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(54) **Title:** PROTEINS WITH IMPROVED HALF-LIFE AND OTHER PROPERTIES

(57) **Abstract:** The invention provides methods and materials for making and using variant serum albumin amino acid sequences which exhibit improved properties compared to wild type serum albumin sequences. The invention further provides methods and materials for making and using fusion proteins in which the variant serum albumin amino acid sequences are fused to a therapeutic or diagnostic agent, such as a therapeutic protein, or a functional fragment or variant thereof that maintains activity, and exhibits improved properties.

PROTEINS WITH IMPROVED HALF-LIFE AND OTHER PROPERTIES

RELATED APPLICATIONS

5 The present application claims priority to U.S. Application Serial No. 61/561785, filed November 18, 2011; U.S. Application Serial No. 61/576491, filed December 16, 2011; and U.S. Application Serial No.61/710476, filed October 5, 2012. The entire content of each of the foregoing applications is hereby incorporated herein by reference.

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SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 16, 2012, is named D2046703.txt and is 43,129 bytes in size.

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FIELD OF THE INVENTION

The present invention relates to the area of therapeutic and diagnostic proteins, materials for producing such proteins, and methods for their production and use.

BACKGROUND

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Serum albumin is an abundant plasma protein that that is essential for maintaining oncotic pressure and is useful in regulating the volume of circulating blood. Serum albumin has therapeutic uses and is indicated, for example, to treat hypovolemia and hypoalbuminemia (e.g., hypoalbuminemia associated with inadequate production, excessive catabolism, hemorrhage, excessive renal excretion, redistribution within the body e.g., due to surgery or inflammatory conditions, burns, adult respiratory distress syndrome, nephrosis), and may be used prior to or during cardiopulmonary bypass surgery or to

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treat hemolytic disease of the newborn. Serum albumin can also be used in association with other agents.

SUMMARY

There is a need for improved variant sequences of serum albumin. Such variant sequences can be used, e.g., to prepare or provide a protein product (e.g., a therapeutic or diagnostic protein product). In

30 embodiments the protein product has improved properties (e.g., higher recycling fraction via an FcRn mechanism and/or improved pharmacokinetic properties, e.g., increased half-life and/or reduced clearance), e.g., as compared with a protein product that is prepared using a corresponding native (e.g., wild type) serum albumin sequence (e.g., a mammalian serum albumin, e.g., human serum albumin or bovine serum

35 albumin). As used herein, a “corresponding native serum albumin” (e.g., a naturally occurring serum

albumin or a wild type serum albumin) refers to a corresponding full length native serum albumin sequence, or fragment thereof, of which the variant serum albumin polypeptide (VSA) is a variant. The disclosure provided herein includes disclosure of proteins that comprise variant serum albumin sequences and methods of using such proteins.

5 In some aspects, the disclosure provides a variant serum albumin polypeptide (VSA). In some embodiments, the variant is a variant of a full-length serum albumin polypeptide sequence (e.g., a human serum albumin polypeptide sequence, or a serum albumin polypeptide sequence from another species, e.g., a bovine serum albumin polypeptide sequence). In other embodiments, the variant is a variant of a fragment of a serum albumin polypeptide sequence (e.g., a fragment of the human serum albumin
10 polypeptide sequence, or a fragment of a serum albumin polypeptide sequence from another species, e.g., a fragment of the bovine serum albumin polypeptide sequence). In some embodiments, the fragment comprises a domain III of a serum albumin polypeptide sequence (e.g., domain III of the human serum albumin polypeptide sequence).

 In some aspects, the present disclosure provides an isolated, recombinant protein that comprises a
15 VSA, e.g., a VSA that is or comprises a variant of domain III of a naturally-occurring serum albumin. In some embodiments, the VSA is in a polypeptide that also includes a heterologous sequence, e.g., a heterologous sequence as described herein. For example, a fusion polypeptide can comprise a VSA and a heterologous sequence.

 In some embodiments, the VSA has or comprises a mutation at one or more of the positions
20 corresponding to V418, T420, V424, E505 and V547 of serum albumin.

