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(54) Title: HUMAN SERUM ALBUMIN BINDING COMPOUNDS AND FUSION PROTEINS THEREOF

(57) Abstract: The present invention relates to a polypeptide binding to human serum albumin and comprising or consisting of an amino acid sequence selected from the group consisting of: (a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 1), wherein (X^1) is A, V, I, L, M, G, P, S, T, N, Q, C, R, H, K, D or E; (X^2) is R, H, K, A, V, I, L, M, G, P, S, T, N, Q or C; (X^3) is R, H, K, S, T, N, Q, C, F, Y, W, A, V, I, L, M, G or P; (X^4) is S, T, N, Q, C, A, V, I, L, M, G, P, R, H, K, F, Y, W, D or E; (X^5) is S, T, N, Q, C, D, E, F, Y, W, A, V, I, L, M, G, P, R, H or K; (X^6) is F, Y, W, A, V, I, L, M, G, P, R, H, K, S, T, N, Q or C; (X^7) is A, V, I, L, M, G, P, R, H or K; (X^8) is S, T, N, Q, C, D or E; (X^9) is S, T, N, Q, C, D, E, A, V, I, L, M, G, P, F, Y or W; (X^{10}) is A, V, I, L, M, G or P; (X^{11}) is F, Y, W, R, H or K; (X^{12}) is S, T, N, Q, C, F, Y or W; (X^{13}) is F, Y, W, R, H, K, S, T, N, Q, C, D, E, A, V, I, L, M, G or P; (X^{14}) is F, Y, W, A, V, I, L, M, G or P; (X^{15}) is D, E, A, V, I, L, M, G or P; (X^{16}) is A, V, I, L, M, G or P; (X^{17}) is D, E, A, V, I, L, M, G, P, R, H or K; (X^{18}) is S, T, N, Q, C, A, V, I, L, M, G or P; (X^{19}) is F, Y, W, S, T, N, Q or C; and (b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

HUMAN SERUM ALBUMIN BINDING COMPOUNDS AND FUSION PROTEINS THEREOF

The present invention relates to a polypeptide binding to human serum albumin and comprising or consisting of an amino acid sequence selected from the group consisting of: (a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 1), wherein (X^1) is A, V, I, L, M, G, P, S, T, N, Q, C, R, H, K, D or E; (X^2) is R, H, K, A, V, I, L, M, G, P, S, T, N, Q or C; (X^3) is R, H, K, S, T, N, Q, C, F, Y, W, A, V, I, L, M, G or P; (X^4) is S, T, N, Q, C, A, V, I, L, M, G, P, R, H, K, F, Y, W, D or E; (X^5) is S, T, N, Q, C, D, E, F, Y, W, A, V, I, L, M, G, P, R, H or K; (X^6) is F, Y, W, A, V, I, L, M, G, P, R, H, K, S, T, N, Q or C; (X^7) is A, V, I, L, M, G, P, R, H or K; (X^8) is S, T, N, Q, C, D or E; (X^9) is S, T, N, Q, C, D, E, A, V, I, L, M, G, P, F, Y or W; (X^{10}) is A, V, I, L, M, G or P; (X^{11}) is F, Y, W, R, H or K; (X^{12}) is S, T, N, Q, C, F, Y or W; (X^{13}) is F, Y, W, R, H, K, S, T, N, Q, C, D, E, A, V, I, L, M, G or P; (X^{14}) is F, Y, W, A, V, I, L, M, G or P; (X^{15}) is D, E, A, V, I, L, M, G or P; (X^{16}) is A, V, I, L, M, G or P; (X^{17}) is D, E, A, V, I, L, M, G, P, R, H or K; (X^{18}) is S, T, N, Q, C, A, V, I, L, M, G or P; (X^{19}) is F, Y, W, S, T, N, Q or C; and (b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

In this specification, a number of documents including patent applications and manufacturer's manuals are cited. The disclosure of these documents, while not considered relevant for the patentability of this invention, is herewith incorporated by reference in its entirety. More specifically, all referenced documents are incorporated by reference to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference.

Many therapeutics and diagnostics, in particular biologicals (i.e. peptide or polypeptide drugs, polynucleotides, etc.) suffer from inadequate serum half-lives *in vivo.* This necessitates the administration of such therapeutics at constant infusions, or high frequencies and/or high doses, or the use of sustained release formulations, in order to maintain the serum levels necessary for therapeutic effects. Frequent systemic administration of drugs is associated with considerable negative side effects. For example, frequent, e.g. daily, systemic injections represent a considerable discomfort to the subject, and pose a high risk of administration related infections, and may require hospitalization or frequent visits to the hospital, in particular when the therapeutic is to be administered intravenously. Moreover, in long term treatments daily intravenous injections can also lead to considerable side effects of tissue scarring and vascular pathologies caused by the repeated puncturing of vessels. Similar problems are known for all frequent systemic administrations of therapeutics, like, for example, the administration of insulin to diabetics, or interferon drugs in patients suffering from multiple sclerosis. All these factors lead to a decreased patient compliance and increased costs for the health system.

One avenue of prolonging the *in vivo* half-life of otherwise short-lived protein therapeutics and molecules represents the fusion of these compounds to polypeptides that bind to human serum albumin. Polypeptides that are capable of binding to serum albumin and uses thereof in polypeptide constructs in order to increase the half-life of therapeutically relevant proteins and polypeptides are known in the art (Dennis MS et al (2002) *J Biol Chem,* 277(38) p. 35035-35043; Jonsson A. et al., (2008) *Protein Eng Des Sel,* 21(8), p. 515-527). Moreover, WO 91/01743, WO 01/45746 and WO 02/076489 describe peptide moieties binding to serum albumin that can be fused to therapeutic proteins and other therapeutic compounds and entities in order to increase the half-life thereof. However, these peptide moieties are of bacterial or synthetic origin, which is less preferred for use in therapeutics. Other binding domains to albumin comprise Nanobodies® (derived from camelids) (WO 04/041865) and single domain antibodies (derived from human antibodies) (Holt L. J. et al. (2008) *Protein Eng Des Sel,* 21, p. 283-288 and Walker, A. et al. (2010) *Protein Eng Des Sel* 23, p. 271-278).

One avenue of obtaining high affinity and specific binding proteins to serum albumin with desired pharmacokinetic and pharmacodynamic properties, represents the use of antibody and alternative binding technologies (the latter termed "scaffolds"). These non-immunoglobulin-derived binding reagents are known in the art and are collectively designated "scaffolds" (Skerra A. (2000) J. Mol. Recognit. 13, 167-187). More than 50 different protein scaffolds have been proposed over the past 10 to 15 years, the most advanced approaches in this field being (as summarized in Gebauer M and Skerra A. (2009) Curr Opinion in Chemical Biology 13:245-255): affibodies® (based on the Z- domain of staphylococcal protein A), Kunitz type domains, adnectins (based on the 10th domain of human fibronectin), anticalins® (derived from lipocalins), DARPins (derived from ankyrin repeat proteins), avimers (based on multimerized LDLR-A), and Fynomers® (Bertschinger J. et al., (2007), Protein Eng Des Sel, **20**(2), p. 57-68 and EP 2054432), which are derived from the human Fyn SH3 domain.

As is evident from the above, there is an ongoing need for further means to prolong the *in vivo* half-life of pharmaceutically and/or diagnostically active proteins or peptides. This need is addressed by the present invention.

Accordingly the present invention relates in a first embodiment to a polypeptide binding to human serum albumin and comprising or consisting of an amino acid sequence selected from the group consisting of:

(a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 1), wherein (X^1) is A, V, I, L, M, G, P, S, T, N, Q, C, R, H, K, D or E; (X^2) is R, H, K, A, V, I, L, M, G, P, S, T, N, Q or C; (X^3) is R, H, K, S, T, N, Q, C, F, Y, W, A, V, I, L, M, G or P; (X^4) is S, T, N, Q, C, A, V, I, L, M, G, P, R, H, K, F, Y, W, D or E; (X^5) is S, T, N, Q, C, D, E, F, Y, W, A, V, I, L, M, G, P, R, H or K; (X^6) is F, Y, W, A, V, I, L, M, G, P, R, H, K, S, T, N, Q or C; (X^7) is A, V, I, L, M, G, P, R, H or K; (X^8) is S, T, N, Q, C, D or E; (X^9) is S, T, N, Q, C, D, E, A, V, I, L, M, G, P, F, Y or W; (X^{10}) is A, V, I, L, M, G or P; (X^{11}) is F, Y, W, R, H or K; (X^{12}) is S, T, N, Q, C, F, Y or W; (X^{13}) is F, Y, W, R, H, K, S, T, N, Q, C, D, E, A, V, I, L, M, G or P; (X^{14}) is F, Y, W, A, V, I, L, M, G or P; (X^{15}) is D, E, A, V, I, L, M, G or P; (X^{16}) is A, V, I, L, M, G or P; (X^{17}) is D, E, A, V, I,

L, M, G, P, R, H or K; (X^{18}) is S, T, N, Q, C, A, V, I, L, M, G or P; (X^{19}) is F, Y, W, S, T, N, Q or C; and (b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

According to a preferred embodiment of the invention the polypeptide comprises or consists of an amino acid sequence selected from the group consisting of: (a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 2), wherein (X^1) is V, T, L, H, Q, E, R, G or M; (X^2) is S, A, R; K, N, M, L or T; (X^3) is N, S, R, H, Q, F, M, K or V; (X^4) is T, Y, N, L, H, R, Q, W, M or E; (X^5) is G, S, A, E, W, L, R or K; (X^6) is F, Y P, W, R or Q; (X^7) is L or R; (X^8) is Q or D; (X^9) is N, D, A, Q, W, M or S; (X^{10}) is L, I or V; (X^{11}) is W or R; (X^{12}) is T or F; (X^{13}) is D, W, N, K, S, E or A; (X^{14}) is W or G; (X^{15}) is E or V; (X^{16}) is A or V; (X^{17}) is E, L or R; (X^{18}) is T, S or M; (X^{19}) is Y or S; and (b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

The term "polypeptide" as used herein describes linear molecular chains of amino acids, including single chain proteins or their fragments, containing more than about 50 amino acids. Polypeptides may further form oligomers consisting of at least two identical or different molecules. The corresponding higher order structures of such multimers are, correspondingly, termed homo- or heterodimers, homo- or heterotrimers etc. Homodimers, trimers etc. also fall under the definition of the term "polypeptide". Furthermore, peptidomimetics of such polypeptides where amino acid(s) and/or peptide bond(s) have been replaced by functional analogs are also encompassed by the invention. Such functional analogues include all known amino acids other than the 20 gene-encoded amino acids, such as selenocysteine. The term "polypeptide" also refers to naturally modified polypeptides where the modification is effected e.g. by glycosylation, acetylation, phosphorylation and similar modifications which are well-known in the art.

Also comprised by the present invention are fragments of the polypeptide of the invention which substantially retain binding to a human serum albumin. In this regard it is preferred with increasing preference that the fragments (which contain in any case less than 50 amino acids) comprise at least 30 amino acids, at least 35 amino acids, at least 40 amino acids, or at least 45 amino acids. It is moreover preferred that in the fragment the amino acid positions corresponding to the RT- and n-Src-loop and the amino acids which are adjacent (e.g. 1, 2, 3, 4, 5, or 6 adjacent amino acid positions) to these loops as defined herein below are retained.

The term "binding to a human serum albumin" requires that the polypeptides of the invention have a specific (*in vivo* and/or *in vitro*) binding affinity to a human serum albumin, and more preferably to the human serum albumin of SEQ ID NO: 46. With increasing preference such polypeptides have a dissociation constant (K_D) to human serum albumin of less than 5000nM, less than 2500nM, and less than 1500nM. Also such polypeptides have with increasing preference a dissociation constant (K_D) to

any protein other than mammalian serum albumins of greater than 50000nM, greater than 100000nM, and greater than 500000nM.

As is evident from the examples herein below, binding can be detected using well-known experiments in the art, such as enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance (SPR) experiments. Such assays may preferably be performed as outlined in the examples herein below.

In accordance with the present invention, the term "percent (%) sequence identity" describes the number of matches ("hits") of identical nucleotides/amino acids of two or more aligned nucleic acid or amino acid sequences as compared to the number of nucleotides or amino acid residues making up the overall length of the template nucleic acid or amino acid sequences. In other terms, using an alignment, for two or more sequences or subsequences the percentage of amino acid residues or nucleotides that are the same (e.g. 90% or 95% identity) may be determined, when the (sub)sequences are compared and aligned for maximum correspondence over a window of comparison, or over a designated region as measured using a sequence comparison algorithm as known in the art, or when manually aligned and visually inspected. The sequences which are compared to determine sequence identity may thus differ by substitution(s), addition(s) or deletion(s) of nucleotides or amino acids. This definition also applies to the complement of a test sequence.

The skilled person is also aware of suitable programs to align nucleic acid sequences. The percentage sequence identity of polypeptide sequences can, for example, be determined with programmes as the above explained programmes CLUSTALW, FASTA and BLAST. Preferably the BLAST programme is used, namely the NCBI BLAST algorithm (Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402).

With regard to the sequence identity as recited in item (b) herein, it is preferred with increasing preference that the sequence identity is at least 95% or at least 98%.

The phrase "the identity determination excludes amino acid positions (X^1) to (X^{19})" as used herein specifies that the calculation of the sequence identity with regard to SEQ ID NOs 1 and 2 (or SEQ ID NOs 41, 42 and 49) does not take into a account amino acid positions (X^1) to (X^{19}) but is confined to the remainder of the amino acids positions of SEQ ID NO: 1 or 2 (or SEQ ID NOs 41, 42 or 49).

It is preferred with regard to the present invention that the sequence calculation according to item (b) above, further excludes the amino acid position D between (X^6) and (X^7) and the amino acid position W between (X^{14}) and (X^{15}), because these amino acids positions are within the RT and the n-Src loop or adjacent to these loops.

The term "serum albumin" (also referred to in the art simply as albumin) refers to a globular protein that in humans is encoded by the *ALB* gene. Serum albumin is the most abundant plasma protein in mammals. Serum albumin is essential for maintaining the oncotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues. It also acts as a plasma carrier by non-specifically binding several hydrophobic steroid hormones and as a transport protein for hemin and fatty acids. Furthermore, serum albumin has a very long half-life of about 19 days, and its metabolism is well-known. Albumin has also been widely used as a protein stabilizer in commercial pharmaceuticals (Sangastino et al. (2012), Blood, 120(12)2405-2411).

