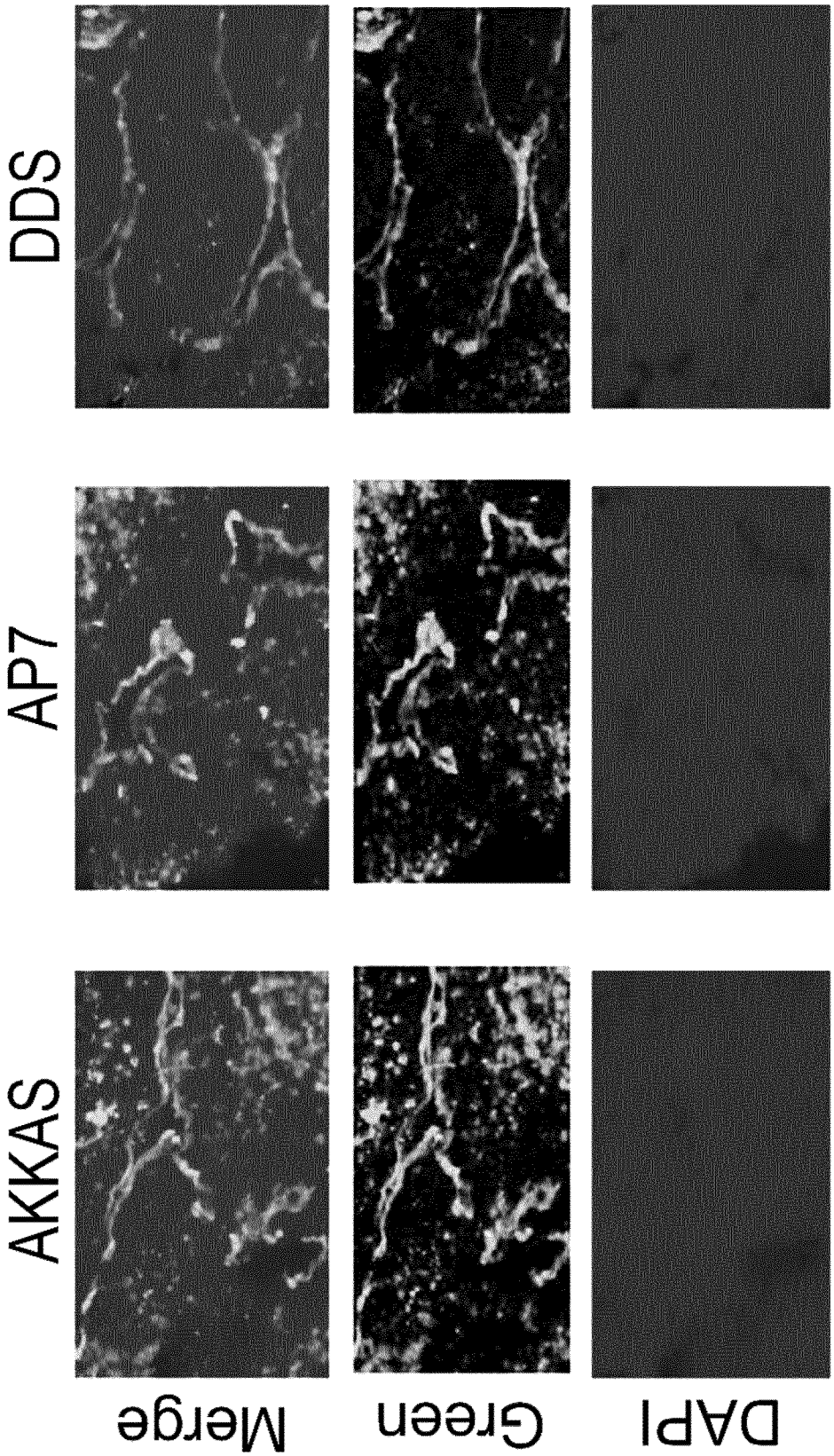


Fig. 5

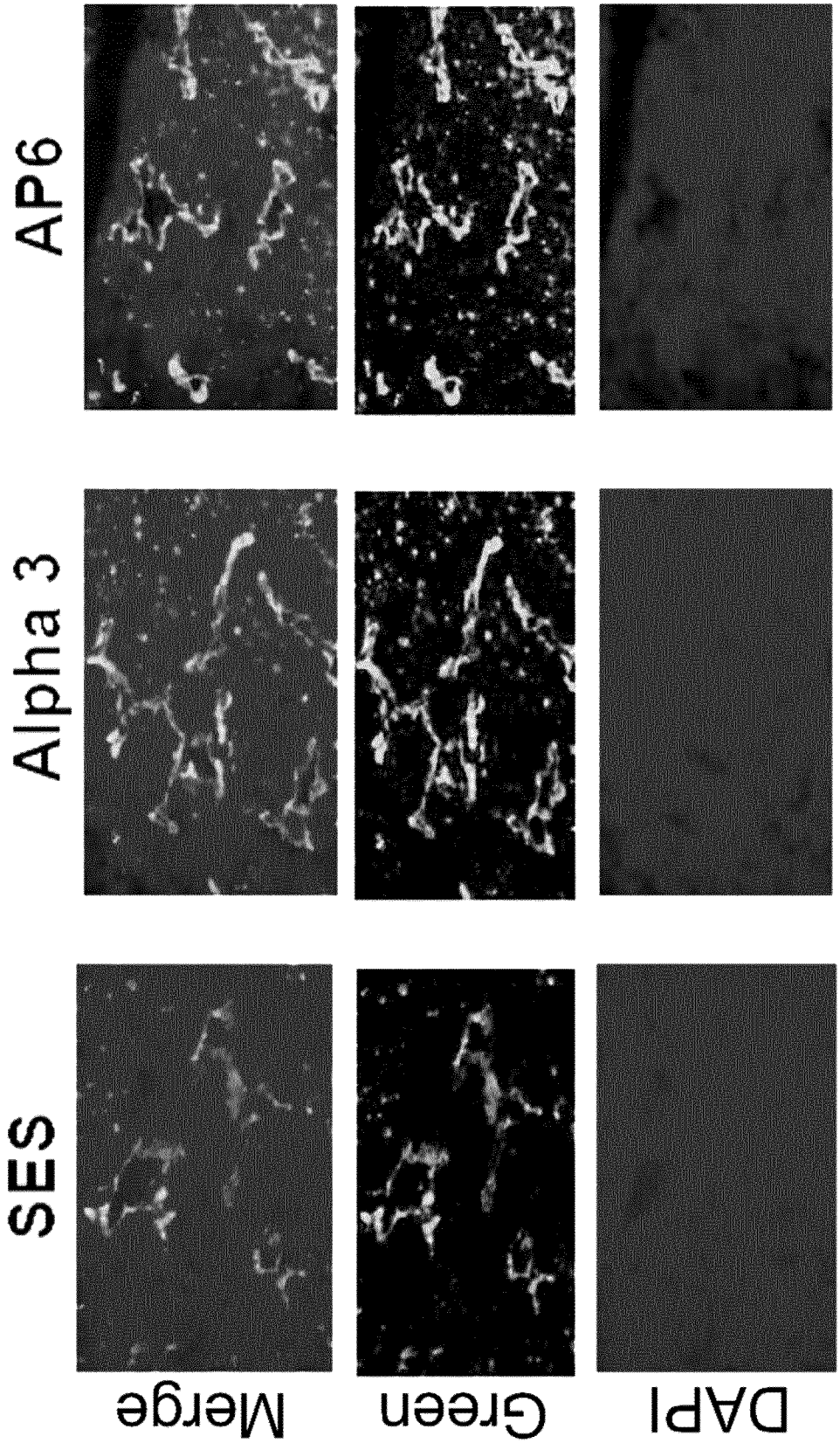