 In some embodiments, the VSA can bind to an FcRn (e.g., human FcRn) at a pH in the range of 5.5 to 6.0 (e.g., at a pH of 5.5, 5.6, 5.7, 5.8, 5.9, or 6.0). In some embodiments, the VSA binds to an FcRn (e.g., human FcRn) with higher affinity than does a corresponding native serum albumin. In some
25 embodiments, the higher affinity binding occurs at a specified pH (e.g., at a pH of 5.5, 5.6, 5.7, 5.8, 5.9, 6.0) or in a specified pH range, e.g., at a pH in the range of 5.5- 6.0 or 6.0-6.5.

 In some embodiments, the ratio of the binding affinity of the VSA, or of the recombinant protein comprising the VSA, at a pH of 5.5 to 6.0 to the binding affinity at a pH of 7.0 to 7.4 is greater than or equal to the ratio for a corresponding native serum albumin (e.g., a corresponding native human serum albumin). In some embodiments, the ratio of the binding affinity of the VSA, or of the recombinant protein
30 comprising the VSA, at a pH of 5.5 to 6.0 to that at a pH of 7.0 to 7.4 is 5; 10; 50; 100; 1000; 10,000; 100,000; or 1 million times that of a corresponding native human albumin.

 In some embodiments, the VSA, or the recombinant protein comprising the VSA, binds to FcRn at a pH in the range of 7.0 to 7.4 with an affinity not greater than a corresponding native human albumin.

 In some embodiments, the disclosure provides an isolated, recombinant protein comprising a VSA
35 that has altered binding properties for an FcRn (e.g., a human FcRn) relative to a corresponding native serum albumin sequence (e.g., the wild-type human serum albumin sequence). The protein can include a heterologous sequence as described herein. In some embodiments, the recombinant protein comprising

aVSA binds to FcRn with an affinity that is altered compared to a recombinant protein comprising a wild type albumin (e.g., a wild type human albumin) and the heterologous sequence. . In some embodiments, the VSA or recombinant protein comprising the VSA binds to FcRn with a dissociation constant (K_D) of less than 50 nM at pH 5.5. In some embodiments, the VSA or recombinant protein has an affinity for FcRn at pH 7.4 that is less than or equal to its affinity for FcRn of a wild type albumin at pH 7.4.

In some embodiments, the VSA has a K_D that is below 100 nM. In some embodiments, the VSA has a K_D that is below 75 nM, below 50 nM, below 40 nM, below 30 nM, below 25 nM, below 20 nM, below 15 nM, below 10 nM, below 9 nM, below 8 nM, below 7 nM, below 6 nM, below 5 nM, or below 4 nM. In some embodiments, the protein has a K_D that is 3 nM or less.

In certain embodiments, the VSA comprises a substitution of V418 with another amino acid, for example, with a methionine. In certain embodiments, the VSA comprises a substitution of T420 with another amino acid, for example, an uncharged amino acid, such as alanine. In certain embodiments, the VSA comprises a substitution of V424 with another amino acid, for example, an uncharged amino acid, such as isoleucine. In certain embodiments, the VSA comprises a substitution of E505 with another amino acid, for example, an uncharged amino acid, such as glycine, or a positively charged amino acid, such as lysine or arginine. In certain embodiments, the VSA comprises a substitution of V547 with another amino acid, for example, an uncharged amino acid, such as alanine.

In some embodiments, the VSA comprises substitutions at two or more (e.g., at two, three, four, or five) of the positions corresponding to V418, T420, V424, E505 and V547. For example, the VSA can comprise two, three, four, or five substitutions selected from V418M, T420A, V424I, E505(R/K/G) and V547A. In a particular embodiment, the VSA comprises the substitutions V418M, T420A and E505R. In another particular embodiment, the VSA comprises the substitutions V418M, T420A, E505G and V547A.

In some embodiments, the VSA comprises one or more additional substitutions at positions selected from N429, M446, A449, T467, and A552. In some embodiments, the substitutions are selected from N429D, M446V, A449V, T467M, and A552T.