SEQ ID NOs 1 and 2 as recited herein above are derived from the amino acid sequence of the SH3 domain of the human Fyn kinase (SEQ ID NO: 3). In more detail, SEQ ID NOs: 4 to 40 are derivatives of the human Fyn kinase (SEQ ID NO: 3) and SEQ ID NO: 2 is a sequence resulting from an alignment of SEQ ID NOs: 4 to 40 (cf. Figure 8). The sequence alignment of SEQ ID NOs 4 to 40 in Figure 8 and the examples herein below show that the amino acids listed for amino acid positions (X^1) to (X^{19}) within the context of SEQ ID NO: 2 confer binding specificity to human serum albumin, in particular to the human serum albumin having SEQ ID NO: 46. In more detail, the sequence alignment of SEQ ID NOs 4 to 40 of the invention in Figure 8 shows that amino acids positions (X^1) to (X^{19}) within the context of SEQ ID NO: 2 can be selected from (X^1) being V, T, L, H, Q, E, R, G or M; (X^2) being S, A, R; K, N, M, L or T; (X^3) being N, S, R, H, Q, F, M, K or V; (X^4) being T, Y, N, L, H, R, Q, W, M or E; (X^5) being G, S, A, E, W, L, R or K; (X^6) being F, Y P, W, R or Q; (X^7) being L or R; (X^8) being Q or D; (X^9) being N, D, A, Q, W, M or S; (X^{10}) being L, I or V; (X^{11}) being W or R; (X^{12}) being T or F; (X^{13}) being D, W, N, K, S, E or A; (X^{14}) being W or G; (X^{15}) being E or V; (X^{16}) being A or V; (X^{17}) being E, L or R; (X^{18}) being T, S or M; (X^{19}) being Y or S. Therefore, it can be expected that all possible combinations of amino acids listed for amino acid positions (X^1) to (X^{19}) in SEQ ID NO: 2 confer binding specificity to human serum albumin.

The further amino acids listed for the variant amino acid positions (X^1) to (X^{19}) within SEQ ID NO: 1 of the invention are conservative substitutions as compared to the concrete amino acids found in SEQ ID NOs 4 to 40 at positions (X^1) to (X^{19}). As further detailed below, a conservative substitution designates the substitution of an amino acid with another amino acid whose side chain has a similar biochemical property. Thus, conservative amino acid substitutions are expected not to change the overall binding properties of the polypeptide of the invention.

For example, in accordance with feature (b) herein, in the polypeptide of SEQ ID NO: 1 or 2 (or SEQ ID NOs 41, 42 or 49) additional amino acid positions outside the RT- and/or n-Src-loop of SH3 domain of the Fyn kinase and outside the amino acid positions adjacent thereto outside amino acid positions (X^1) to (X^{19}) may be exchanged or deleted, or further amino acids may be added, without substantially interfering with the binding specificity to human serum albumin. If amino acids are exchanged, conservative exchanges are preferred.

A conservative substitution comprises the substitution of an amino acid with another amino acid having a chemical property similar to the amino acid that is substituted. Preferably, the conservative substitution is a substitution selected from the group consisting of: (i) a substitution of a basic amino acid with a different basic amino acid; (ii) a substitution of an acidic amino acid with a different acidic amino acid; (iii) a substitution of an aromatic amino acid with a different aromatic amino acid; (iv) a substitution of a non-polar, aliphatic amino acid with a different non-polar, aliphatic amino acid; and (v) a substitution of a polar, uncharged amino acid with a different polar, uncharged amino acid. A basic amino acid is selected from the group consisting of arginine, histidine, and lysine. An acidic amino acid is selected from aspartate or glutamate. An aromatic amino acid is selected from the group consisting of phenylalanine, tyrosine and tryptophane. A non-polar, aliphatic amino acid is selected from the group consisting of glycine, alanine, valine, leucine, methionine, isoleucine and proline. A polar, uncharged amino acid is selected from the group consisting of serine, threonine, cysteine, asparagine and glutamine. In contrast to a conservative amino acid substitution, a non-conservative amino acid substitution is the exchange of one amino acid with any amino acid that does not fall under the above-outlined conservative substitutions (i) through (v).

Thus, also encompassed by the invention are amino acid sequences which are with increasing preference at least 90%, at least 95%, or at least 98% identical to the amino acid sequence of SEQ ID NO: 1 or 2 (or SEQ ID NOs 41, 42 or 49), wherein (X^1) to (X^{19}) are selected from the distinct amino acids shown above, or an amino acid sequence which is with increasing preference at least 90%, at least 95%, or at least 98% identical to the amino acid sequence of SEQ ID NO: 1 or 2 (or SEQ ID NOs 41, 42 or 49), wherein (X^1) to (X^{19}) are selected from the distinct amino acids shown above, and wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

SEQ ID NO: 3 is the amino acid sequence of the SH3 domain of the human Fyn kinase (aa 83-145 of Fyn kinase as reported by Kawakami et al. and Semba et al. in 1986). The amino acid sequence of Fyn SH3 is fully conserved among man, mouse, rat and monkey (gibbon). Chicken Fyn SH3 differs in one, the one of *Xenopus laevis* in two amino acid positions from the corresponding human domain. Just as other SH3 domains the Fyn SH3 is composed of two antiparallel β-sheets and contains two flexible loops (called RT and n-Src-loops) in order to interact with other proteins. In general, SH3 domains are present in a large variety of proteins participating in cellular signal transduction (Musacchio et al. (1994) Prog. Biophys. Mol. Biol. 61; 283-297). These domains do not occupy a fixed position within proteins and can be expressed and purified independently. More than 1000 occurrences of the domain are known with about 300 human SH3 domains (Musacchio A. (2003) Advances in Protein Chemistry. 61; 211-268). Although there is great sequence diversity among SH3 domains, they all share a conserved fold: a compact beta barrel formed by two anti-parallel beta-sheets (Musacchio A. (2003) Advances in Protein Chemistry. 61; 211-268). Typically, SH3 domains bind to proline-rich peptides containing a PXXP core-binding motif (Ren et al. (1993) Science 259; 1157-1161), but examples of unconventional SH3 binding sites have also been described (Karkkainen et al. (2006) EMBO Rep. 7;186-191). Most of the SH3 domains sequenced so far have an overall length of approximately 60 to 65 amino acids, but

some of them may feature as many as 85 amino acids due to inserts into the loops connecting the main conservative elements of the secondary structure (Koyama et al. (1993) Cell 72(6); 945-952). An alignment of different SH3 domains revealed conserved amino acid residues responsible for the proper structure formation as well as for the canonical proline-rich motif recognition (Larson et al. (2000) Protein Science 9; 2170- 2180). SEQ ID NO: 3 reads:

GVTLFVALYDYEARTEDDLSFHKGEKFQILNSSEGDWWEARSLTTGETGYIPSNYVAPVDSIQ (SEQ ID NO: 3)

In SEQ ID NO: 3 as shown above, the sequences of the RT and the n-Src loop are underlined and double-underlined, respectively. Erpel et al. ("Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions." Embo J. 14(5): 963-75,1995) investigated the influence of mutations in the RT and n-Src loops of Src SH3 domains and demonstrated that mutations in both loops which are adjacent to the hydrophobic surface could influence the ability of this domain to participate in inter- and intramolecular associations with naturally-occurring SH3-ligands. Moreover, EP 2054432 shows that mutations in and adjacent to the RT- and/or the n-Src loop determine the binding specificity of an SH3 domain. In more detail, it was demonstrated that the Fyn SH3 domain is an attractive scaffold ("Fynomer®") for the generation of binding proteins because it (i) can be expressed in bacteria in soluble form in high amounts, (ii) is monomeric and does not aggregate when stored in solution, (iii) is very stable (Tm 70.5 °C), (iv) lacks cysteine residues, and (v) is of human origin featuring an amino acid sequence completely conserved from mouse to man and, hence, are essentially non- immunogenic (Grabulovski et al. (2007) JBC, 282, p. 3196-3204; EP 2054432). The term "Fyn SH3-derived polypeptide", used interchangeably herein with the term "Fynomer®", refers in general to a non-immunoglobulin-derived binding (poly)peptide (e.g. a so-called scaffold as described above) derived from the human Fyn SH3 domain.

In the past, the isolation of Fynomers® binding to mouse serum albumin have been described (Bertschinger J. et al., (2007), Protein Eng Des Sel, **20**(2), p. 57-68 and EP 2054432). However, to the best knowledge of the inventors, so far no Fynomer®-based binders have been described in the art which bind to human serum albumin and not to unrelated proteins, such as ovalbumin (see Example 4). In addition, to the best knowledge of the inventors, so far no Fynomer®-based binders have been described in the art which bind to human serum albumin and rodent serum albumin and not to unrelated proteins, such as ovalbumin (see Example 4).

Thus, in another preferred embodiment the polypeptide of the invention further binds to rodent serum albumin and comprises or consists of an amino acid sequence selected from the group consisting of: (a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 41), wherein (X^1) is A, V, I, L, M, G, P, S, T, N, Q, C, R, H, K, D or E; (X^2) is R, H, K, A, V, I, L, M, G, P, S, T, N, Q or C; (X^3) is R, H, K, S, T, N, Q, C, F, Y, W, A, V, I, L, M, G or P; (X^4) is S, T, N, Q, C, A, V, I, L, M, G, P, R, H, K, F, Y or W; (X^5) is S, T, N,

Q, C, D, E, F, Y, W, A, V, I, L, M, G, P, R, H or K; (X^6) is F, Y, W, A, V, I, L, M, G, P, R, H, K, S, T, N, Q or C; (X^7) is A, V, I, L, M, G, P, R, H or K; (X^8) is S, T, N, Q, C, D or E; (X^9) is S, T, N, Q, C, D or E; (X^{10}) is A, V, I, L, M, G or P; (X^{11}) is F, Y or W; (X^{12}) is S, T, N, Q or C; (X^{13}) is F, Y, W, R, H, K, S, T, N, Q, C, D, E, A, V, I, L, M, G or P; (X^{14}) is F, Y, W, A, V, I, L, M, G or P; (X^{15}) is D, E, A, V, I, L, M, G or P; (X^{16}) is A, V, I, L, M, G or P; (X^{17}) is D, E, A, V, I, L, M, G, P, R, H or K; (X^{18}) is S, T, N, Q, C, A, V, I, L, M, G or P; (X^{19}) is F, Y, W, S, T, N, Q or C; and (b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

According to a more preferred embodiment the polypeptide of the invention further binds to rodent serum albumin and comprises or consists of an amino acid sequence selected from the group consisting of: (a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G (X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 42), wherein (X^1) is V, T, L, H, Q, E, R, G or M; (X^2) is S, A, R; K, N, M, L or T; (X^3) is N, S, R, H, Q, F, M, K or V; (X^4) is T, Y, N, L, H, R, Q, W or M; (X^5) is G, S, A, E, W, L or R; (X^6) is F, Y P, W, R or Q; (X^7) is L or R; (X^8) is Q or D; (X^9) is N or D; (X^{10}) is L or I; (X^{11}) is W; (X^{12}) is T; (X^{13}) is D, W, N, K, S, E or A; (X^{14}) is W or G; (X^{15}) is E or V; (X^{16}) is A or V; (X^{17}) is E, L or R; (X^{18}) is T, S or M; (X^{19}) is Y or S; and (b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

In accordance with this preferred and more preferred embodiment it is preferred that positions (X^8) to (X^{12}) are either "QDLWT" (SEQ ID NO: 43) or "QNLWT" (SEQ ID NO: 44) or "DDIWT" (SEQ ID NO: 45). Positions (X^8) to (X^{12}) correspond to the n-Src-loop of the Fyn SH3 domain of SEQ ID NO: 2. As it is evident from the sequence alignment of SEQ ID NOs 4 to 32 in Figure 8, all these polypeptides have a n-Src-loop of either "QDLWT" (SEQ ID NO: 43) or "QNLWT" (SEQ ID NO: 44) or "DDIWT" (SEQ ID NO: 45). Hence, in particular such n-Src-loop confers binding specificity to human and rodent serum albumin.

Non-limiting examples of rodents are mice, rats, squirrels, porcupines, beavers, guinea pigs, and hamsters. In another preferred embodiment the rodent is rat. In a further preferred embodiment the rodent is mouse. In a different preferred embodiment the rodent comprises mouse and rat.

According to an even more preferred embodiment the polypeptide of the invention further binds to rodent serum albumin and the amino acid sequence is selected from the amino acid sequences of SEQ ID NOs: 4 to 32.

The sequence alignment of SEQ ID NOs 4 to 32 of the invention in Figure 8 shows that amino acids positions (X^1) to (X^{19}) within the context of SEQ ID NO: 42 can be selected from (X^1) being V, T, L, H, Q, E, R, G or M; (X^2) being S, A, R; K, N, M, L or T; (X^3) being N, S, R, H, Q, F, M, K or V; (X^4) being T, Y,

N, L, H, R, Q, W or M; (X^5) being G, S, A, E, W, L or R; (X^6) being F, Y P, W, R or Q; (X^7) being L or R; (X^8) being Q or D; (X^9) being N or D; (X^{10}) being L or I; (X^{11}) being W; (X^{12}) being T; (X^{13}) being D, W, N, K, S, E or A; (X^{14}) being W or G; (X^{15}) being E or V; (X^{16}) being A or V; (X^{17}) being E, L or R; (X^{18}) being T, S or M; and (X^{19}) being Y or S. Therefore, it can be expected that all possible combinations of amino acids listed for amino acid positions (X^1) to (X^{19}) in SEQ ID NO: 42 confer binding specificity to human serum albumin as well as rodent serum albumin.

The further amino acids listed for the variant amino acid positions (X^1) to (X^{19}) within SEQ ID NO: 41 of the invention are conservative substitutions as compared to the concrete amino acids found in SEQ ID NOs 4 to 32 at positions (X^1) to (X^{19}). As further detailed above, a conservative substitution designates the substitution of an amino acid with another amino acid whose side chain has a similar biochemical property. Thus, conservative amino acid substitutions are expected not to change the overall binding properties of the polypeptide of the invention.

The term "binding to a rodent serum albumin" requires that the polypeptides of the invention have a specific (*in vivo* and/or *in vitro*) binding affinity to a rodent serum albumin, and more preferably the mouse serum albumin of SEQ ID NO: 47 and the rat serum albumin of SEQ ID NO: 48. With increasing preference such polypeptides have a dissociation constant (K_D) to rodent serum albumin of less than 5000nM, less than 2500nM, and less than 1500nM. Also such polypeptides have with increasing preference a dissociation constant (K_D) to any protein other than mammalian serum albumins of greater than 50000nM, greater than 100000nM, and greater than 500000nM.