Fig. 5 ctd'

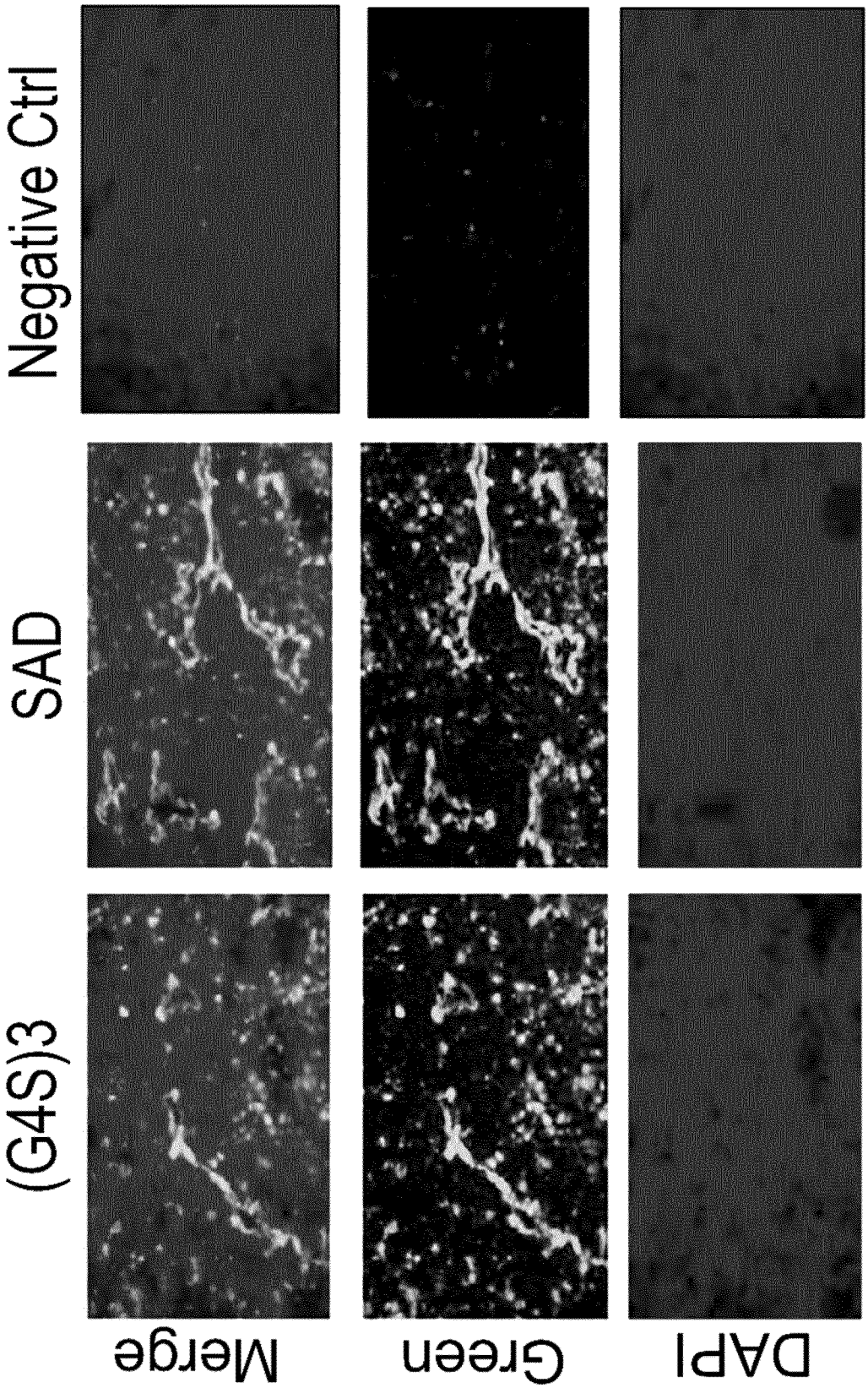


Fig. 5 ctd'