In some embodiments, a protein provided herein includes a VSA that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, but less than 100% identical to domain III of a naturally occurring serum albumin, e.g., a human serum albumin. In certain embodiments, the protein includes a VSA that is at least 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, but less than 100% identical to domain III of human serum albumin.

In some embodiments, a protein provided herein includes a VSA that differs by at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 residues from a corresponding native serum albumin, e.g., a corresponding human serum albumin sequence. In some embodiments, a protein provided herein includes a VSA that differs by at least 1, but no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or 20 residues from a corresponding native serum albumin e.g., a corresponding human serum albumin sequence. In embodiments the differences are in domain III, e.g., in domain III of human serum albumin.

In some aspects, the disclosure provides an agent in association with a VSA, e.g., a VSA as described herein. In some embodiments, the agent is a therapeutic or diagnostic agent, e.g., an agent as described herein. In some embodiments, the agent is fused to the VSA or to a protein that comprises the VSA, e.g., in the form of a fusion protein. For example, a VSA can be fused to a heterologous sequence using recombinant genetic techniques. A heterologous sequence can comprise, e.g., the agent, a protein that comprises the agent, or a sequence to which the agent is attached or can be attached.

In some aspects, the disclosure provides an isolated, recombinant protein that comprises a VSA as described herein and a heterologous sequence, e.g., a heterologous sequence as described herein (e.g., the heterologous sequence can be a cytokine, immunoglobulin, cell surface receptor, coagulation protein, or functional fragment of any of these). The protein can also include more than one heterologous sequence. In some embodiments, the VSA is a variant of domain III of a naturally-occurring serum albumin. In some embodiments the recombinant protein is a fusion protein.

In some embodiments, the heterologous sequence comprises a cytokine domain, for example, an amino acid sequence originating or derived from an interleukin-2. In some embodiments, the heterologous sequence comprises an immunoglobulin single-chain variable domain, e.g., an scFv. In some embodiments, the heterologous domain is a scaffold, e.g., an anti-calix, a DARPin, a Surrobody™, Adnectin™, a Domain antibody, Affibody, or fragment of any of the foregoing. In some embodiments, the heterologous sequence comprises a soluble fragment of a cell surface receptor. In some embodiments, the heterologous sequence comprises an enzyme. In still other embodiments, the heterologous sequence comprises a component of a coagulation protein, such as an amino acid sequence originating or derived from coagulation factor VII (FVII) or coagulation factor VIII (FVIII). In some embodiments, the heterologous sequence comprises two or more of the foregoing.

In certain aspects, the protein comprises a first and a second heterologous sequence. In some embodiments, the first and the second heterologous sequence are identical. In some embodiments, the first and second heterologous sequence are in tandem. In other embodiments, the first heterologous sequence is located N-terminal to the variant sequence and the second heterologous sequence is located C-terminal to the variant sequence.

In another aspect, the present disclosure provides an isolated recombinant DNA molecule and/or nucleotide sequence that encodes a polypeptide, peptide, protein or recombinant protein disclosed herein. The recombinant DNA molecule and/or nucleotide sequence comprises a variant nucleotide sequence that encodes a VSA. In some embodiments, the VSA includes at least a variant of domain III of a naturally-occurring serum albumin.

In other aspects, the present disclosure provides a recombinant host cell (e.g., a recombinant CHO cell, or a *Saccharomyces cerevisiae* cell) that has been transformed with a recombinant DNA molecule or nucleotide sequence that encodes a polypeptide, peptide, protein or recombinant protein as disclosed herein. Unless otherwise noted, the term protein includes polypeptides and peptides.

In some aspects, the disclosure provides a method of treatment or diagnosis, the method comprising administering to a subject (e.g., a human or non-human animal) a VSA (e.g., a VSA as described herein), or a pharmaceutical composition comprising a VSA, e.g., a VSA as described herein. In some embodiments, the subject is suffering from a disease or condition in which albumin is indicated. In some embodiments the disease or condition is selected from hypovolemia, hypoalbuminemia, a burn, adult respiratory distress syndrome, nephrosis, and hemolytic disease of the newborn.