As discussed herein above, binding can be detected using well-known experiments in the art, such as an enzyme-linked immunosorbent assay (ELISA) or surface plasmon resonance (SPR) experiments. Such assays may preferably be performed as outlined in the examples herein below.

All polypeptides of the invention bind to human serum albumin, which is a prerequisite for being suited as a therapeutic or diagnostic for human beings. Already a dissociation constant (K_D) to human serum albumin of less than 5000nM (5μM) is believed to be sufficient to ensure that the polypeptides upon administration effectively bind to human serum albumin *in vivo*. For example Holt et al. (2008), Protein Engineering, Design & Selection; 21(5)283–288, provides evidence that serum albumin serum binders having a K_D in the two-digit nanomolar range as well as the one-digit micromolar range increases the serum half-life of a protein to a similar extent. Polypeptides which are cross-reactive to human serum albumin as well as rodent serum albumin are particularly advantageous, because drug discovery is facilitated. In more detail, the polypeptide may be tested in well-established and widely used animal models such as mouse and rat as well as in humans. Human (SEQ ID NO: 46) and mouse serum albumin (SEQ ID NO: 47) share an amino acid sequence identity of above 70%.

The polypeptide of the invention may bind to human serum albumin and not bind to rodent serum albumin, and comprise or consist of an amino acid sequence selected from the group consisting of:

GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 49), wherein (X^1) is T, H or Q; (X^2) is S or A; (X^3) is R or H; (X^4) is T, L, H or E; (X^5) is G or K; (X^6) is F or Y; (X^7) is L; (X^8) is D; (X^9) is N, D, A, Q, W, M or S; (X^{10}) is L, I or V; (X^{11}) is R; (X^{12}) is F; (X^{13}) is D; (X^{14}) is W; (X^{15}) is E; (X^{16}) is A; (X^{17}) is E; (X^{18}) is T; (X^{19}) is Y; and (b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

Furthermore, the polypeptide of the invention may bind to human serum albumin and not bind to rodent serum albumin, and the amino acid sequence may be selected from the amino acid sequences of SEQ ID NOs: 33 to 40.

The sequence alignment of SEQ ID NOs 33 to 40 of the invention in Figure 8 shows that amino acids positions (X^1) to (X^{19}) within the context of SEQ ID NO: 49 can be selected from (X^1) being T, H or Q; (X^2) being S or A; (X^3) being R or H; (X^4) is T, L, H or E; (X^5) is G or K; (X^6) is F or Y; (X^7) is L; (X^8) is D; (X^9) is N, D, A, Q, W, M or S; (X^{10}) being L, I or V; (X^{11}) being R; (X^{12}) being F; (X^{13}) being D; (X^{14}) being W; (X^{15}) being E; (X^{16}) being A; (X^{17}) being E; (X^{18}) is T; and (X^{19}) is Y.

The term "does not bind to a rodent serum albumin" requires that the polypeptides of the invention do not have a specific (*in vivo* and/or *in vitro*) binding affinity to a rodent serum albumin and in particular to mouse serum albumin of SEQ ID NO: 47 and rat serum albumin of SEQ ID NO: 48. With increasing preference such polypeptides have a dissociation constant (K_D) to rodent serum albumin of greater than 50000nM, greater than 75000nM and greater than 100000nM. Also such polypeptides preferably have a dissociation constant (K_D) to any protein other than mammalian serum albumins of greater than 100000nM.

As mentioned herein above, binding can be detected using well-known experiments in the art, such as enzyme-linked as an immunosorbent assay (ELISA) or surface plasmon resonance (SPR) experiments. Such assays may preferably be performed as outlined in the examples herein below.

A further embodiment of the present invention relates to a fusion protein comprising the polypeptide of the invention fused to a pharmaceutically and/or diagnostically active protein or peptide.
In the fusion protein the polypeptide of the invention is intended to enhance the *in vivo* serum half-life of the pharmaceutically and/or diagnostically active protein or peptide. Hence, the pharmaceutically and/or diagnostically active protein or peptide is preferably a short-lived pharmaceutically and/or diagnostically active protein or peptide having per se with increasing preference a half-life of less than 5 days, less than 2 days, less than 1 day, less than 12 hours, less than 10 hours and less than 5 hours. For example, insulin only has a half-life of 4 to 6 min. As discussed above, serum albumin has a half-life of 19 days (Dennis et al. (2002), *J Biol Chem*, 277(38), p. 35035–35043).

The term "fusion protein" as used herein is in general terms directed to a polypeptide construct generated through the joining and expression of two or more genes which code for separate polypeptides. In other words, translation of this fusion gene results in a single polypeptide with functional properties derived from each of the original polypeptides. The polypeptides may either be directly fused or via a linker, e.g. a short peptide sequence. In general, fusion proteins are generated artificially by recombinant DNA technology well known to the skilled person (e.g. Alberts et al., Molecular Biology of the Cell, 4th ed. Garland Science, p. 518-519). However, polypeptides and fusion proteins of the invention may be prepared by any of the many conventional and well known techniques such as plain organic synthetic strategies, solid phase-assisted synthesis techniques or by commercially available automated synthesizers. Fusion proteins may be used in biological research or therapeutics.

A pharmaceutically active protein or peptide is a protein or peptide having a biologically activity upon administration to a subject, which brings about a beneficial effect for the subject. This term also encompasses prodrugs. A prodrug is a protein or peptide that is administered in an inactive (or less than fully active) form to a subject, and is subsequently converted to a pharmaceutically active or pharmaceutically fully active protein or peptide through metabolic processes in the subject. The pharmaceutically (fully) active protein or peptide is preferably a protein or peptide suitable for the treatment or prevention of a disease.

A diagnostically active protein or peptide is a protein or peptide having an activity upon administration to a subject, which allows to determine or identify a possible disease or disorder. A diagnostically active protein or peptide fused to a polypeptide of the invention can in particular be used to determine or identify a specific disease. For example, the location of diseased tissue within the body can be detected or identified by the polypeptide of the invention fused to the diagnostically active protein or peptide of the invention.

According to one preferred embodiment the polypeptide of the invention is directly fused to the pharmaceutically and/or diagnostically active protein or peptide.

According to another preferred embodiment the polypeptide of the invention is fused to the pharmaceutically and/or diagnostically active protein or peptide via a linker.

The fusion construct may be (directly) fused to the C- or N-terminus of the pharmaceutically and/or diagnostically active protein or peptide, more specifically by the formation of a peptide bond between the carboxy group of the C-terminal amino acid and the amino group of the N-terminal amino acid, or may be connected to the C- or N-terminus of the pharmaceutically and/or diagnostically active protein or peptide via a linker.

Suitable linkers are at the skilled person's disposal. The linker according to the invention is preferably selected from the group consisting of alkyl with 1 to 30 carbon atoms, polyethylene glycol with 1 to 20

ethylene moieties, polyalanine with 1 to 20 residues, caproic acid, substituted or unsubstituted poly-p-phenylene and triazol. Preference is also given to peptidic linkers, more specifically to oligopeptides having a length from 2 to 30 amino acids. Use of a single amino acid is also deliberately envisaged. Preferred length ranges are from 5 to 15 amino acids. Other preferred lengths are 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19 or 20 amino acids.

Particularly preferred are linkers which are peptides which consist of at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or 100% of small amino acids such as glycine, serine and alanine. Particularly preferred are linkers consisting of glycines and serines only. Most preferred are the linkers of SEQ ID NOs: 50 to 52 with special preference given to a linker being a peptide consisting of the sequence of SEQ ID NO: 52.

In accordance with a preferred embodiment the pharmaceutically and/or diagnostically active protein or peptide is selected from the group consisting of a recombinant protein, an antibody, a blood factor, a hormone, an anticoagulans, a thrombolytic, a cytokine, a chemokine, and an interferon.

Non-limiting examples of pharmaceutically and/or diagnostically active molecules which can be fused to the polypeptide of the invention binding to human serum albumin thereby prolonging their *in vivo* half-life comprise recombinant blood factors (such as factor VIII, factor VIIa, factor IX, thrombin, antithrombin), thrombolytics and anticoagulants (such as tissue plasminogen activator, hirudin, protein C), insulin, growth hormones (such as human growth hormone (hGH), follicle-stimulating hormone), glucagon, parathyroid hormone, calcitonin, lutropin, parathyroid hormone, thyrotrophin-alpha, choriogonadotropin-alpha, growth factors (such as erythropoetin, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), keratinocyte growth factor), interferons (such as interferon-alpha, interferon-beta, interferon-gamma), cytokines, chemokines, recombinant IL-1 receptor antagonist and bispecific T-cell engaging molecules (BITEs®).

The chemokine is preferably selected from the group consisting of IL-8, GRO alpha, GRO beta, GRO gamma, ENA-78, LDGF-PBP, GCP-2, PF4, Mig, IP-10, SDF-1alpha/beta, BUNZO/STRC33, I-TAC, BLC/BCA-1, MIP-1alpha, MIP-1 beta, MDC, TECK, TARC, RANTES, HCC-1, HCC-4, DC-CK1, MIP-3 alpha, MIP-3 beta, MCP-1-5, eotaxin, Eotaxin-2, I-309, MPIF-1, 6Ckine, CTACK, MEC, lymphotactin and fractalkine.

The cytokine is preferably selected from the group consisting of IL-2, IL-12, TNF-alpha, IFN alpha, IFN beta, IFN gamma, IL-10, IL-15, IL-24, GM-CSF, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-13, LIF, CD80, B70, TNF beta, LT-beta, CD-40 ligand, Fas-ligand, TGF-beta, IL-1alpha and IL-1beta. As it is well-known in the art, cytokines may favour a pro-inflammatory or an anti-inflammatory response of the immune system. Thus, depending on the disease to be treated either fusion constructs with a pro-inflammatory or an anti-inflammatory cytokine may be favored. For example, for the treatment of inflammatory diseases in general fusion constructs comprising anti-inflammatory cytokines are

preferred, whereas for the treatment of cancer in general fusion constructs comprising pro-inflammatory cytokines are preferred.

The term “antibody” as used in accordance with the present invention comprises, for example, polyclonal or monoclonal antibodies. Furthermore, also derivatives or fragments thereof, which still retain the binding specificity, are comprised in the term "antibody". Antibody fragments or derivatives comprise, inter alia, Fab or Fab' fragments, Fd, $F(ab')_2$, Fv or scFv fragments, single domain V_H or V-like domains, such as VhH or V-NAR-domains, as well as multimeric formats such as minibodies, diabodies, tribodies, tetrabodies or chemically conjugated Fab'-multimers (see, for example, Altshuler et al., 2010., Holliger and Hudson, 2005). The term "antibody" also includes embodiments such as chimeric (human constant domain, non-human variable domain), single chain and humanized (human antibody with the exception of non-human CDRs) antibodies.

Various techniques for the production of antibodies and fragments thereof are well known in the art and described, e.g. in Altshuler et al., 2010. Thus, polyclonal antibodies can be obtained from the blood of an animal following immunisation with an antigen in mixture with additives and adjuvans and monoclonal antibodies can be produced by any technique which provides antibodies produced by continuous cell line cultures. Examples for such techniques are described, e.g. Harlow and Lane (1988) and (1999) and include the hybridoma technique originally described by Köhler and Milstein, 1975, the trioma technique, the human B-cell hybridoma technique (see e.g. Kozbor, 1983; Li et al., 2006) and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985). Furthermore, recombinant antibodies may be obtained from monoclonal antibodies or can be prepared *de novo* using various display methods such as phage, ribosomal, mRNA, or cell display. A suitable system for the expression of the recombinant (humanized) antibodies or fragments thereof may be selected from, for example, bacteria, yeast, insects, mammalian cell lines or transgenic animals or plants (see, e.g., US patent 6,080,560; Holliger and Hudson, 2005). Further, techniques described for the production of single chain antibodies (see, inter alia, US Patent 4,946,778) can be adapted to produce single chain antibodies specific for the target of this invention. Surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies

The term “monoclonal” or “polyclonal antibody” (see Harlow and Lane, (1988), loc. cit.) also relates to derivatives of said antibodies which retain or essentially retain their binding specificity. Whereas particularly preferred embodiments of said derivatives are specified further herein below, other preferred derivatives of such antibodies are chimeric antibodies comprising, for example, a mouse or rat variable region and a human constant region.

The term "scFv fragment" (single-chain Fv fragment) is well understood in the art and preferred due to its small size and the possibility to recombinantly produce such fragments.

The antibody may be a humanized antibody. The term "humanized antibody" means, in accordance with the present invention, an antibody of non-human origin, whose protein sequences has been modified to increase its similarity to antibody variants produced naturally in humans. Creation of a humanized antibody may be accomplished, for example, by inserting the appropriate CDR coding segments (responsible for the desired binding properties), such as CDR 3 and preferably all 6 CDRs, into a human antibody "scaffold". Methods for the production of humanized antibodies are described in, e.g., EP-A1 0 239 400 and WO 90/07861.

The term "antibody light chain" designates the small polypeptide subunit of an antibody while the term "antibody heavy chain" designates the large polypeptide subunit of an antibody. A typical antibody is composed of two immunoglobulin (Ig) heavy chains and two Ig light chains. Each light chain is composed of two tandem immunoglobulin domains; one constant (C_L) domain and one variable domain (V_L) that is important for binding antigen. The heavy chain determines the class or isotype of an antibody. Each heavy chain has two regions, namely a constant region (which is the same for all immunoglobulins of the same class but differs between classes) and a variable region that differs between different B cells, but is the same for all immunoglobulins produced by the same B cell or B cell clone. The variable domain of any heavy chain is composed of a single immunoglobulin domain.

A "functional Fc domain" of an antibody is a term well known to the skilled artisan and defined on the basis of papain cleavage of antibodies. Depending on the amino acid sequence of the constant region of their heavy chains, immunoglobulins are divided in the classes: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG1, IgG2, IgG3, and IgG4, IgA1, and IgA2. According to the heavy chain constant regions the different classes of immunoglobulins are called [alpha], [delta], [epsilon], [gamma], and [mu], respectively. The functional Fc domain of an antibody is directly involved in ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement-dependent cytotoxicity) based on complement activation, C1q binding and Fc receptor binding. The four human IgG isotypes bind different receptors, such as the neonatal Fc receptor, the activating Fc gamma receptors, FcγRI, FcγRIIa, and FcγRIIIa, the inhibitory receptor FcγRIIb, and C1q with different affinities, yielding very different activities. It is known that the affinities to activating and inhibiting receptors of an Fc domain of a human antibody can be engineered and modified (see Strohl W. (2009) Curr Opin Biotechnol, 20, p. 685-691).