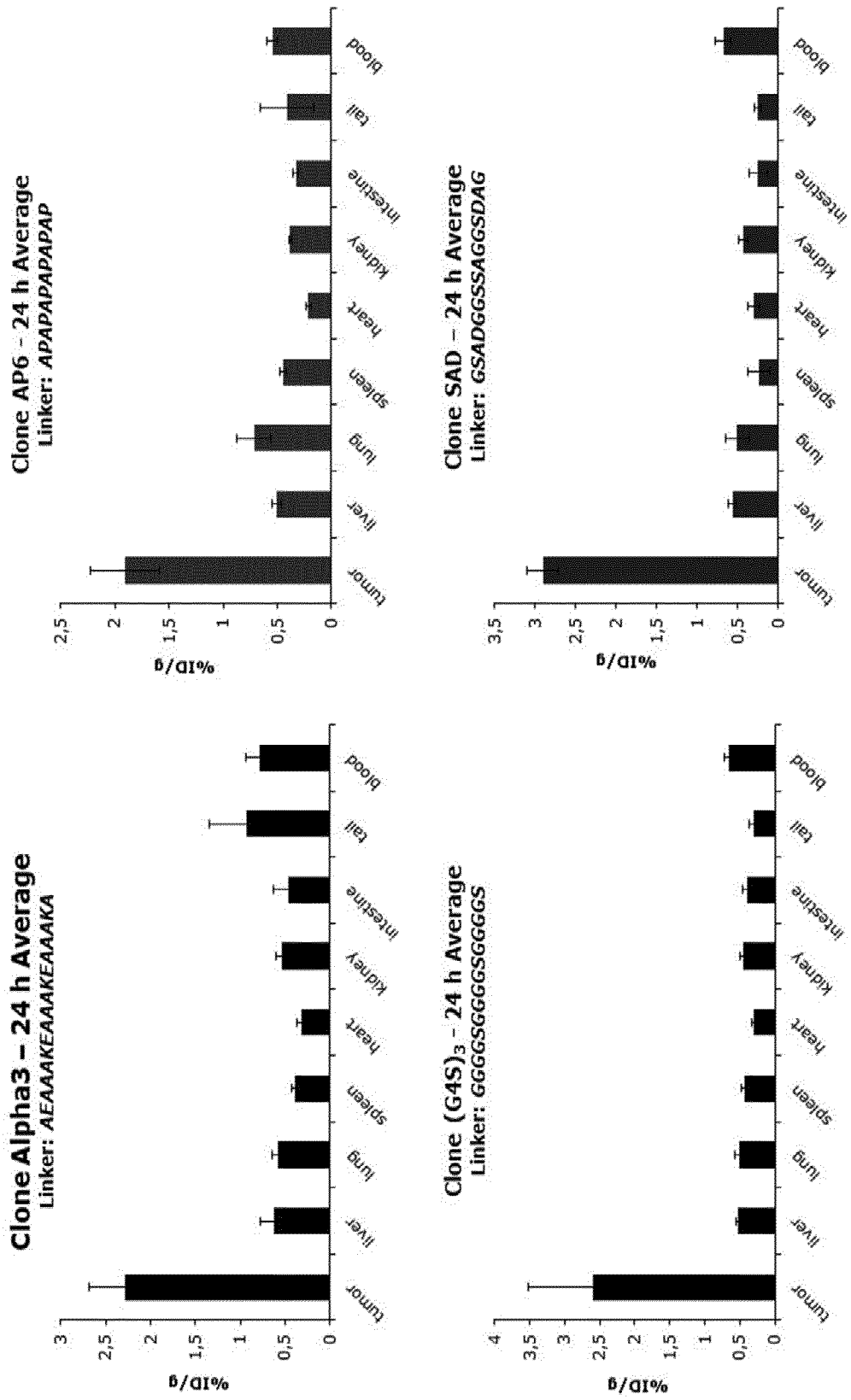

Clone AP6 – 24 h Average
Linker: APAPAPAPAP
Clone SAD – 24 h Average
Linker: GSADGGSSAGGSDAG
Clone Alpha3 – 24 h Average
Linker: AEAAAKEAAAKEAAAKA
Clone (G4S)3 – 24 h Average
Linker: GGGGSGGGGSGGGGS
%ID/g
tumor
liver
lung
spleen
heart
kidney
intestine
tail
blood