In some aspects, the disclosure provides a method of treatment and/or a method of diagnosis, wherein the method comprises administering to a subject an agent (e.g., a therapeutic or diagnostic agent) in association with a VSA (e.g., a VSA as described herein). In some embodiments, the method is a method of treatment that comprises administering to a subject a therapeutic agent in association with a VSA, e.g., a VSA as described herein. In some embodiments, the method is a method of diagnosis that comprises administering to a subject a diagnostic agent in association with a VSA, e.g., a VSA as described herein.

The agent can be any biologically or pharmaceutically active moiety (e.g., a biologic, such as a peptide or nucleotide sequence or a chemical entity such as a small molecule). Typically, the agent is administered in an effective amount and at an effective frequency, e.g., in an amount and frequency that is effective for the desired therapeutic or diagnostic purpose. In some embodiments, the agent is a known therapeutic or diagnostic agent. In some embodiments, the agent comprises an entire known protein, nucleotide sequence or chemical entity. In some embodiments, the agent comprises a fragment or variant of a known protein, nucleotide sequence or chemical entity. The fragment or variant can have the same or similar activity as does the known protein, nucleotide sequence or chemical entity, or the fragment or variant can have altered activity, e.g., an increased desirable activity or a reduced undesirable activity compared with the known protein, nucleotide sequence or chemical entity.

In some embodiments, administering the agent in association with the VSA results in an improved function of the agent. In some embodiments, the improved function is an improved pharmacokinetic property (e.g., increased half- life and/or reduced clearance) of the agent. In some embodiments, the improved function is a reduced effective dosage and/or a reduced effective frequency of administration of the agent compared with the agent when it is administered without the association with VSA. In some embodiments, the improved function is a more desirable delivery level of the agent (e.g., a higher, more consistent, and/or less variable level of delivery). An improved function of the agent can be established, e.g., by comparing the function of the agent when it is administered in association with the VSA with the same function of the agent when it is administered without the association with the VSA (e.g., when the agent is administered alone or when it is administered in association with a corresponding native serum albumin sequence).

In some embodiments in which the agent (e.g., therapeutic or diagnostic agent) is in association with the VSA, the agent is fused to the VSA. In some embodiments, the agent is fused to the VSA via a covalent bond. In some embodiments, the agent is fused to the VSA via a peptide bond. In some

embodiments, the agent is fused to the VSA via a non-peptide bond. In some embodiments, the agent is fused to the VSA via a linker, e.g., a linker as described herein. In some embodiments, the agent is fused to the VSA using recombinant genetic methods. In some embodiments, the agent is fused to the VSA using chemical methods.

5 In some embodiments in which the agent (e.g., therapeutic or diagnostic agent) is in association with the VSA, the agent is not fused to the VSA. In some embodiments, the agent is not fused to the VSA and is stably associated with the VSA. "Stably associated" means that the VSA and the agent can be or remain physically associated at a pH at which they are intended to function, e.g., at an endosomal pH. Typically, the agent and VSA also can be or remain physically associated at a neutral pH. In embodiments,
10 the agent can be associated with the VSA by any means known in the art. For example, the agent can be conjugated to a moiety that is capable of binding the VSA. In some embodiments, the moiety is an albumin binding protein. In some embodiments the moiety is a fatty acid. In some embodiments, the agent is non-covalently bound to the VSA. In embodiments wherein the agent is not fused to the VSA, the agent can be administered before, after, or concurrently with the VSA. In some embodiments, the agent is administered
15 concurrently with the VSA. In some embodiments, the agent is administered at the same frequency as is the VSA. In some embodiments, the agent is administered more or less frequently than the VSA. An advantage of using a VSA can be that a VSA can retain lipophilic features of an albumin, thereby improving solubility of a molecule (e.g., a therapeutic agent).