The polypeptides of the invention can be fused either to the N- or C-terminus of one or more functional Fc domains or to both the N- and the C-terminus of one or more Fc domains. It is preferred that the fusion proteins of the invention comprise multimers, preferably tetramers, trimers or most preferably dimers of the polypeptides of the invention fused to at least one side, preferably to the N-terminus of one or more, preferably one Fc domain.

In accordance with a more preferred embodiment the antibody of the invention is a bispecific T-cell engaging antibody (also-called BITE® molecule).

BITE® molecules are a class of artificial bispecific fusion proteins consisting of two single-chain variable fragments (scFv), whereas one of the scFvs binds to T cells via the CD3 receptor, and the other to a tumor cell via a tumor specific molecule (Baeuerle PA. et al., (2003) *Curr Opin Mol Ther.*, **5**(4) p.413-419; Wolf E. et al., (2005) *Drug Discov Today*, **10**(18), p. 1237-1244). Recently, it has been reported that two BITE® antibodies are currently being tested in clinical trials (Bauerle PA and Reinhardt C., (2009), *Cancer Res*, **69**(12), p. 4941-4944): Blinatumomab (also known as MT103) is bispecific for CD3 and CD19. It is currently being tested in a phase 1 trial in patients with late stage, relapsed non-Hodgkin's lymphoma (NHL), and in a phase 2 trial in patients with B-precursor acute lymphoblastic leukemia (B-ALL) having minimal residual disease in their bone marrow. CD19 is a surface antigen suitable to address a wide range of B-cell malignancies. The other BITE® antibody in clinical development is called MT110 and is bispecific for CD3 and epithelial cell adhesion molecule (EpCAM). It is currently being tested in a phase 1 trial with lung and gastrointestinal cancer patients. EpCAM is very frequently expressed on human adeno- and some squamous cell carcinoma, and also on cancer stem cells. Another BITE® molecule described in the literature is MT112 which is bispecific for CD3 and PSMA (Friedrich M. et al., (2012) *Mol Cancer Ther.*, **11**, p. 2664-2673). While showing excellent antitumoral efficacy, one of the major limitations often described for the BITE® molecules is their short *in vivo* half-life of only several hours. The short half-life of Blinatumomab and MT110, for example, makes a continuous intravenous infusion by portable minipumps for 4 to 8 weeks necessary (Bauerle PA and Reinhardt C., (2009), *Cancer Res*, **69**(12), p. 4941-4944). More convenient administration regimens of the BITE® molecules would be beneficial for the patients.

As is evident from the Examples herein below it was surprisingly found that the fusion of a Fynomer® to a BITE® molecule (anti-albumin-CD3-PSMA) resulted in a Fynomer-BITE fusion protein with cytotoxic activity and with prolonged *in vivo* half-life compared to the unmodified BITE® (anti-CD3-PSMA).

The present invention relates in a further embodiment to a nucleic acid molecule encoding the polypeptide of the invention or the fusion protein of the invention.

Nucleic acid molecules, in accordance with the present invention, include DNA, such as cDNA, and mRNA. Further included are nucleic acid mimicking molecules known in the art such as synthetic or semisynthetic derivatives of DNA or RNA and mixed polymers, both sense and antisense strands. They may contain additional non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. In a preferred embodiment the polynucleotide or the nucleic acid molecule(s) is/are DNA. Such nucleic acid mimicking molecules or nucleic acid derivatives according to the invention include phosphorothioate nucleic acid, phosphoramidate nucleic acid, 2'-O-methoxyethyl ribonucleic acid, morpholino nucleic acid, hexitol nucleic acid (HNA) and locked nucleic acid (LNA) (see, for example, Braasch and Corey, Chemistry & Biology 8, 1-7 (2001)). LNA is an RNA derivative in which the ribose ring is constrained by a methylene linkage between the 2'-oxygen and the 4'-carbon.

For the purposes of the present invention, a peptide nucleic acid (PNA) is a polyamide type of DNA analog. The monomeric units for the corresponding derivatives of adenine, guanine, thymine and

cytosine are available commercially (for example from Perceptive Biosystems). PNA is a synthetic DNA-mimic with an amide backbone in place of the sugar-phosphate backbone of DNA or RNA. As a consequence, certain components of DNA, such as phosphorus, phosphorus oxides, or deoxyribose derivatives, are not present in PNAs.

PNA chimera according to the present invention are molecules comprising one or more PNA portions. The remainder of the chimeric molecule may comprise one or more DNA portions (PNA-DNA chimera) or one or more (poly)peptide portions (peptide-PNA chimera). Peptide-DNA chimera according to the invention are molecules comprising one or more (poly)peptide portions and one or more DNA portions. Molecules comprising PNA, peptide and DNA portions are envisaged as well. The length of a portion of a chimeric molecule may range from 1 to n-1 bases, equivalents thereof or amino acids, wherein „n" is the total number of bases, equivalents thereof and amino acids of the entire molecule.

The term "derivatives" in conjunction with the above described PNAs, (poly)peptides, PNA chimera and peptide-DNA chimera relates to molecules wherein these molecules comprise one or more further groups or substituents different from PNA, (poly)peptides and DNA. All groups or substituents known in the art and used for the synthesis of these molecules, such as protection groups, and/or for applications involving these molecules, such as labels and (cleavable) linkers are envisaged.

In those embodiments where the nucleic acid molecule comprises (rather than have) the recited sequence, additional nucleotides extend over the specific sequence either on the 5' end or the 3' end or both. Those additional sequence may be of heterologous or homologous nature and may comprise stretches of about 50 to 500 nucleotides although higher or lower values are not excluded. In the case of homologous sequences, those embodiments do not include complete genomes and are generally confined to about 1500 additional nucleotides at the 5' and/or the 3' end.

Additional heterologous sequences may include heterologous promoters which are operatively linked to the coding sequences of above molecules. Hence, preferably the nucleic acid molecule is operably linked to a promoter, and more preferably linked to a promoter selected from the group of prokaryotic promoters consisting of T5 promoter/lac operator element, T7 promotor/lac operator element, or from the group of eukaryotic promoters consisting of hEF1-HTLV, CMV enh/hFerL promoter.

The present invention also relates to a vector comprising the nucleic acid molecule of the invention.

Preferably, the vector is a plasmid, cosmid, virus, bacteriophage or another vector used e.g. conventionally in genetic engineering.

The nucleic acid molecule of the invention may be inserted into several commercially available vectors. Non-limiting examples include prokaryotic plasmid vectors, such as the pUC-series, pBluescript (Stratagene), the pET-series of expression vectors (Novagen) or pCRTOPO (Invitrogen) and vectors

compatible with an expression in mammalian cells like pREP (Invitrogen), pcDNA3 (Invitrogen), pCEP4 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2neo, pBPV-1, pdBPVMMTneo, pRSVgpt, pRSVneo, pSV2-dhfr, plZD35, pLXIN, pSIR (Clontech), plRES-EGFP (Clontech), pEAK-10 (Edge Biosystems) pTriEx-Hygro (Novagen) and pCINeo (Promega). Plant expression vectors comprise pGEM-T (Promega), pCAMBIA 1391 (Cambia), GATEWAY (Invitrogen), pGreen and pGreenII (PGREEN). Examples for plasmid vectors suitable for Pichia pastoris comprise e.g. the plasmids pAO815, pPIC9K and pPIC3.5K (all Intvitrogen).

The nucleic acid molecule referred to above may also be inserted into vectors such that a translational fusion with another polynucleotide is generated. The other polynucleotide may encode a protein which may e.g. increase the solubility, half-life and/or facilitate the purification of the fusion protein. The vectors may also contain an additional expressible polynucleotide coding for one or more chaperones to facilitate correct protein folding. For vector modification techniques, see Sambrook and Russel (2001), loc. cit. Generally, vectors can contain one or more origin of replication (ori) and inheritance systems for cloning or expression, one or more markers for selection in the host, e. g., antibiotic resistance, and one or more expression cassettes. Suitable origins of replication (ori) include, for example, the Col E1, the SV40 viral and the M 13 origins of replication.

The coding sequences inserted in the vector can e.g. be synthesized by standard methods, or isolated from natural sources. Ligation of the coding sequences to transcriptional regulatory elements and/or to other amino acid encoding sequences can be carried out using established methods. Transcriptional regulatory elements (parts of an expression cassette) ensuring expression in prokaryotes or eukaryotic cells are well known to those skilled in the art. These elements comprise regulatory sequences ensuring the initiation of transcription (e. g., translation initiation codon, promoters, such as naturally-associated or heterologous promoters and/or insulators), internal ribosomal entry sites (IRES) (Owens, Proc. Natl. Acad. Sci. USA 98 (2001), 1471-1476) and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers. Preferably, the polynucleotide of the invention is operatively linked to such expression control sequences allowing expression in prokaryotes or eukaryotic cells. The vector may further comprise nucleotide sequences encoding secretion signals as further regulatory elements. Such sequences are well known to the person skilled in the art. Furthermore, depending on the expression system used, leader sequences capable of directing the expressed polypeptide to a cellular compartment may be added to the coding sequence of the polynucleotide of the invention. Such leader sequences are well known in the art.

Furthermore, it is preferred that the vector comprises a selectable marker. Examples of selectable markers include neomycin, ampicillin, and hygromycine, kanamycin resistance and the like. Specifically-designed vectors allow the shuttling of DNA between different hosts, such as bacteria- fungal cells or bacteria-animal cells (e. g. the Gateway® system available from Invitrogen).

An expression vector according to this invention is capable of directing the replication, and the expression, of the polynucleotide and encoded enzyme of this invention. Suitable expression vectors which comprise the described regulatory elements are known in the art.

The nucleic acid molecules as described herein above may be designed for direct introduction or for introduction via liposomes, phage vectors or viral vectors (e.g. adenoviral, retroviral) into a cell. Additionally, baculoviral systems or systems based on Vaccinia Virus or Semliki Forest Virus can be used as eukaryotic expression systems for the nucleic acid molecules of the invention.

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Moreover, elements such as origin of replication, drug resistance gene, regulators (as part of an inducible promoter) may also be included. The *lac* promoter is a typical inducible promoter, useful for prokaryotic cells, which can be induced using the lactose analogue *isopropylthiol-b-D-galactoside*. ("IPTG"). For recombinant expression and secretion, the polynucleotide of interest may be ligated between e.g. the PelB leader signal, which directs the recombinant protein in the periplasm and the gene III in a phagemid called pHEN4 (described in Ghahroudi et al, 1997, FEBS Letters 414:521-526). Additional elements might include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from retroviruses, e.g., RSV, HTLVI, HIVI, and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Alternatively, the recombinant polypeptide can be expressed in stable cell lines that contain the gene construct integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells. The transfected nucleic acid can also be amplified to express large amounts of the encoded polypeptide. As indicated above, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria.

The present invention furthermore relates to an isolated cell comprising the nucleic acid molecule of the invention or the vector of the invention.

Suitable prokaryotic host cells comprise e.g. bacteria of the species Escherichia, Bacillus, Streptomyces and Salmonella typhimurium. Suitable eukaryotic host cells are e.g. fungal cells, inter alia, yeasts such as Saccharomyces cerevisiae or Pichia pastoris or insect cells such as Drosophila S2 and Spodoptera Sf9 cells and plant cells as well as mammalian cells.

Mammalian host cells that could be used include, human Hela, HEK293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

In a more preferred embodiment, said cell is a primary cell or primary cell line. Primary cells are cells which are directly obtained from an organism. Suitable primary cells are, for example, mouse embryonic fibroblasts, mouse primary hepatocytes, cardiomyocytes and neuronal cells as well as mouse muscle stem cells (satellite cells) and stable, immortalized cell lines derived thereof. Also within the scope of the present invention are primary mammalian cells such as mouse embryonic fibroblasts (MEF). Alternatively, the recombinant (poly)peptide can be expressed in stable cell lines that contain the gene construct integrated into a chromosome.

Appropriate culture media and conditions for the above-described host cells are well-known in the art.

The one or more host cells may be produced by introducing the one or more nucleic acid molecules or one or more vectors of the invention into the one or more host cells which upon their presence mediates the expression of the polypeptides encoded by said nucleic acid molecules or vectors. The host cells are preferably isolated host cell, meaning that the cells are not within the context of a living organism. The host may be any prokaryotic or eukaryotic cell. A suitable eukaryotic host may be a mammalian cell, an amphibian cell, a fish cell, an insect cell, a fungal cell or a plant cell. A eukaryotic cell may be an insect cell such as a *Spodoptera frugiperda* cell, a yeast cell such as a *Saccharomyces cerevisiae* or *Pichia pastoris* cell, a fungal cell such as an *Aspergillus* cell or a vertebrate cell. In the latter regard, it is preferred that the cell is a mammalian cell such a human cell. The cell may be a part of a cell line.

Another embodiment of the present invention relates to a method of producing the polypeptide of any one of the invention, or the fusion protein of the invention comprising (a) culturing the isolated cell of the invention, and (b) isolating the produced polypeptide or fusion protein.

Suitable conditions for culturing a prokaryotic or eukaryotic host are well known to the person skilled in the art. For example, suitable conditions for culturing bacteria are growing them under aeration in Luria Bertani (LB) medium. To increase the yield and the solubility of the expression product, the medium can be buffered or supplemented with suitable additives known to enhance or facilitate both. *E. coli* can be cultured from 4 to about 37 °C, the exact temperature or sequence of temperatures depends on the molecule to be overexpressed. In general, the skilled person is also aware that these conditions may have to be adapted to the needs of the host and the requirements of the polypeptide expressed. In case an inducible promoter controls the nucleic acid of the invention in the vector present in the host cell, expression of the polypeptide can be induced by addition of an appropriate inducing agent. Suitable expression protocols and strategies are known to the skilled person.

Depending on the cell type and its specific requirements, mammalian cell culture can e.g. be carried out in RPMI or DMEM medium containing 10% (v/v) FCS, 2mM L-glutamine and 100 U/ml penicillin/streptomycine. The cells can be kept at 37 °C in a 5% CO_2, water saturated atmosphere. Suitable media for insect cell culture is e.g. TNM + 10% FCS or SF900 medium. Insect cells are usually grown at 27 °C as adhesion or suspension culture. Suitable expression protocols for eukaryotic cells are well known to the skilled person and can be retrieved e.g. from in Sambrook J, Russell DW, (2001), Molecular Cloning: A laboratory manual, 3rd ed, Cold Spring Harbor Laboratory Press, New York.

Methods of isolation of the polypeptide produced are well-known in the art and comprise without limitation method steps such as ion exchange chromatography, gel filtration chromatography (size exclusion chromatography), affinity chromatography, high pressure liquid chromatography (HPLC), reversed phase HPLC, disc gel electrophoresis or immunoprecipitation, see, for example, in Sambrook J, Russell DW, (2001), Molecular Cloning: A laboratory manual. 3rd ed, Cold Spring Harbor Laboratory Press, New York.