Fig. 6

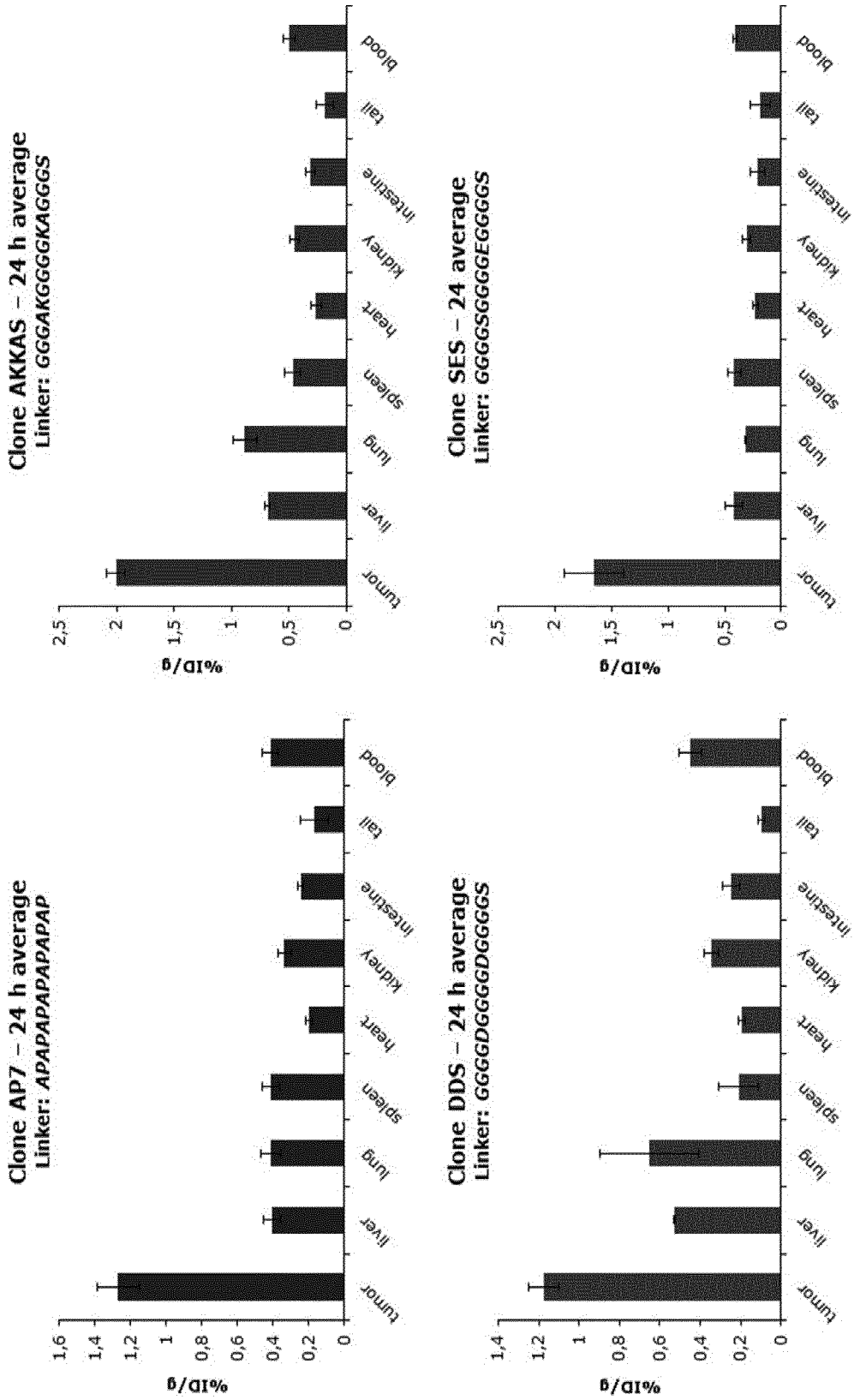

Clone AKKAS – 24 h average
Linker: GGGAKGGGGGKAGGGS
Clone SES – 24 average
Linker: GGGGSGGGGGEGGGGS
Clone AP7 – 24 h average
Linker: APAPAPAPAPAPAP
Clone DDS – 24 h average
Linker: GGGGDGGGGDGGGGS
%ID/g
tumor
liver
lung
spleen
heart
kidney
intestine
tail
blood

Fig. 6 ctd‘

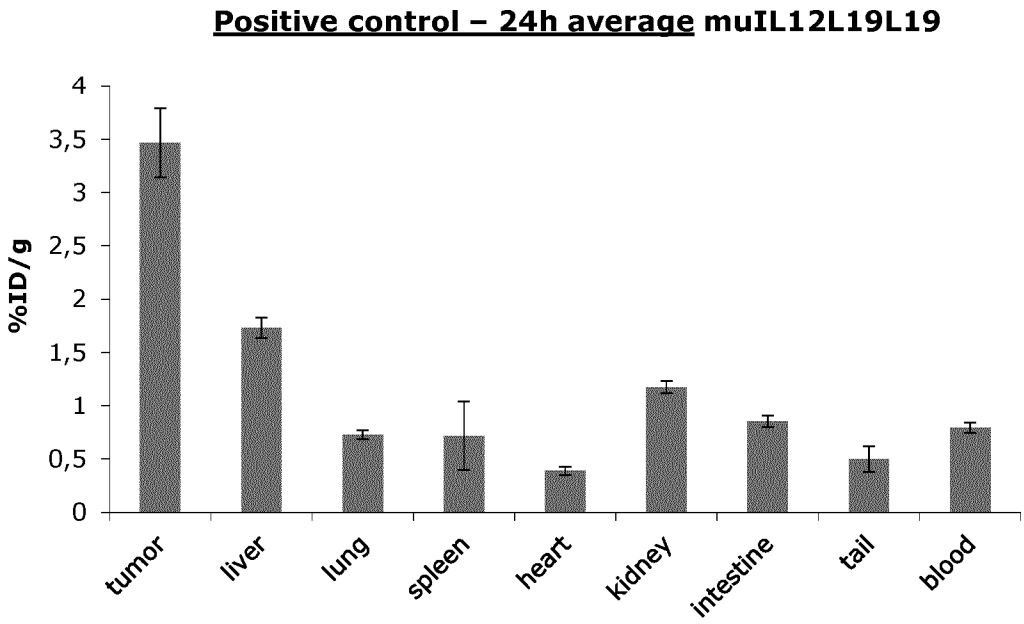

Positive control – 24h average muIL12L19L19
%ID/g
4
3,5
3
2,5
2
1,5
1
0,5
0
tumor
liver
lung
spleen
heart
kidney
intestine
tail
blood

Fig. 6 ctd‘

[prior art for illustrative purposes]

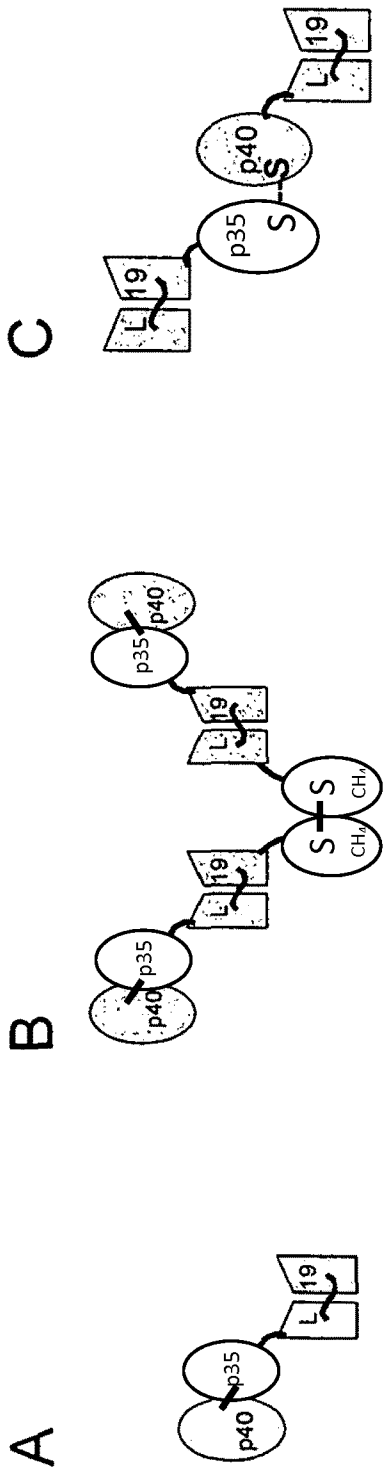


Fig. 7

[prior art for illustrative purposes]