In some aspects, the disclosure provides a method of treating a subject, the method comprising
20 administering to the subject an effective amount of a therapeutic agent in association with a VSA (e.g., a VSA as described herein), such that the dosage and/or frequency of administration at which the agent produces a therapeutic effect is reduced relative to the dosage and/or frequency of administration at which the agent produces a therapeutic effect when it is not in association with the albumin protein. In some
25 embodiments, In some embodiments, the agent comprises a polypeptide component that is fused to the VSA or to a protein comprising the VSA. In some embodiments, the polypeptide component and the VSA or protein that comprises the VSA are separated by a linker sequence. In some embodiments, the polypeptide component and the VSA or protein that comprises the VSA are covalently linked by a non-peptide bond. In some embodiments, the polypeptide component and the VSA or protein that comprises
the VSA are non-covalently and stably associated.

30 In additional aspects, the present disclosure provides a method of engineering a VSA associated therapeutic agent or a VSA associated diagnostic agent. The method comprises providing a biologically or pharmaceutically active agent (e.g., an agent as described herein) and associating the agent with a VSA as described herein to provide a VSA associated agent; i.e., a VSA associated therapeutic agent or a VSA associated diagnostic agent. "Associating" the agent with the VSA can be done by any means known in the
35 art or any means described herein, e.g., by fusing the agent to the VSA using recombinant genetic and/or chemical means, by conjugating the agent to a moiety that is capable of binding the VSA, or by non-covalent methods.

In some embodiments, the method further comprises formulating the albumin modified agent, or providing a formulation or pharmaceutical composition, for administration to a subject, such as a human subject. In embodiments including an albumin modified diagnostic agent, the method may further comprise administering the albumin modified diagnostic agent to a subject and detecting the albumin modified diagnostic agent, for example, a method wherein the subject is imaged. In embodiments the formulation is in a storage or delivery device, e.g., a syringe.

In some aspects, the present disclosure provides a formulation that comprises a biologically or pharmaceutically active agent (e.g., an agent as described herein) in association with VSA.

Calculations of “homology” or “sequence identity” between two sequences (the terms are used interchangeably herein) can be performed as follows. The sequences are aligned according to the alignments provided herein, or, in the absence of an appropriate alignment, the optimal alignment determined as the best score using the Needleman and Wunsch algorithm as implemented in the Needle algorithm of the EMBOSS package using a Blosum 62 scoring matrix with a gap penalty of 10, and a gap extend penalty of 1. (See Needleman, S. B. and Wunsch, C. D. (1970) *J. Mol. Biol.* 48: 443-453; Kruskal, J. B. (1983) An overview of sequence comparison In D. Sankoff and J. B. Kruskal, (Eds.), Time Warps, String Edits and Macromolecules: the Theory and Practice of Sequence Comparison, pp. 1-44, Addison Wesley, and tools available from the European Bioinformatics Institute (Cambridge UK), described in Rice, P. et al. (2000) *Trends in Genetics* 16(6): 276-277 and available online at www.ebi.ac.uk/Tools/emboss/align/index.html and emboss.open-bio.org/wiki/Appdoc:Needle.) The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid “identity” is equivalent to amino acid or nucleic acid “homology”). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences. To determine collective identity of one sequence of interest to a group of reference sequences, a position is considered to be identical if it is identical to at least one amino acid at a corresponding position in any one or more of the group of reference sequences. With respect to lists of segments, features, or regions, identity can be calculated collectively for all members of such list to arrive an overall percentage identity.

Provided herein are sequences that are at least 60, 65, 70, 75, 80, 82, 85, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to sequences disclosed herein.

As used herein, the singular forms “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise.

All patents, patent applications, scientific publications and other references cited in this specification are hereby incorporated herein for all purposes, and for the disclosure for which they have been cited.

DRAWINGS

FIG. 1 is a set of graphs depicting the results of SPR experiments determining the binding of the variant serum albumin polypeptides (VSAs) HSA-5, HSA-11, HSA-7, and HSA-13 to human FcRn at pH 5.5, pH 6.0, pH 6.5, and pH 7.4.

5 FIG. 2 is a table depicting the K_{DS} as determined by ELISA of variant serum albumin polypeptides (VSAs) at pH 5.5.

FIG. 3 is a set of graphs depicting the results of ELISA that determining the binding of VSAs and wild type HSA to human FcRn at pH 5.5, 6.0, and 7.4.