The present invention furthermore relates to pharmaceutical and/or diagnostic composition comprising the fusion protein of any one of claims of the invention.

The pharmaceutical composition is preferably administered to mammals such as domestic and pet animals. Most preferred it is administered to humans. The pharmaceutical compositions described herein will be administered to the subject at a suitable dose.

The pharmaceutical composition for use in accordance with the present invention can be formulated in conventional manner according to methods found in the art, using one or more physiological carriers or excipient, see, for example Ansel et al., "Pharmaceutical Dosage Forms and Drug Delivery Systems", 7th edition, Lippincott Williams & Wilkins Publishers, 1999. The pharmaceutical composition may, accordingly, be administered orally, parenterally, such as subcutaneously, intravenously, intramuscularly, intraperitoneally, intrathecally, transdermally, transmucosally, subdurally, locally or topically via iontopheresis, sublingually, by inhalation spray, aerosol or rectally and the like in dosage unit formulations optionally comprising conventional pharmaceutically acceptable excipients. Also diagnostic compositions of the invention may be manufactured in any conventional manner.

The pharmaceutical composition of the invention may be administered as the sole active ingredient or in conjunction with, e.g. as an adjuvant to or in combination with, other drugs, e.g. immunosuppressive or immune modulating agents or other anti-inflammatory agents.

Pharmaceutical compositions of the invention preferably comprise a pharmaceutically acceptable carrier or excipient. By "pharmaceutically acceptable carrier or excipient" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Examples of pharmaceutically acceptable carriers or excipients are described, e.g., in Ansel et al., "Pharmaceutical

Dosage Forms and Drug Delivery Systems", 7th edition, Lippincott Williams & Wilkins Publishers, 1999.

The diagnostic composition of the invention is useful in the detection of an undesired physiological condition or a disease. Accordingly, the diagnostic composition of the invention may be used for assessing the onset or the disease status and in particular a disease state. The diagnostic composition of the invention can be administered as sole active agent or can be administered in combination with other agents, if the diagnostic composition is, for example, used to identify sites of undesired physiological condition or a disease.

The present invention furthermore relates to a kit comprising the fusion protein of the invention, wherein the fusion protein comprises a diagnostically active protein or peptide.

The various components of the kit may be packaged in one or more containers such as one or more vials. The vials may, in addition to the components, comprise preservatives or buffers for storage. In addition, the kit may contain instructions for use.

The figures show:

Fig. 1 shows the SDS-PAGE characterization of albumin-binding polypeptides of the invention: Lane M: molecular weight standard; Lane A: Fynomer® C1 (SEQ ID NO: 4); Lane B: Fynomer® 17H (SEQ ID NO: 5); Lane C: WT Fyn-SH3 (SEQ ID NO: 3).

Fig. 2 shows the SDS-PAGE characterization of the unmodified BITE® polypeptide (SEQ ID NO: 53, lane 1) and the Fynomer®-BITE® fusion protein COVA406 (SEQ ID NO: 54, lane 2), consisting of the albumin binding Fynomer® 17H and the BITE® molecule. The molecular weight standard is shown in Lane M.

Fig. 3 shows the size exclusion chromatogram (SEC) of COVA406 (SEQ ID NO: 54).

Fig. 4 depicts a FACS binding experiment with COVA406 (SEQ ID NO: 54) using cells expressing CD3 (Jurkat E6-1), cells expressing PSMA (22Rv1 cells) and an irrelevant cell line expressing neither CD3 nor PSMA (LS174T cells). Binding is expressed as mean fluorescence intensity. CD3+: CD3-positive cells; CD3-: CD3-negative cells; PSMA+: PSMA-positive cells; PSMA-: PSMA-negative cells; COVA406: COVA406 is used as binding reagent; PBS: negative control, phosphate buffered saline is added instead of COVA406. COVA406 recognizes both antigens CD3 and PSMA expressed on cells.

Fig. 5 depicts the analysis of redirected cell lysis of PSMA positive cells (22Rv1 cells) or PSMA negative cells (HT29 cells) by COVA406 (SEQ ID NO: 54) using human PBMCs as effector cells. The target cells 22Rv1 and HT29 were pre-labeled with Calcein AM and then incubated with human

PBMCs (at effector cell to target cell ratio (E:T) of 25:1) and different concentrations of COVA406 (SEQ ID NO: 54) for 5 hours. The percentage of specific tumor cell lysis was measured by detection of calcein-release into the supernatant. Triplicates of 3 wells are shown ± SEM.

Fig. 6 shows the serum concentrations of COVA406 (SEQ ID NO: 54) and the BITE® protein (SEQ ID NO: 53) at different time-points after a single i.v. injection into C57BL/6 mice. The concentration in serum was determined by ELISA. Mean values of 5 mice are shown ± SD

Fig. 7 Specificity ELISA of prior art Fyn SH3 variants isolated after affinity selections. MSA = mouse serum albumin, HSA = human serum albumin, RSA = rat serum albumin, BSA = bovine serum albumin

Fig. 8 Sequence alignment of SEQ ID NOs 4 to 40.

The examples illustrate the invention.

Example 1: Fyn SH3 derived polypeptides bind to human serum albumin

Methods

1) Lysate ELISA on human serum albumin protein

Using the Fynomer® phage libraries described in Schlatter et al. (Schlatter et al. (2012) mAbs, **4**(4) p. 497-50) Fyn-SH3 derived binding proteins specific to human serum albumin were isolated using human serum albumin (Sigma-Aldrich, cat. no A3782) and serum albumin from a rodent species (rat serum albumin, Sigma-Aldrich, cat. no A6414) as antigens and standard phage display as selection technology (Grabulovski D. et al., (2007) J Biol Chem 282, p. 3196-3204, Viti, F. et al. (2000) Methods Enzymol. 326, 480-505).

After naïve and affinity maturation selections, enriched Fyn SH3-derived polypeptides were screened for binding to human serum albumin and/or serum albumin from a rodent species (mouse/rat) by lysate ELISA. DNA encoding the Fyn SH3-derived binding proteins was cloned into the bacterial expression vector pQE12 (Qiagen) so that the resulting constructs carried a C-terminal myc-hexahistidine tag as described in Grabulovski et al. (Grabulovski et al. (2007) JBC, 282, p. 3196-3204). The polypeptides were expressed in the cytosol of *E.coli* bacteria in a 96-well format and 200 µl of cleared lysate per well was prepared essentially as described in Bertschinger et al. (Bertschinger et al. (2007) Protein Eng Des Sel 20(2): p. 57-68). Briefly, transformed bacterial colonies were picked from agar plates and grown in a round bottom 96-well plate (Nunc, cat. no. 163320) in 200µl 2xYT medium containing 100 µg/ml ampicillin and 0.1% (w/v) glucose. Protein expression was induced after growth for 3 h at 37°C and 200 r.p.m. by adding 1 mM IPTG (Applichem, Germany). Proteins were expressed overnight in a rotary shaker (200 r.p.m., 30° C). Subsequently, the 96-well plate was centrifuged at 1800 g for 10 min and the

supernatant was discarded. The bacterial pellets were resuspended in 65 µl Bugbuster containing Benzonase Nuclease (VWR, cat. No. 70750-3) and incubated at RT for 30 minutes. Afterwards, the monoclonal bacterial lysates were cleared by centrifugation (1800 g for 10 min), diluted with 170 µL PBS and filtered using a multiscreen filter plate (0.45 µm pore size; Millipore cat. No. MSHVN4510). Monoclonal bacterial lysates were used for ELISA: human serum albumin was immobilized on maxisorp F96 wells (Nunc, cat. no 439454) overnight at room temperature. Plates were then blocked with PBS, 4% (w/v) milk (Rapilait, Migros, Switzerland). Subsequently, 20 µl of PBS, 10% milk containing 25 µg/ml anti-myc antibody 9E10 and 80 µl of bacterial lysate were applied (resulting in a final anti-myc antibody concentration of 5 µg/ml). After incubating for 1 h and washing, bound Fyn SH3-derived polypeptides were detected with anti-mouse-HRP antibody conjugate (Sigma) at a final concentration of 5 µg/ml. The detection of peroxidase activity was done by adding 100 µL per well BM blue POD substrate (Roche) and the reaction was stopped by adding 50 µl 1 M H_2SO_4. The DNA sequence of the specific binders was verified by DNA sequencing. Cross-reactivity towards serum albumin from a rodent species was detected by monoclonal lysate ELISA using mouse serum albumin (Sigma-Aldrich, cat. no A3139) as an antigen and the protocol described above. Alternatively, cross-reactivity towards mouse and rat serum albumin was confirmed surface plasmon resonance experiments (see below).

2) Expression and purification of Fyn SH3-derived polypeptides in *E.coli*

Fyn SH3-derived albumin-binding polypeptides were expressed in the cytosol of TG1 *E.coli* bacteria as well as purified as described in Grabulovski et al. (Grabulovski et al. (2007) JBC, 282, p. 3196-3204).

3) Affinity Measurements

Affinity measurements were performed using a Biacore T200 instrument (GE Healthcare). For the interaction analysis between serum albumin, derived from mouse, rat or human, and Fyn SH3-derived albumin-binding polypeptides, a Series S CM5 chip (GE Healthcare) was used with albumin proteins immobilized using the amine coupling kit (GE healthcare). Serum albumin proteins from different species (mouse, rat or human) were immobilized (2000-3000 RU) on different flow cells of the chip whereas a blank-immobilized flow cell served as a reference flow cell. The running buffer was PBS containing 0.05% Tween 20 at pH 7.4. The interactions were measured at a flow of 30 µl/min and 25°C and different concentrations of Fyn SH3-derived albumin-binding polypeptides were injected. All kinetic data of the interaction was evaluated using Biacore T200 evaluation software.

Results

1) The amino acid sequences of ELISA positive Fyn SH3-derived polypeptides binding to human serum albumin is presented in SEQ ID NOs: 4 to 40 as appended in the sequence listing. In addition, Fyn SH3-derived polypeptides (SEQ ID NOs: 4 to 32) also showed binding to mouse serum albumin as confirmed by lysate ELISA and/or Biacore affinity measurements.

2) The expression yields of two selected Fyn SH3-derived albumin-binding polypeptides of the invention from bacterial cultures under non-optimized conditions in shake flasks is depicted in Table 1. The yield was in the same range as the expression yield of the WT Fyn-SH3 polypeptide. High protein-purity was confirmed by SDS-PAGE analysis and the gel is depicted in Figure 1.

Table 1. Expression yields of Fyn SH3-derived albumin-binding polypeptides produced in TG1 *E.coli* bacteria

Fynomer®	SEQ ID NO.	Yield (mg/l)
17H	5	10
C1	4	25
WT Fyn-SH3	3	10

3) The binding properties were analyzed by real-time interaction analysis on a Biacore chip revealing the following dissociation constants (K_D) for selected albumin-binding polypeptides against albumin derived from either rat (RSA), mouse (MSA) or human (HSA) (depicted in Table 2).

Table 2. Dissociation constants of Fyn SH3-derived serum albumin-binding polypeptides to RSA, MSA and HSA.

Fynomer®	SEQ ID NO.	K_D (nM) RSA	K_D (nM) MSA	K_D (nM) HSA
C1	4	72	408	1290
17H	5	17	96	455

Example 2: Albumin-binding Fyn SH3 derived polypeptides have a prolonged serum half-life in mice

Methods

The pharmacokinetic profile of albumin-binding Fyn-SH3 derived polypeptides was investigated in BALB/c mice (Charles River) and compared to the WT Fyn-SH3 molecule. Fynomer® C1 (SEQ ID NO: 4), Fynomer® 17H (SEQ ID NO: 5) and WT Fyn-SH3 (SEQ ID NO: 3) were radiolabeled using Iodine-125 (Perkin Elmer cat no. NEZ033A001MC) and Chloramine T (Sigma-Aldrich cat NO 31224). The labeling reaction was carried out for two minutes at room temperature before removal of labeling reagents using PD MiniTrap G-25 columns (GE Healthcare cat.no 28-9180-07). Three BALB/c mice were injected i.v. with 13.5 µg of either radiolabeled Fynomer® C1 (SEQ ID NO: 4), Fynomer® 17H (SEQ ID NO: 5) or WT Fyn-SH3 (SEQ ID NO: 3). After 10 minutes, 2.5, 4, 6, 9, 25, 35 hours, blood was collected into EDTA coated microvettes (Sarstedt) and centrifuged for 10 min at 9300 g. Radioactivity

was counted by mixing the serum with Supermix Perkin Elmer Scintillation Fluid and quantification of beta-emission of each sample with a 1450 MicroBeta Trilux scintillation counter and serum levels were calculated (results expressed as % injected dose (ID)/ml of blood). From the serum levels of Fynomer® C1, Fynomer® 17H and WT Fyn-SH3 determined in serum at different time points and the resulting slope k of the elimination phase (plotted in a semi-logarithmic scale), the half-lives were calculated using the formula $t_{1/2} = \ln 2/-k$.

Results

As depicted in Table 3, Fynomer® C1 (SEQ ID NO: 4) and Fynomer® 17H (SEQ ID NO: 5) show a significantly better terminal half-life as the WT Fyn-SH3 protein (SEQ ID NO: 3). Time-points used for half-life calculation: Fynomer® C1 and Fynomer® 17H: 2.5-35 h; WT Fyn-SH3: 2.5-25 h)

Table 3. Terminal half-life of Fyn SH3-derived serum albumin-binding polypeptides in mice compared to the WT Fyn-SH3 protein.

Fynomer®	SEQ ID NO:	$t_{1/2}$ (h)
C1	4	10.5
17H	5	21.3
WT Fyn-SH3	3	4.4

Example 3: Albumin-binding Fyn SH3 derived polypeptides can extend serum half-life of BITE® molecules

Methods:

1) Expression and purification of an albumin binding Fyn-SH3 fusion protein

The Fynomer® 17H (Seq ID NO: 5) has been genetically fused to the N-terminus of the CD3-PSMA specific BITE® (Seq ID NO: 53) via a 15 amino acid linker (linker SEQ ID NO: 52) yielding the trispecific anti-albumin/PSMA/CD3 protein COVA406 (SEQ ID NO: 54). The BITE® protein (SEQ ID NO: 53) and the fusion molecule of the invention COVA406 (SEQ ID NO: 54) carrying a C-terminal penta-his-tag were transiently transfected into FreeStyle CHO-S cells and expressed in serum-free/animal component-free media for 6 days. The proteins were purified from the supernatants by Protein L affinity chromatography (Thermo Scientific, cat. No. 89928) with an ÄKTA Purifier instrument (GE Healthcare). Concentrations were determined by absorbance measurement at 280 nm. Yields are listed in Table 4. The SDS PAGE of both proteins is shown in Figure 2.