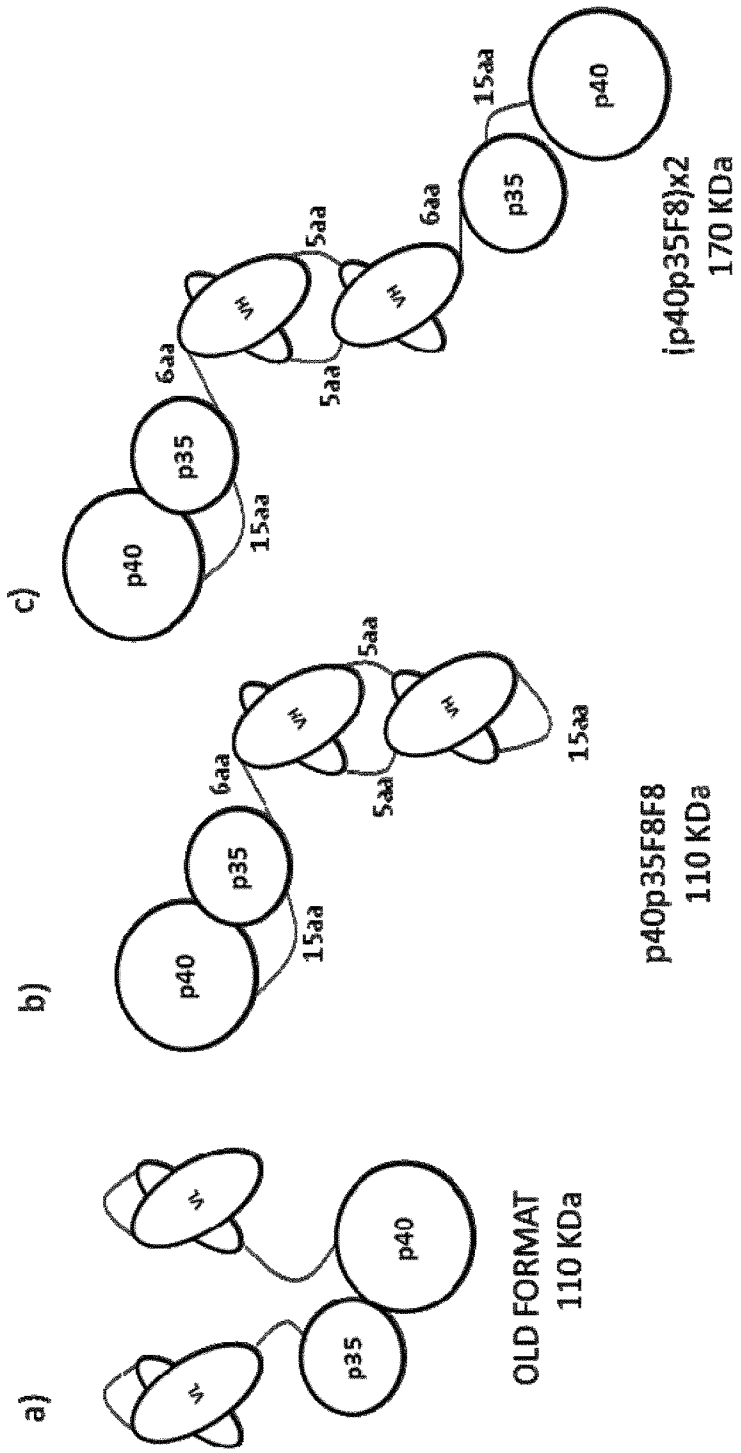
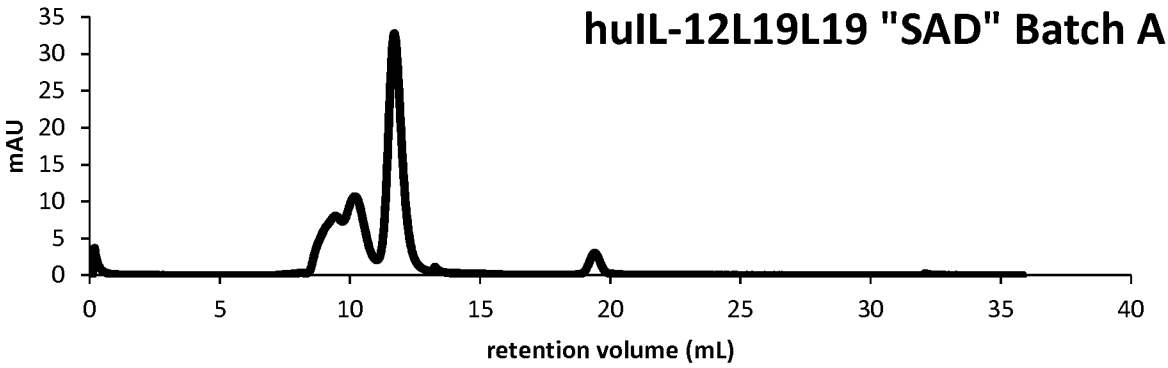


Fig. 8

A


huIL-12L19L19 "SAD" Batch A
mAU
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
40
retention volume (mL)