10 FIG. 4 is a set of graphs depicting the pharmacokinetics of VSAs (HSA-5, HSA-11, and HSA-13) and wild type human serum albumin (wt-HSA) in wild type mice. The left panel shows plasma concentration over time. The right upper panel shows half-life, and the right lower panel shows clearance.

FIG. 5 is a set of graphs that depicting the pharmacokinetics of VSAs (HSA-5, HSA-11, and HSA-13) and wild type HSA in human FcRn transgenic mice. The left panel shows plasma concentration over time. The right upper panel shows half-life, and the right lower panel shows clearance.

15 FIG. 6 is a table depicting the PK parameters of a wild type HSA (HSA-wt) and a VSA (HSA-7) in a primate.

FIG. 7 is a graph and a table depicting the pharmacokinetics of IL-2 fused to wild type HSA and to a VSA (HSA-13) in C57Bl/6J mice.

20 DETAILED DESCRIPTION

One limitation of certain agents (e.g., therapeutic or diagnostic agents) can be their suboptimal pharmacokinetics (PK), particularly when administered systemically. Relatively short PKs (as assessed, e.g., by plasma half-life of the agents) can mean, for example, that a therapeutic must be administered at a relatively high frequency. The present disclosure provides VSAs that have improved functional properties, e.g., improved pharmacokinetics (e.g., increased half-life, reduced clearance, and/or reduced beta phase clearance). The VSAs can be useful themselves, e.g., for therapeutic or diagnostic uses. The VSAs can be used to improve the functional properties, e.g., the pharmacokinetics, of other agents.

The present disclosure provides variant serum albumin polypeptides (VSAs). As used herein, a variant serum albumin polypeptide (VSA) can refer to a variant of a full length native serum albumin or to a variant of a fragment of a native serum albumin (e.g., a variant of a functional fragment of a native serum albumin). Typically, a VSA that is a variant of a fragment of a native serum albumin has a minimal length that provides functionality (e.g., an ability to bind FcRn at endosomal pH). In some embodiments, the variant comprises or consists of a sequence that is a variant of the domain III sequence of a native serum albumin. In some embodiments, the variant comprises a sequence that is a variant of the domain III sequence of a native human serum albumin sequence. In some embodiments, the affinity of a VSA for an FcRn is increased at an endosomal pH (e.g., pH 5.5 or 6.0) compared to the affinity of wild type albumin corresponding to the VSA. In certain embodiments, the affinity of a VSA for an FcRn is increased at an

endosomal pH (e.g., pH 5.5 or 6.0) compared to the affinity of wild type albumin corresponding to the VSA and the affinity of the VSA for FcRn at a neutral pH (e.g., pH 7.0 or 7.4) is the same or less than the affinity of a corresponding wild type albumin for an FcRn. In some cases, Applicants have found that in selecting a VSA that is useful, e.g., as a therapeutic, it is necessary to evaluate the affinity for FcRn at both endosomal and neutral pH, noting that a VSA with the greatest affinity for FcRn at endosomal pH may be unsuitable for use because it also has an increased affinity at a neutral pH.

In some embodiments, a VSA comprises an amino acid substitution or deletion at one or more (e.g., 1, 2, 3, 4, 5, or more) positions disclosed herein, e.g., at the positions that were mutated in the experiments disclosed in the Examples and Figures. In some embodiments, a VSA comprises a substitution described herein, e.g., one or more substitutions of a specific amino acid at a specific position as described herein, e.g., as disclosed in the Examples and Figures.

In some embodiments, a VSA has one or more of the following mutations (amino acid residue numbers defined in accordance with the mature human amino acid sequence of SEQ ID NO:2): V418M; T420A; V424I; N429D; M446V; A449V; T467M; E505(R/K/G); V547A; and A552T, wherein "(X/Z)" means that the amino acid sequence may alternatively comprise amino acid X or amino acid Z at that residue. In certain embodiments, the VSA can include one or more of the following mutations: V418M; T420A; V424I; E505(R/K/G); and V547A.