After purification size exclusion chromatography has been performed with COVA406 using an ÄKTA FPLC system and a Superdex G200, 30/100 GL column (GE Healthcare) (see Figure 3).

2) FACS binding experiment with a BITE® fusion protein of the invention

The polypeptide COVA406 (SEQ ID NO: 54, final concentration 300 nM) was mixed with 100 µl cell suspension containing either (i) 1 x 10^5 Jurkat E6-1 cells (CD3 positive cells), (ii) 1 x 10^5 22Rv1 prostate carcinoma cells (PSMA positive cells) or (iii) 1 x 10^5 LS174T colorectal adenocarinoma cells (PSMA and CD3 negative, ATCC cat. No. CL-188) in PBS/1% BSA/0.2 % sodium azide. As a negative control, the same cells were incubated with PBS/1% BSA/0.2 % sodium azide instead of COVA406 (PBS control). After 60 min incubation on ice, cells were washed, and bound protein was detected by incubation with 10 µg/ml mouse anti tetra-HIS antibody (Qiagen, cat no. 34670), followed by incubation with anti-mouse IgG – Alexa488 conjugate (Invitrogen) at a concentration of 10 ug/mL. Finally cells were washed three times and stained cells were then analyzed on a Guava easyCyte™ (Millipore) flow cytometer.

3) Redirected T-cell mediated cell cytotoxicity analysis

The polypeptide COVA406 (SEQ ID NO: 54) was tested in a redirected T-cell mediated cell cytotoxicity assay using a protocol adapted from Dreier *et.al.* (2002) Int. J. Cancer: 100, 690–697.

Human PBMCs were used as effector cells. On the day before the experiment PBMCs were isolated from fresh buffy coat preparations by Ficoll Plaque plus (GE Healthcare) and density gradient centrifugation using standard procedures. Isolated PBMCs were then incubated over night at a cell concentration of 4x10^6 cells/ml in 10% FCS, RPMI and 37°C, 5% CO_2.

For the cell kill experiment PBMCs were centrifuged and resuspended in 10% FCS, RPMI at a cell concentration of 2.5x10^7 cells/ml.

Target cells were labeled with Calcein AM by incubating cells at a final Calcein AM concentration of 10 µM for 30 min at 37°C, 5% CO_2. Subsequently excess dye was removed by washing cells twice with approx. 15 mL Medium. Finally target cell number was adjusted to 1*10^6 cells/ml. Target tumor cells were either 22Rv1 cells (PSMA positive, ATCC cat. No. CRL-2505) or HT29 colon carcinoma cells (PSMA negative, DSMZ cat. No. ACC-299).

Effector molecules were diluted in 10% FCS, RPMI to a maximum concentration of 1200 ng/mL. A dilution series of 1/10 dilutions was prepared.

Finally target cell suspension, effector cell suspension and the different concentrations of the polypeptide COVA406 (SEQ ID NO: 54) were then mixed in equal amounts. A total of 50000 target cells were added per well and the effector to target cell ratio was 25/1, The final maximal concentration of effector molecules was 400 ng/µl. Cell lysis was measured after 5 hours incubation at 37°C and 5% CO_2. After incubation, the cell suspension was centrifuged and cell lysis was quantified by detection of Calcein AM fluorescence in the supernatant using a fluorescence reader.

The amount of redirected cell lysis was normalized to the maximum lysis control (cells lysed by the addtion of 1% Triton X-100) and spontaneous lysis (target cells incubated with PMBCs in the absence of effector molecules). Percentage of cell lysis was calculated according to the following formula:

% lysis= (((fluorescence sample)-(fluorescence spontaneous lysis control)) / ((fluorescence maximum lysis control)-(fluorescence spontaneous lysis control))) x 100

All measurements were done in triplicates. Specific cell lysis was plotted versus the concentration of COVA406 and evaluated using Prism 5 (GraphPad Software) by fitting a sigmoidal dose-response.

4) Comparison of the pharmacokinetic profiles of COVA406 and the BITE® molecule

The pharmacokinetic profile of COVA406 in C57BL/6 mice (Charles River) was investigated and compared to the parental BITE® molecule. Five C57BL/6 mice were injected i.v. each with 48 µg COVA406 (SEQ ID NO: 54) or BITE® (SEQ ID NO: 53). After 10 and 30 min, 1, 3, 5, 7, 9, 12, 24, 28, 33 and 48 hours, blood was collected into EDTA coated microvettes (Sarstedt), centrifuged for 10 min at 9300 g and the serum levels of COVA406 or BITE® were determined by ELISA. Briefly, black maxisorp microtiter plates (Nunc) were coated with 10 µg/ml of a peptide derived from CD3 (Sequence: QDGNEEMGGITQTPYKVSISGTTVILT; SEQ ID NO: 55) (expressed as Fc-fusion) and incubated over night at 4°C. After blocking with 4 % milk (Rapilait, Migros, Switzerland) in PBS, serum samples at appropriate dilutions were applied, resulting in a final buffer concentration of 2% mouse serum (Sigma) and 4% milk. After incubation for 1 hr, wells were washed with PBS, and bound COVA406 or BITE® were detected with Penta-His-biotin (Qiagen) followed by Streptavidin-HRP conjugate (Sigma). The assay was developed with QuantaRed fluorogenic substrate (Pierce). The reaction was stopped after 3 min incubation and the fluorescence intensity was measured at 544 nm (excitation) and 590 nm (emission). The serum levels of COVA406 and BITE® were determined using a standard curve of COVA406 and BITE® (diluted to 333 - 0.5 ng/ml each). From the concentrations of COVA406 and BITE® determined in serum at different time points and the resulting slope k of the elimination phase (plotted in a semi-logarithmic scale), the half-lives were calculated using the formula $t_{1/2}$ = ln2/-k. Timepoints used for half-life calculation: COVA406: 1-48 h; BITE®: 1-12 h.

Result:

COVA406 (SEQ ID NO: 54) expressed with a similar yield as the BITE® molecule (SEQ ID NO: 53) (Table 4).

Table 4. Purification yields of the BITE® and Fyn-SH3 derived albumin-binding polypeptide fusions produced in transiently transfected CHO-S cells.

	SEQ ID NO:	Yield (mg/l)
BITE®	53	8.1
COVA406	54	5.0

The size exclusion chromatography (SEC) profile after purification demonstrated that COVA406 eluted as a single, monomeric peak showing that the fusion protein has excellent biophysical properties (Figure 3). Specific binding to PSMA-positive cells (22Rv1 cells) and CD3-positive cells (Jurkat E6-1, CD3 positive) was validated in a FACS experiment. Mean fluorescence intensities (MFI) of the stainings are depicted in Figure 4. Redirected T-cell mediated cell cytotoxicity was validated in a Calcein release assay using PBMCs as effector cells. Specific redirected cell-lysis of PSMA-positive cells with COVA406 (EC_{50} = 4.35 ng/ml) is shown in Figure 5. Cells with no PSMA expression (HT29 cells) were not lysed under the same conditions, showing that COVA406 is able to kill specifically PSMA positive cells. An improved pharmacokinetic profile of COVA406 (SEQ ID NO: 54) compared to the BITE® protein (SEQ ID NO: 53) was observed in mice. Figure 6 shows the serum concentrations (ng/ml) and terminal elimination phase of COVA406 and the parental BITE®. COVA406 shows a significantly better half-life (14.3 hours) compared to the BITE® (1.5 hours). This example shows that serum albumin binding proteins of the invention are able to prolong the *in vivo* half-life of otherwise short-lived molecules, in particular of BITE® molecules.

Example 4: Prior art Fynomers® which bind to serum albumin:

For Material and Methods, see Publications EP2054432 and "Grabulovski, Dragan: The SH3 domain of fyn kinase as a scaffold for the generation of new binding proteins. ETH Dissertation Nr 17216 (May 2007). http://dx.doi.org/10.3929/ethz-a-005407897".

Figure 7 shows specificity ELISA of Fyn SH3 variants isolated after affinity selections. None of the Fynomers® binds to HSA or HSA/rodent serum albumin, except for C3. However, C3 cross-reacts also with the non-related ovalbumin (hen egg white albumin). Therefore, C3 is considered as an unspecific binding protein.

CLAIMS

1. A polypeptide binding to human serum albumin and comprising or consisting of an amino acid sequence selected from the group consisting of:

(a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 1), wherein

(X^1) is A, V, I, L, M, G, P, S, T, N, Q, C, R, H, K, D or E;

(X^2) is R, H, K, A, V, I, L, M, G, P, S, T, N, Q or C;

(X^3) is R, H, K, S, T, N, Q, C, F, Y, W, A, V, I, L, M, G or P;

(X^4) is S, T, N, Q, C, A, V, I, L, M, G, P, R, H, K, F, Y, W, D or E;

(X^5) is S, T, N, Q, C, D, E, F, Y, W, A, V, I, L, M, G, P, R, H or K;

(X^6) is F, Y, W, A, V, I, L, M, G, P, R, H, K, S, T, N, Q or C;

(X^7) is A, V, I, L, M, G, P, R, H or K;

(X^8) is S, T, N, Q, C, D or E;

(X^9) is S, T, N, Q, C, D, E, A, V, I, L, M, G, P, F, Y or W;

(X^{10}) is A, V, I, L, M, G or P;

(X^{11}) is F, Y, W, R, H or K;

(X^{12}) is S, T, N, Q, C, F, Y or W;

(X^{13}) is F, Y, W, R, H, K, S, T, N, Q, C, D, E, A, V, I, L, M, G or P;

(X^{14}) is F, Y, W, A, V, I, L, M, G or P;

(X^{15}) is D, E, A, V, I, L, M, G or P;

(X^{16}) is A, V, I, L, M, G or P;

(X^{17}) is D, E, A, V, I, L, M, G, P, R, H or K;

(X^{18}) is S, T, N, Q, C, A, V, I, L, M, G or P;

(X^{19}) is F, Y, W, S, T, N, Q or C; and

(b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

2. The polypeptide of claim 1, wherein the polypeptide comprises or consists of an amino acid sequence selected from the group consisting of:

(a) GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 2), wherein

(X^1) is V, T, L, H, Q, E, R, G or M;

(X^2) is S, A, R, K, N, M, L or T;

(X^3) is N, S, R, H, Q, F, M, K or V;

(X^4) is T, Y, N, L, H, R, Q, W, M or E;

(X^5) is G, S, A, E, W, L, R or K;

(X^6) is F, Y, P, W, R or Q;
(X^7) is L or R;
(X^8) is Q or D;
(X^9) is N, D, A, Q, W, M or S;
(X^{10}) is L, I or V;
(X^{11}) is W or R;
(X^{12}) is T or F;
(X^{13}) is D, W, N, K, S, E or A;
(X^{14}) is W or G;
(X^{15}) is E or V;
(X^{16}) is A or V;
(X^{17}) is E, L or R;
(X^{18}) is T, S or M;
(X^{19}) is Y or S; and

(b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

3. The polypeptide of claim 1, wherein the polypeptide further binds to rodent serum albumin and comprises or consists of an amino acid sequence selected from the group consisting of:

(a)
GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 41), wherein
(X^1) is A, V, I, L, M, G, P, S, T, N, Q, C, R, H, K, D or E;
(X^2) is R, H, K, A, V, I, L, M, G, P, S, T, N, Q or C;
(X^3) is R, H, K, S, T, N, Q, C, F, Y, W, A, V, I, L, M, G or P;
(X^4) is S, T, N, Q, C, A, V, I, L, M, G, P, R, H, K, F, Y or W;
(X^5) is S, T, N, Q, C, D, E, F, Y, W, A, V, I, L, M, G, P, R, H or K;
(X^6) is F, Y, W, A, V, I, L, M, G, P, R, H, K, S, T, N, Q or C;
(X^7) is A, V, I, L, M, G, P, R, H or K;
(X^8) is S, T, N, Q, C, D or E;
(X^9) is S, T, N, Q, C, D or E;
(X^{10}) is A, V, I, L, M, G or P;
(X^{11}) is F, Y or W;
(X^{12}) is S, T, N, Q or C;
(X^{13}) is F, Y, W, R, H, K, S, T, N, Q, C, D, E, A, V, I, L, M, G or P;
(X^{14}) is F, Y, W, A, V, I, L, M, G or P;
(X^{15}) is D, E, A, V, I, L, M, G or P;
(X^{16}) is A, V, I, L, M, G or P;
(X^{17}) is D, E, A, V, I, L, M, G, P, R, H or K;
(X^{18}) is S, T, N, Q, C, A, V, I, L, M, G or P;

(X^{19}) is F, Y, W, S, T, N, Q or C; and

(b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

4. The polypeptide of claim 3, wherein the polypeptide comprises or consists of an amino acid sequence selected from the group consisting of:

(a)
GVTLFVALYDY(X^1)(X^2)(X^3)(X^4)(X^5)(X^6)D(X^7)SFHKGEKFQIL(X^8)(X^9)(X^{10})(X^{11})(X^{12})G(X^{13})(X^{14})W(X^{15})(X^{16})RSLTTG(X^{17})(X^{18})G(X^{19})IPSNYVAPVDSIQ (SEQ ID NO: 42), wherein

(X^1) is V, T, L, H, Q, E, R, G or M;

(X^2) is S, A, R, K, N, M, L or T;

(X^3) is N, S, R, H, Q, F, M, K or V;

(X^4) is T, Y, N, L, H, R, Q, W or M;

(X^5) is G, S, A, E, W, L or R;

(X^6) is F, Y, P, W, R or Q;

(X^7) is L or R;

(X^8) is Q or D;

(X^9) is N or D;

(X^{10}) is L or I;

(X^{11}) is W;

(X^{12}) is T;

(X^{13}) is D, W, N, K, S, E or A

(X^{14}) is W or G;

(X^{15}) is E or V;

(X^{16}) is A or V;

(X^{17}) is E, L or R;

(X^{18}) is T, S or M;

(X^{19}) is Y or S; and

(b) an amino acid sequence which is at least 90% identical to the amino acid sequence of (a), wherein the identity determination excludes amino acid positions (X^1) to (X^{19}).

5. The polypeptide of claim 4, wherein the amino acid sequence is selected from the amino acid sequences of SEQ ID NOs: 5, 4 or 6 to 32.

6. A fusion protein comprising the polypeptide of any one of claims 1 to 5 fused to a pharmaceutically and/or diagnostically active protein or peptide.