B

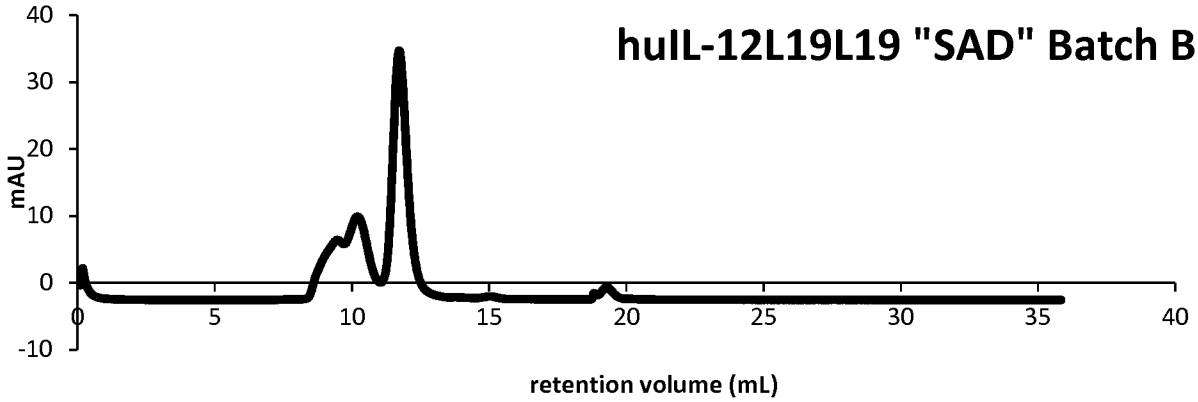
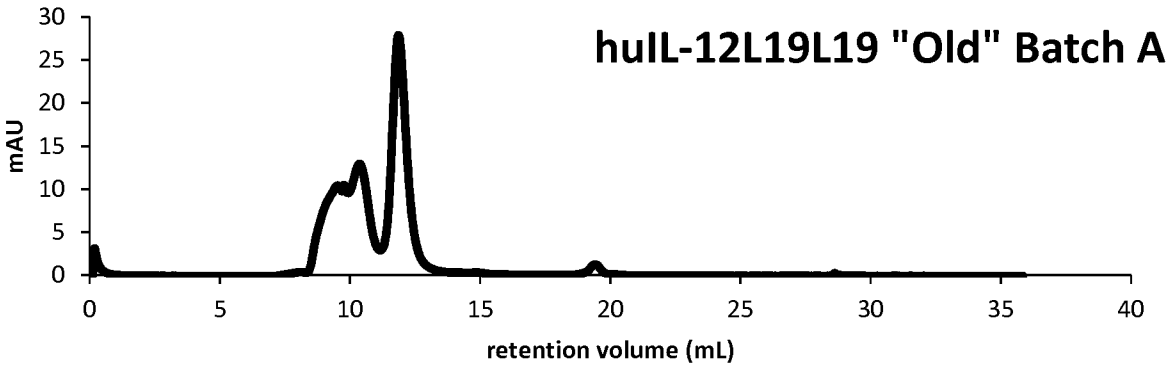

huIL-12L19L19 "SAD" Batch B
mAU
40
30
20
10
0
-10
0
5
10
15
20
25
30
35
40
retention volume (mL)

Fig. 9

C


huIL-12L19L19 "Old" Batch A
mAU
0
5
10
15
20
25
30
0
5
10
15
20
25
30
35
40
retention volume (mL)

D

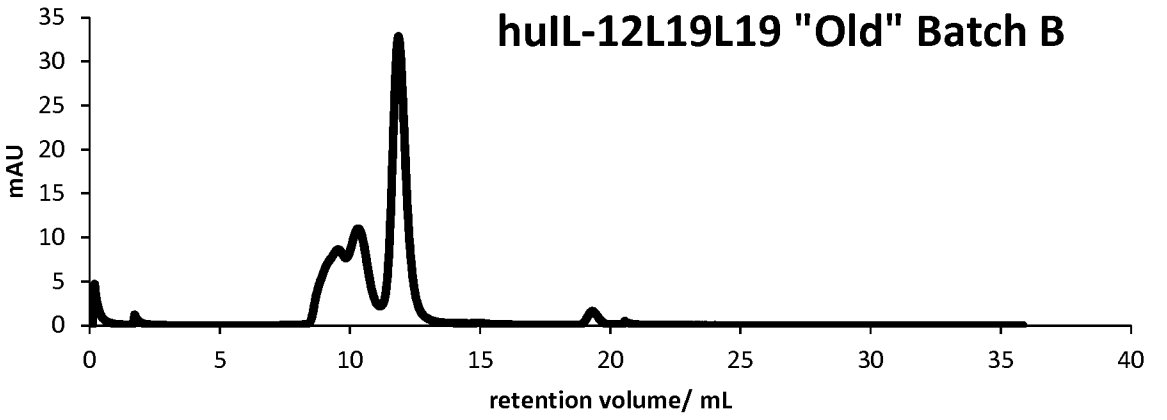

huIL-12L19L19 "Old" Batch B
mAU
0
5
10
15
20
25
30
35
0
5
10
15
20
25
30
35
40
retention volume/ mL