In some embodiments, a VSA comprises a mutation that is present in a VSA disclosed herein, e.g., a VSA selected from one or more of the following variants described herein: HSA-15, HSA-13, HSA-12, HSA-7, HSA-21, HSA-11, HSA-14, HSA-5, HSA-10, HSA-6, HSA-9, and HSA-18. In some embodiments, a VSA includes each of the mutations that are present in a variant described herein, e.g., a variant selected from HSA-15, HSA-13, HSA-12, HSA-7, HSA-21, HSA-11, HSA-2, HSA-14, HSA-5, HSA-10, HSA-6, HSA-9, and HSA-18. In some embodiments, a VSA has the sequence of HSA-15, HSA-13, HSA-12, HSA-7, HSA-21, HSA-11, HSA-14, HSA-5, HSA-10, HSA-6, HSA-9, or HSA-18.

Typically, the VSAs provided herein have improved functional properties (e.g., higher affinity for FcRn at endosomal pH and/or extended pharmacokinetics, e.g., increased half-life and/or reduced clearance) compared with a corresponding native serum albumin sequence. Such VSAs can be useful for therapeutic or diagnostic applications. For example, VSAs can be useful for treating, or for producing formulations that are useful for treating, conditions for which serum albumin is indicated. A VSA that has improved properties compared with a corresponding native serum albumin sequence has therapeutic advantages. For example, a VSA that has extended PK compared with a corresponding native serum albumin polypeptide can have therapeutic advantages, such as less frequent and/or reduced dosing and/or more consistent delivery levels. A VSA can also be less expensive to administer because fewer doses are required.

Native human serum albumin (HSA) binds to human FcRn at pH 6.0 with an affinity of about 1-10 micromolar, and about 400 micromolar affinity at pH 7.4. The VSAs described herein can have improved binding to FcRn at endosomal pH, e.g., at pH 6.0 compared with a native serum albumin (e.g., a

corresponding native serum albumin, e.g., the corresponding full length native serum albumin or fragment thereof of which the VSA is a variant).

In some embodiments, the VSA binds to FcRn with a K_D that is less than a value selected from 2 micromolar, 1.5 micromolar, 1 micromolar, and 0.5 micromolar. In some embodiments, the VSA binds to FcRn with a K_D that is less than a value selected from 100 nM, 95 nM, 90 nM, 85 nM, 80 nM, 75 nM, 70 nM, 65 nM, 60 nM, 55 nM, 50 nM, 45 nM, 40 nM, 35 nM, 30 nM, 25 nM, 20 nM, 15 nM, 10 nM, and 5 nM. The K_D can be determined as described herein or using any method known in the art.

The K_D is pH dependent. In some embodiments, the VSA exhibits improved binding to FcRn at a specified pH, e.g., at pH 5.0, 5.2, 5.5, 5.6, 5.7, 5.8, 5.9, 6.2, 6.0, or 6.2. In some embodiments, the VSA exhibits improved binding to FcRn at an endosomal pH, for example at pH 5.0-6.2, pH 5.0-6.2, pH 5.0-6.0, or pH 5.5-6.0. In some embodiments, the VSA exhibits improved binding to FcRn at pH 5.5 or 6.0. In some embodiments, the VSA binds to FcRn at an endosomal pH, e.g., at pH 5.5 or pH 6.0, with a K_D that is less than a value selected from 2 micromolar, 1.5 micromolar, 1 micromolar, and 0.5 micromolar. In some embodiments, the VSA binds to FcRn at an endosomal pH, e.g., at pH 5.5 or pH 6.0 with a K_D that is less than a value selected from 100 nM, 95 nM, 90 nM, 85 nM, 80 nM, 75 nM, 70 nM, 65 nM, 60 nM, 55 nM, 50 nM, 45 nM, 40 nM, 35 nM, 30 nM, 25 nM, 20 nM, 15 nM, 10 nM, and 5 nM.

In some embodiments, the serum albumin variants have a K_D for FcRn at pH 5.5 that is less than 23 nM. In some embodiments, the serum albumin variants have a K_D for FcRn at pH 5.5 that is less or equal to 75 nM, 50 nM, 25 nM, 15 nM, 10 nM, 5 nM, or 3 nM.