7. The fusion protein of claim 6, wherein the polypeptide is directly fused to the pharmaceutically and/or diagnostically active protein or peptide.

8. The fusion protein of claim 6, wherein the polypeptide is fused to the pharmaceutically and/or diagnostically active protein or peptide via a linker.

9. The fusion protein of any one of claims 6 to 8, wherein the pharmaceutically and/or diagnostically active protein or peptide is selected from the group consisting of a recombinant protein, an antibody, a blood factor, a hormone, an anticoagulans, a thrombolytic, a cytokine, a chemokine, and an interferon.

10. The fusion protein of claims 9, wherein the antibody is a bispecific T-cell engaging antibody.

11. A nucleic acid molecule encoding the polypeptide of any one of claims 1 to 5 or the fusion protein of any one of claims 6 to 10.

12. A vector comprising the nucleic acid molecule according to claim 11.

13. An isolated cell comprising the nucleic acid molecule of claim 11 or the vector of claim 12.

14. A method of producing the polypeptide of any one of claims 1 to 5, or the fusion protein of any one of claims 6 to 10 comprising
(a) culturing the isolated cell of claim 13, and
(b) isolating the produced polypeptide or fusion protein.

15. A pharmaceutical and/or diagnostic composition comprising the fusion protein of any one of claims 6 to 10.

Figure 1:

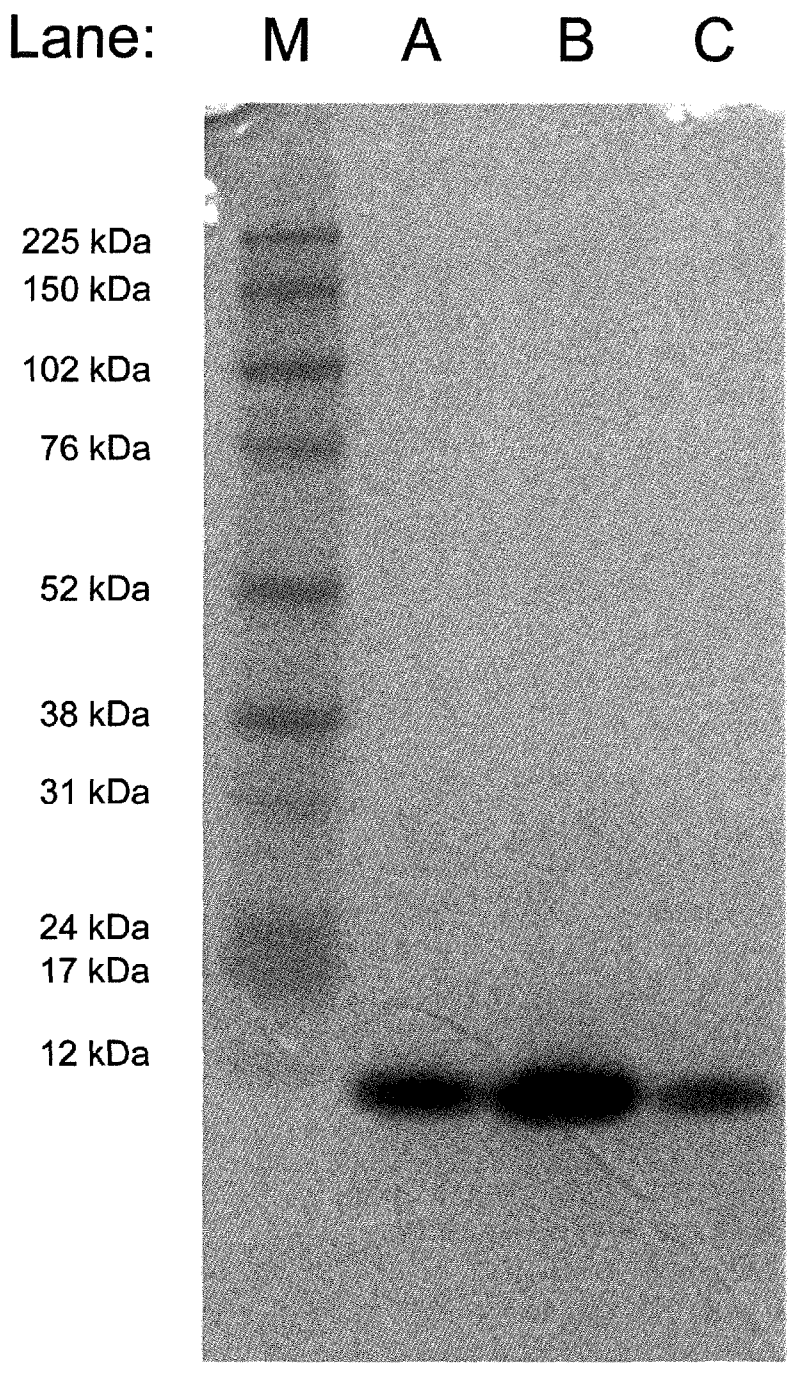

Lane: M A B C
225 kDa
150 kDa
102 kDa
76 kDa
52 kDa
38 kDa
31 kDa
24 kDa
17 kDa
12 kDa

Figure 2:

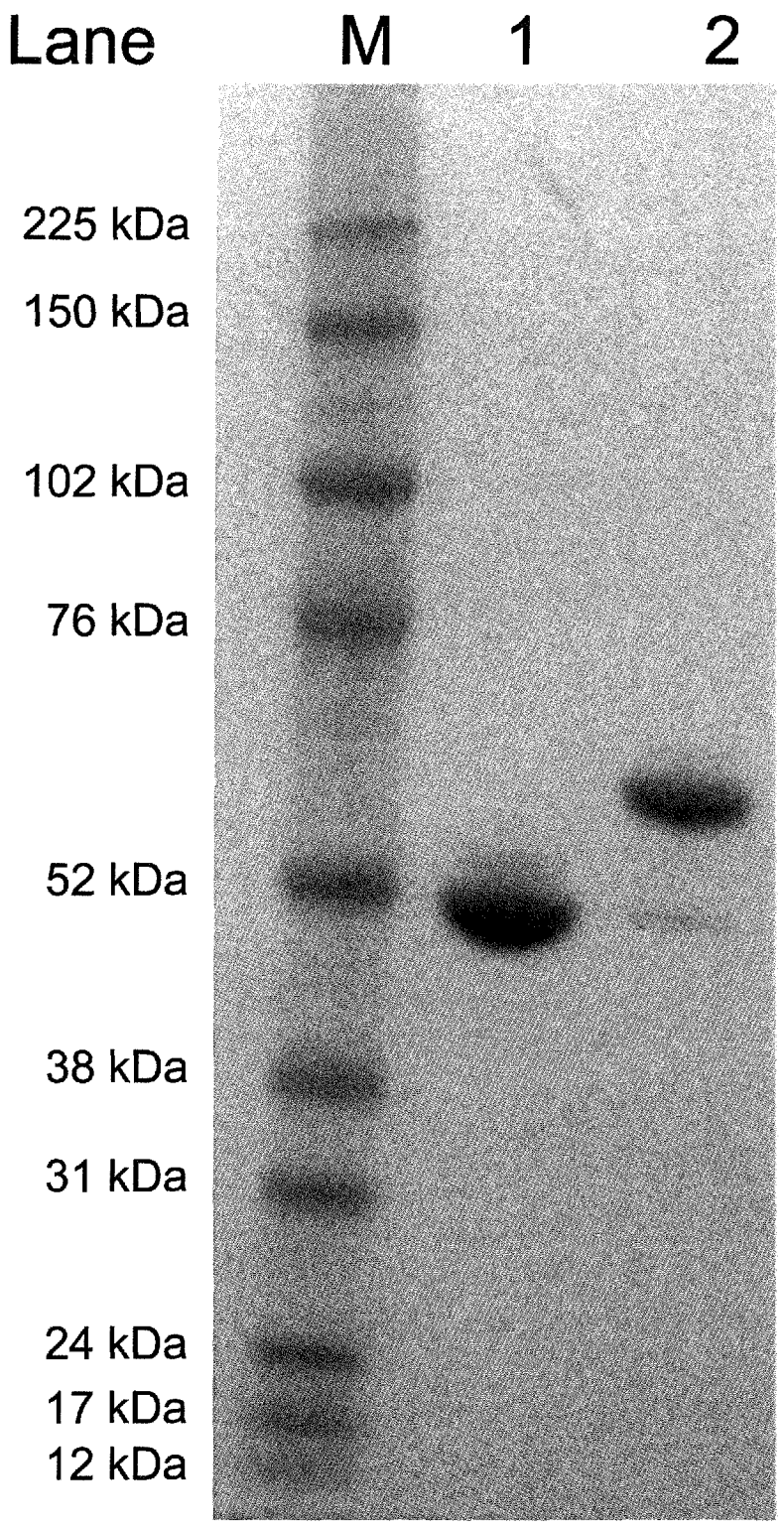

Lane
M
1
2
225 kDa
150 kDa
102 kDa
76 kDa
52 kDa
38 kDa
31 kDa
24 kDa
17 kDa
12 kDa

Figure 3:

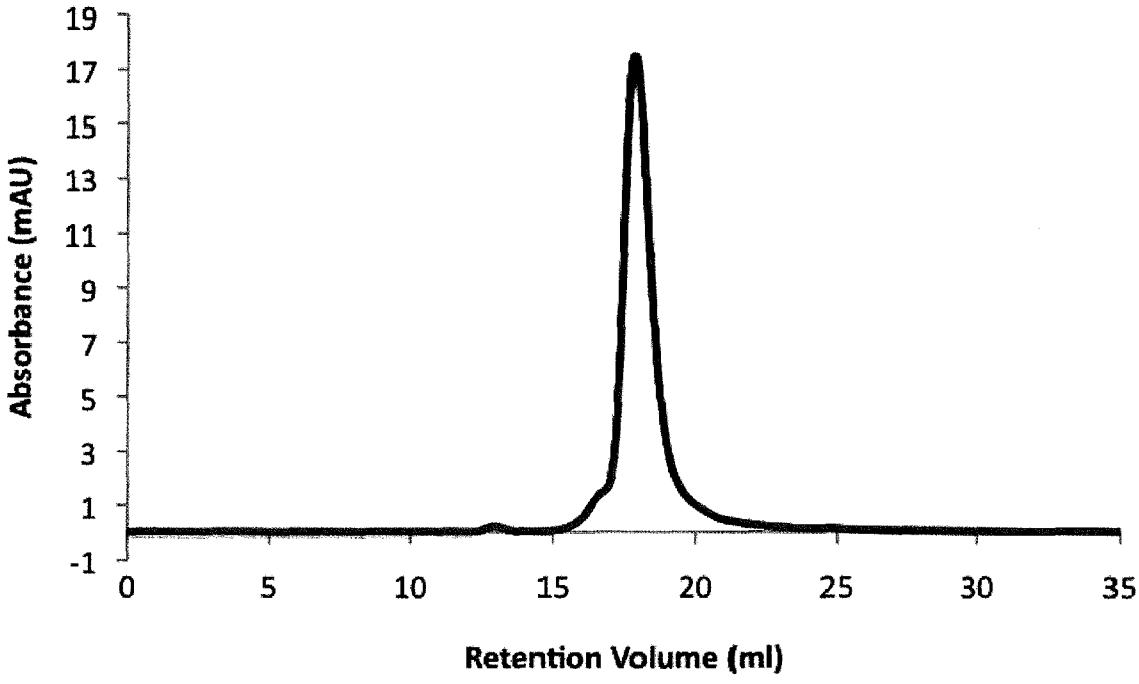
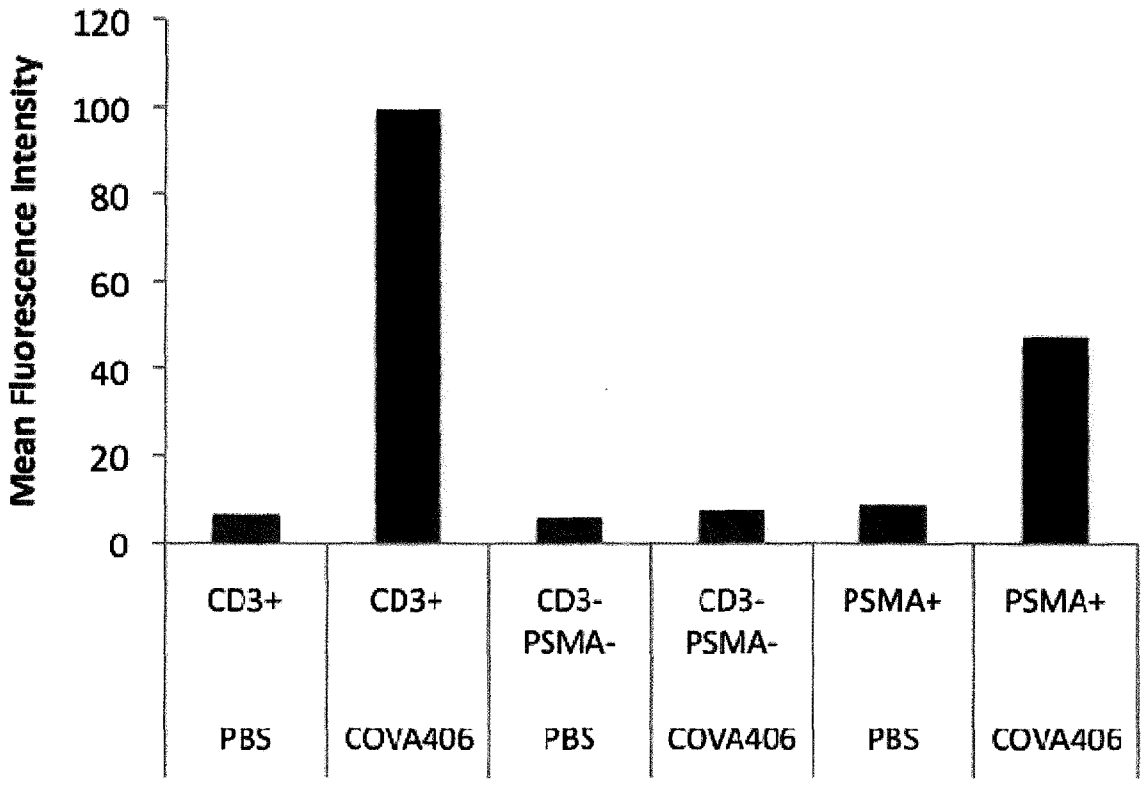
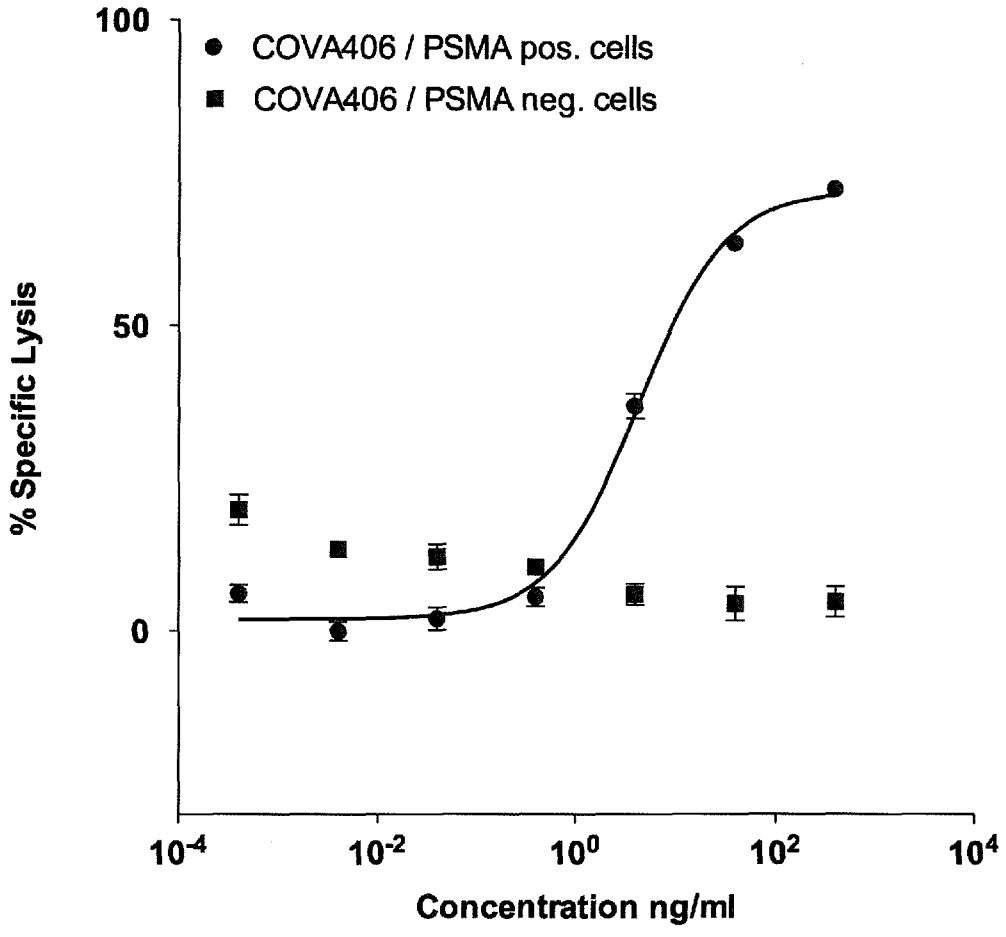