Fig. 9 ctd‘

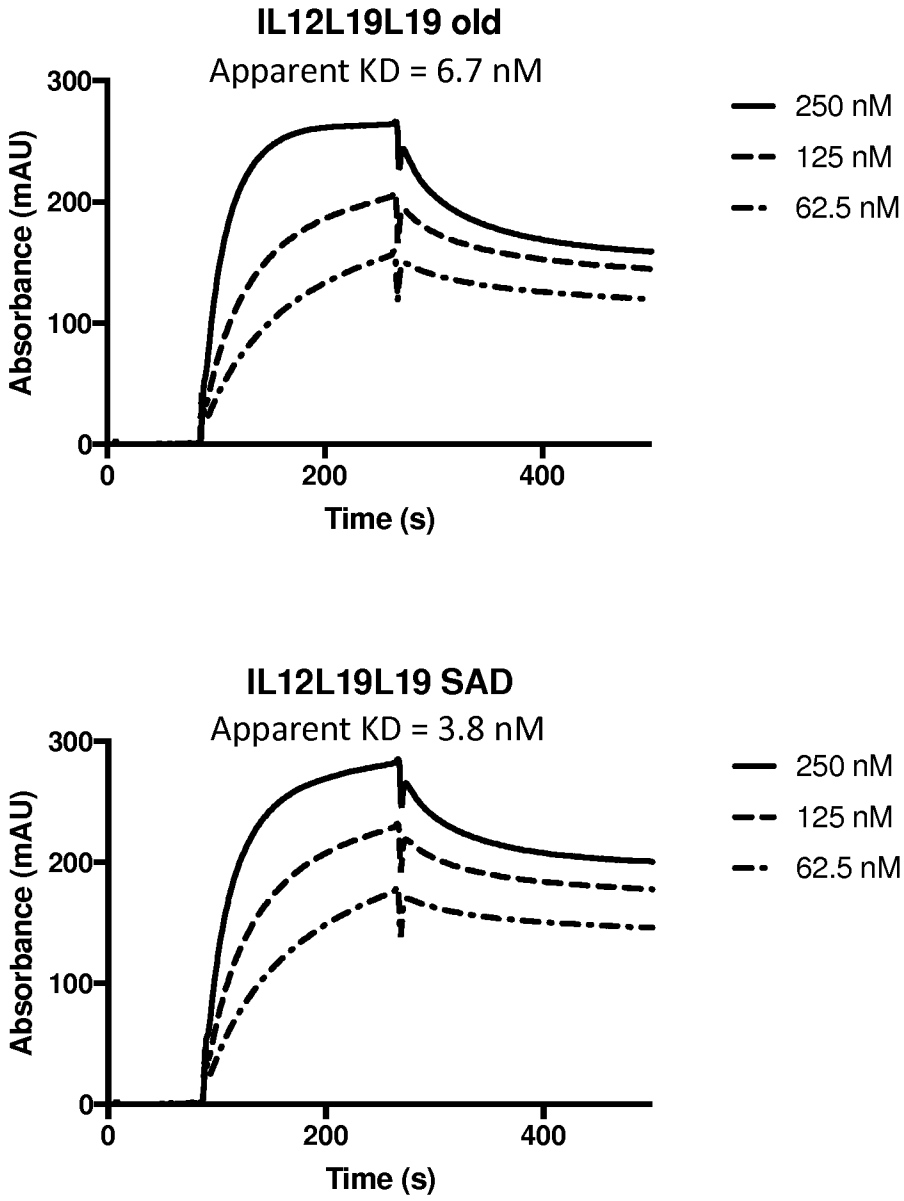
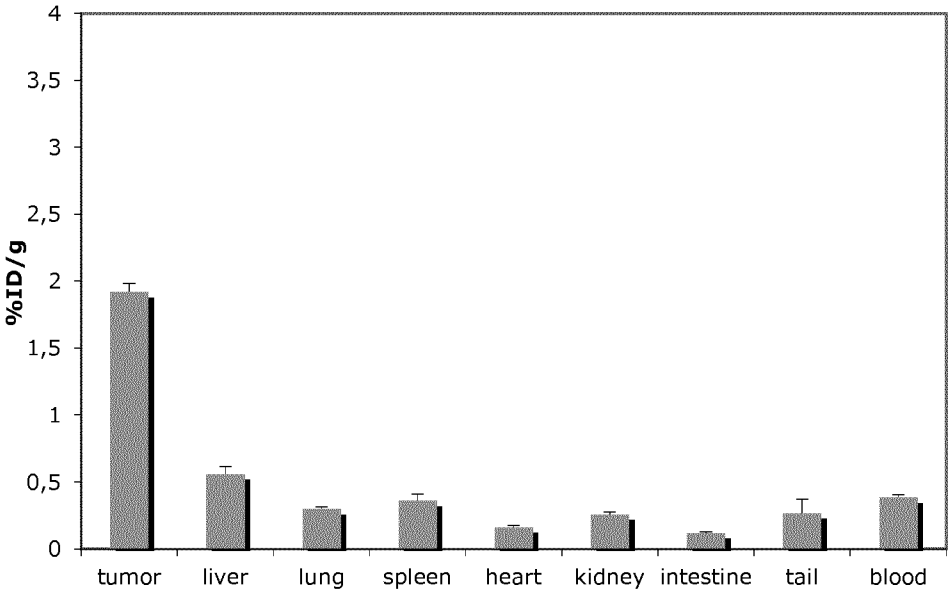

IL12L19L19 old
Apparent KD = 6.7 nM
Absorbance (mAU)
300
200
100
0
0
200
400
Time (s)
250 nM
125 nM
62.5 nM
IL12L19L19 SAD
Apparent KD = 3.8 nM
Absorbance (mAU)
300
200
100
0
0
200
400
Time (s)
250 nM
125 nM
62.5 nM

Fig. 10

24 Hours tumor targeting
with «Old» variant



24 Hours tumor targeting
with «SAD» variant

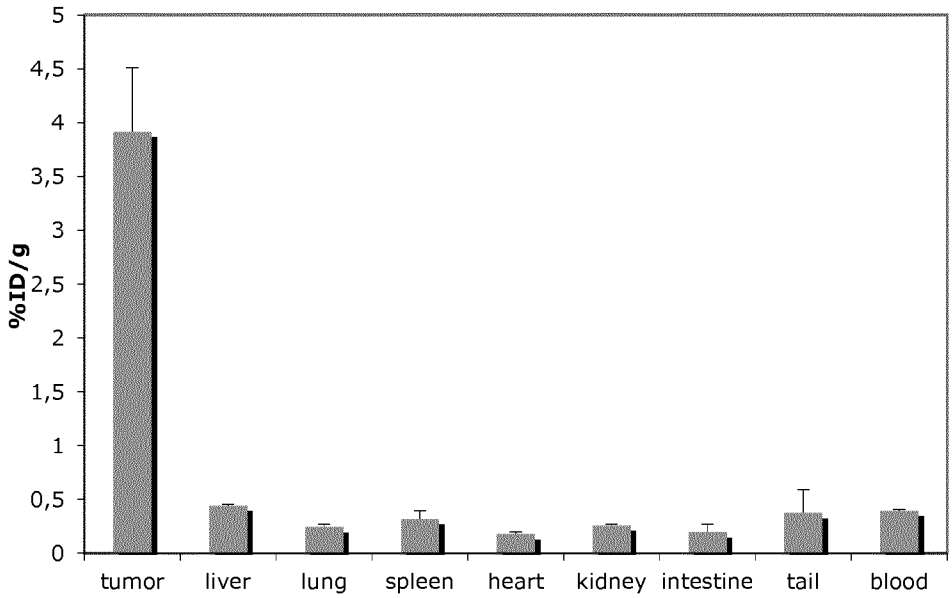


Fig.11

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/053136

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/68 A61P35/00
ADD. C07K14/54 C07K16/18