19
17
15
13
11
9
7
5
3
1
-1
Absorbance (mAU)
0
5
10
15
20
25
30
35
Retention Volume (ml)

Figure 4:


Mean Fluorescence Intensity
120
100
80
60
40
20
0
CD3+
PBS
CD3+
COVA406
CD3-
PSMA-
PBS
CD3-
PSMA-
COVA406
PSMA+
PBS
PSMA+
COVA406

5

Figure 5:


100
50
0
% Specific Lysis
COVA406 / PSMA pos. cells
COVA406 / PSMA neg. cells
10-4
10-2
100
102
104
Concentration ng/ml

5

Figure 6:

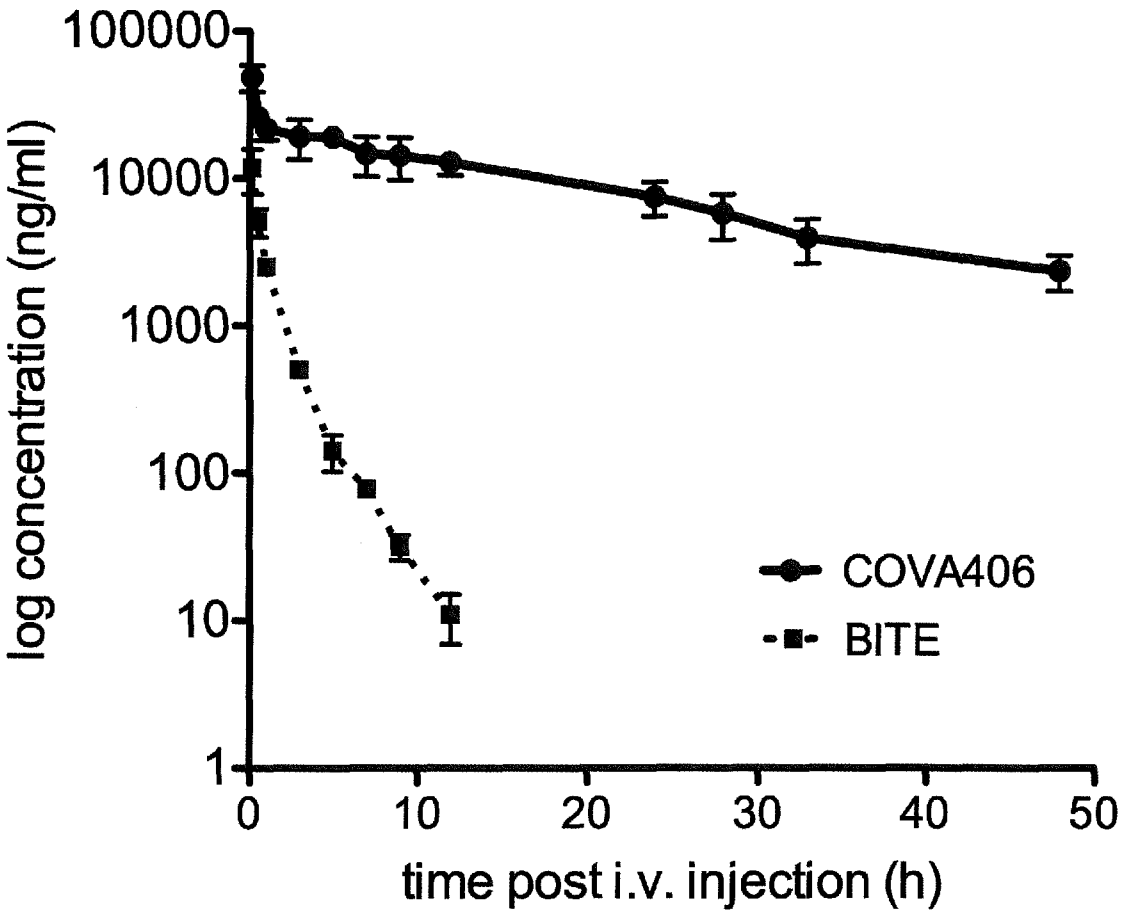
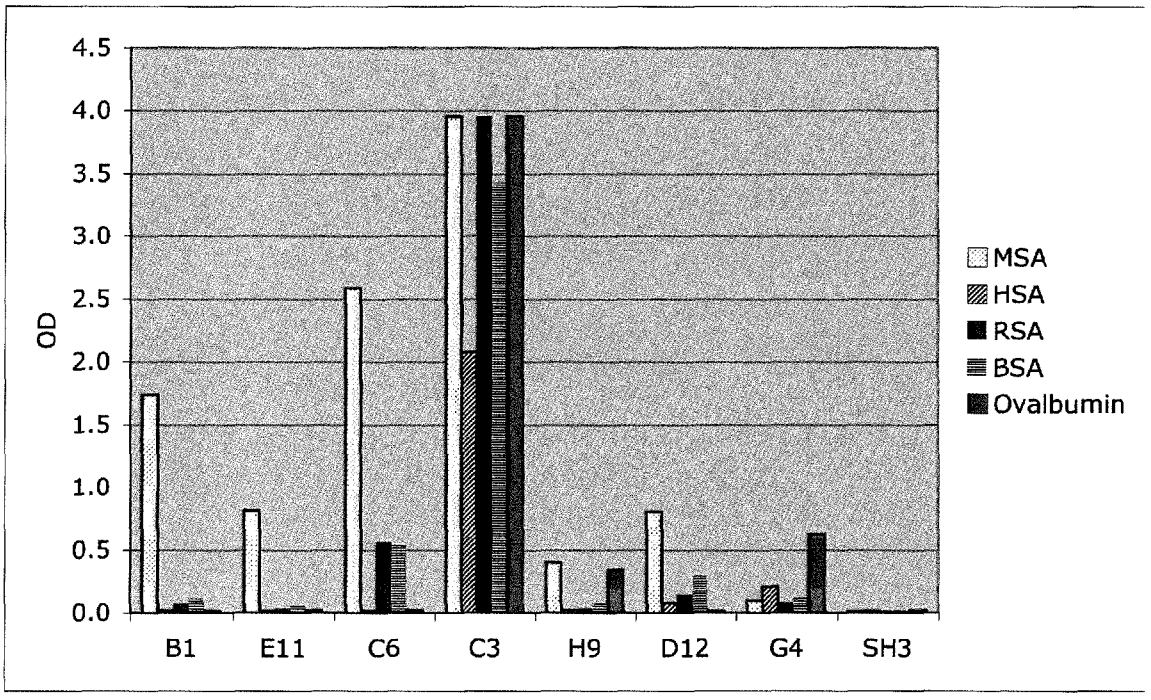

100000
10000
1000
100
10
1
log concentration (ng/ml)
0
10
20
30
40
50
time post i.v. injection (h)
COVA406
BITE

Figure 7: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4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OD
B1
E11
C6
C3
H9
D12
G4
SH3
MSA
HSA
RSA
BSA
Ovalbumin

Figure 8:

```
CLUSTAL W (1.83) multiple sequence alignment
C1 (SEQ ID NO: 4)
GVTLFVALYDYHAHLGYDLSFHKGEKFQILQDLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

17H (SEQ ID NO: 5)
GVTLFVALYDYQSRHGFDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

AB15F (SEQ ID NO: 6)
GVTLFVALYDYVSNTGFDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

AB19C4 (SEQ ID NO: 7)
GVTLFVALYDYTASYGYDLSFHKGEKFQILDDIWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM5H5 (SEQ ID NO: 8)
GVTLFVALYDYLRRNSPDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM7G4 (SEQ ID NO: 9)
GVTLFVALYDYHAHLGYDLSFHKGEKFQILQDLWTGALWRARSLTTGRMGSIPSNYVAPVDSIQ

SAM23C5 (SEQ ID NO: 10)
GVTLFVALYDYQSRHGFDLSFHKGEKFQILQNLWTGEWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM22A2 (SEQ ID NO: 11)
GVTLFVALYDYQSRHGFDLSFHKGEKFQILQNLWTGSWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM23F4 (SEQ ID NO: 12)
GVTLFVALYDYQSRHGFDLSFHKGEKFQILQNLWTGKWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM11C1 (SEQ ID NO: 13)
GVTLFVALYDYEKQHAWDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM11E6 (SEQ ID NO: 14)
GVTLFVALYDYRSSYEWDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM23C9 (SEQ ID NO: 15)
GVTLFVALYDYQSRHGFDLSFHKGEKFQILQNLWTGNWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM19G2 (SEQ ID NO: 16)
GVTLFVALYDYHAHLGYDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM19F9 (SEQ ID NO: 17)
GVTLFVALYDYGNFRWRDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM20F11 (SEQ ID NO: 18)
GVTLFVALYDYQSRHGFDLSFHKGEKFQILQNLWTGWGWVARSLTTGLSGYIPSNYVAPVDSIQ

SAM21D10 (SEQ ID NO: 19)
GVTLFVALYDYTSMRGYDLSFHKGEKFQILQDLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM21E1 (SEQ ID NO: 20)
GVTLFVALYDYQSKHGYDLSFHKGEKFQILQDLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM21G3 (SEQ ID NO: 21)
GVTLFVALYDYLMKTLADLSFHKGEKFQILQDLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM22B3 (SEQ ID NO: 22)
GVTLFVALYDYTLRQWKDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ
```

Figure 8 - continued:

SAM22B8 (SEQ ID NO: 23)
GVTLFVALYDYERQHGFDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM22B7 (SEQ ID NO: 24)
GVTLFVALYDYESRHGYDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM22E3 (SEQ ID NO: 25)
GVTLFVALYDYQSKQGFDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM28B1 (SEQ ID NO: 26)
GVTLFVALYDYLLQWRQDLSFHKGEKFQILQDLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM28B9 (SEQ ID NO: 27)
GVTLFVALYDYTSSHGFDLSFHKGEKFQILQDLWTGDWWEVRSLTTGETGYIPSNYVAPVDSIQ

SAM30H2 (SEQ ID NO: 28)
GVTLFVALYDYEAHLGYDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM30D6 (SEQ ID NO: 29)
GVTLFVALYDYTAVHGYDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM30B8 (SEQ ID NO: 30)
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SAM30B12 (SEQ ID NO: 31)
GVTLFVALYDYMSQMGYDLSFHKGEKFQILQNLWTGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM31H9 (SEQ ID NO: 32)
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AB8F11 (SEQ ID NO: 33)
GVTLFVALYDYTARTGYDLSFHKGEKFQILDAVRFGDWWEARSLTTGETGYIPSNYVAPVDSIQ

AB18G9 (SEQ ID NO: 34)
GVTLFVALYDYQSREKFDLSFHKGEKFQILDQLRFGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM14D1 (SEQ ID NO: 35)
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SAM14C3 (SEQ ID NO: 36)
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SAM17F1 (SEQ ID NO: 37)
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SAM17B3 (SEQ ID NO: 38)
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SAM17F3 (SEQ ID NO: 39)
GVTLFVALYDYQSRHGFDLSFHKGEKFQILDMLRFGDWWEARSLTTGETGYIPSNYVAPVDSIQ

SAM17H4 (SEQ ID NO: 40)
GVTLFVALYDYHAHLGYDLSFHKGEKFQILDSLRFGDWWEARSLTTGETGYIPSNYVAPVDSIQ

********** * **********: : * * .****** * **********

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/077227

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/765 C07K14/47 C12N1/21 C12N1/15 C12N5/10
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

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, WPI Data, BIOSIS, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 892 248 A1 (EIDGENOESS TECH HOCHSCHULE [CH]) 27 February 2008 (2008-02-27) cited in the application Whole document, especially example 2 and figure 5 -----	1-15
A	EP 2 524 927 A1 (COVAGEN AG [CH]) 21 November 2012 (2012-11-21) Whole document, especially claim 1 -----	1-15
	-/--	

[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

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"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

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Date of the actual completion of the international search	Date of mailing of the international search report
7 February 2014	17/02/2014

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	Kools, Patrick

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