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, COMPENDEX, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/014149 A1 (PHILOGEN SPA [IT]; WULHFARD SARAH [CH]) 31 January 2013 (2013-01-31) cited in the application	1-20, 40-46
Y	page 3, lines 1-3,26-31 page 5, lines 19-25,31-34 page 10, lines 19-24 page 13, lines 3-16,30-34 page 14, lines 3-6 page 15, lines 4-11; sequence 20 page 15, lines 23-35 page 16, line 25 - page 18, line 18 page 19, lines 9-17 figures 1a,2,7 sequence 8 claims ----- -/--	21-39

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2019	17/05/2019

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Villard, Anne-Laure

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GAFNER V ET AL: "An engineered antibody-interleukin-12 fusion protein with enhanced tumor vascular targeting properties", INTERNATIONAL JOURNAL OF CANCER, JOHN WILEY & SONS, INC, US, vol. 119, no. 9, 1 November 2006 (2006-11-01), pages 2205-2212, XP002405179, ISSN: 0020-7136, DOI: 10.1002/IJC.22101	1-20, 40-46
Y	abstract figure 3 page 2211, right-hand column, paragraph 2	21-39

X	WO 2006/119897 A2 (PHILOGEN SPA [IT]; NERI DARIO [CH]; GAFNER VERENA [CH]; HALIN CORNELIA) 16 November 2006 (2006-11-16) cited in the application	1-20, 40-46
Y	page 3, lines 13-24 page 5, line 10; sequence 15 page 16, line 22 - page 17, line 5 page 19, lines 1-29 page 22, lines 5-13 page 25, lines 23-30 figures 1,5,7	21-39

X	WO 01/62298 A2 (PHILOGEN SRL [IT]; ZARDI LUCIANO [IT] ET AL.) 30 August 2001 (2001-08-30) page 6, line 27 - page 8, line 2 page 13, lines 6-14 page 17, line 22 - page 18, line 4 page 18, lines 9-14 page 19, lines 15-19 examples 4,5	1-20, 40-46

X	WO 2011/020783 A2 (ROCHE GLYCART AG [CH]; HOSSE RALF [CH] ET AL.) 24 February 2011 (2011-02-24) page 18, lines 29-32 page 34, lines 1-5 page 50, lines 28-32 pages 72-73; sequences 104,105 page 128, lines 24-33 figures 20(A),20(B)	1-3, 6-20, 40-46

	-/--	

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	TIZIANO ONGARO ET AL: "A novel anti-cancer L19-interleukin-12 fusion protein with an optimized peptide linker efficiently localizes in vivo at the site of tumors", JOURNAL OF BIOTECHNOLOGY, vol. 291, 1 February 2019 (2019-02-01), pages 17-25, XP055584521, AMSTERDAM, NL ISSN: 0168-1656, DOI: 10.1016/j.jbiotec.2018.12.004 the whole document -----	1-46
A,P	WO 2018/153865 A1 (PHILOGEN SPA [IT]) 30 August 2018 (2018-08-30) figure 1 -----	1-46

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/053136

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013014149 A1	31-01-2013	CA 2842053 A1	31-01-2013
		EP 2736535 A1	04-06-2014
		EP 3437662 A1	06-02-2019
		ES 2706428 T3	28-03-2019
		US 2014170109 A1	19-06-2014
		WO 2013014149 A1	31-01-2013
WO 2006119897 A2	16-11-2006	AT 459648 T	15-03-2010
		AU 2006246061 A1	16-11-2006
		BR PI0609100 A2	17-02-2010
		CA 2607954 A1	16-11-2006
		CN 101258162 A	03-09-2008
		EA 200702460 A1	30-06-2008
		EP 1891102 A2	27-02-2008
		ES 2341126 T3	15-06-2010
		HK 1113579 A1	30-04-2010
		JP 2008543278 A	04-12-2008
		KR 20080009161 A	24-01-2008
		US 2009035255 A1	05-02-2009
		WO 2006119897 A2	16-11-2006
WO 0162298 A2	30-08-2001	AT 333900 T	15-08-2006
		AT 526039 T	15-10-2011
		AU 3947001 A	03-09-2001
		CA 2399866 A1	30-08-2001
		CY 1112168 T1	09-12-2015
		DE 60121733 T2	09-08-2007
		DK 1257297 T3	20-11-2006
		DK 1719528 T3	09-01-2012
		EP 1257297 A2	20-11-2002
		EP 1719528 A2	08-11-2006
		ES 2269361 T3	01-04-2007
		ES 2373966 T3	10-02-2012
		JP 4917232 B2	18-04-2012
		JP 2003523406 A	05-08-2003
		JP 2012036216 A	23-02-2012
		PT 1257297 E	29-12-2006
		PT 1719528 E	06-01-2012
		US 2004013640 A1	22-01-2004
		US 2010316602 A1	16-12-2010
		US 2014348784 A1	27-11-2014
		WO 0162298 A2	30-08-2001
WO 2011020783 A2	24-02-2011	AR 077879 A1	28-09-2011
		AU 2010285071 A1	02-02-2012
		CA 2769619 A1	24-02-2011
		CN 102573922 A	11-07-2012
		CO 6430436 A2	30-04-2012
		CR 20120042 A	22-03-2012
		EC SP12011685 A	30-03-2012
		EP 2467165 A2	27-06-2012
		ES 2534085 T3	17-04-2015
		HK 1169318 A1	15-07-2016
		JP 5764127 B2	12-08-2015
		JP 2013502209 A	24-01-2013
		KR 20120053042 A	24-05-2012
		KR 20150088328 A	31-07-2015
		MA 33717 B1	01-11-2012

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/053136

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
		PE 06302012 A1	26-05-2012
		RU 2012110127 A	27-09-2013
		SG 178443 A1	27-04-2012
		TW 201119672 A	16-06-2011
		US 2011064751 A1	17-03-2011
		WO 2011020783 A2	24-02-2011
		ZA 201200767 B	30-07-2014
WO 2018153865 A1	30-08-2018	NONE	