In some embodiments, the VSA binds to FcRn at an endosomal pH, e.g., pH 5.5 or pH 6.0, but does not bind to FcRn at pH 7.4. In some embodiments, the VSA binds to FcRn at an endosomal pH, e.g., pH 5.5 or pH 6.0, and shows reduced binding to FcRn at pH 7.4 compared with its binding to FcRn at pH 5.5 (e.g., the binding to FcRn at pH 7.4 is less than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or 60% of the level observed at pH 5.5).

In some embodiments, the VSA binds to FcRn at pH 5.5 and shows reduced binding to FcRn at pH 7.4 compared with its binding to FcRn at an endosomal pH, e.g., pH 5.5 or pH 6.0 (e.g., the binding to FcRn at pH 7.4 is less than 1%, 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or 60% of the level observed at pH 5.5), and the VSA has improved pharmacokinetics (e.g., increased plasma half-life and/or reduced clearance, e.g., reduced beta phase clearance) compared with a corresponding native serum albumin.

In some embodiments, the VSA has a decreased K_D for FcRn at an endosomal pH, e.g. pH5.5 or pH 6.0, compared to the K_D of a wild type albumin (e.g., an HSA) at endosomal pH, and has the same or increased K_D for FcRn at a neutral pH, e.g., pH 7.0 or pH 7.4.

The ratio of the K_D s of wild type HSA to FcRn at pH 7.4 and 5.5 is 40-400, depending on the report. In some embodiments, the ratio of K_D s of the VSA to FcRn at pH 7.4 and 5.5 is greater than for wild type HSA (e.g., a ratio of 500; 1000; 5000; 10,000; 100,000; or 1 million). In some embodiments, the

binding of VSA to FcRn is assessed using SPR. In some embodiments, the binding of VSA to FcRn is assessed using ELISA.

In some embodiments, the half life (T1/2) of the VSA is greater than the T1/2 of a corresponding native serum albumin. In some embodiments, the T1/2 of the VSA is increased by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% compared with the T1/2 of a corresponding native serum albumin. In some embodiments, the T1/2 of the VSA is at least two times, three times, or four times as long as the T1/2 of a corresponding native serum albumin. In some embodiments, the T1/2 of the VSA is at least twice as long as the T1/2 of a corresponding native serum albumin.

Wild type HSA has a half-life (T1/2) in plasma of about 15-20 days in humans. A variant serum albumin described herein can have a longer serum half-life than a corresponding albumin, e.g., T1/2 at least about 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 days. In some embodiments, the VSA. In some embodiments, the VSA has a longer serum T1/2 in an animal compared to the corresponding wild type VSA, e.g., a VSA has a longer serum T1/2 in a monkey than the T1/2 of a human wild type albumin in a monkey.

Wild type HSA (UniProt DB Accession No. P02768; SEQ ID NO:1 below) is a 609 amino acid protein, comprising an 18 amino acid signal peptide; a 6 amino acid propeptide, extending from amino acid 19 to 24; Domain 1, extending from amino acid 25 to 210; Domain 2, extending from amino acid 211 to 403; and Domain 3, extending from amino acid 404 to 601 or amino acid 404 to 609 of Sequence ID No. 1. An exemplary sequence of a human serum albumin (including its leader sequence) is as follows:

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20      10      20      30      40      50      60
MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA FAQYLQQCPF

25      70      80      90     100     110     120
EDHVKLVNEV TEFAKTCVAD ESAENC DKSL HTLFGDKLCT VATLRETYGE MADCCAKQEP

      130     140     150     160     170     180
ERNECF LQHK DDNPNL PRLV RPEVDV MCTA FHDNEETFLK KYLYEIARRH PYFYAPELLEF

30      190     200     210     220     230     240
FAKRYKAAFT ECCQAADKAA CLLPKLDEL R DEGKASSAKQ RLKCASLQKF GERA FKAWAV

      250     260     270     280     290     300
ARLSQRFPKA EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLL

35      310     320     330     340     350     360
ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF LGMFLYEYAR

40      370     380     390     400     410     420
RHPDYSVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE FKPLVEEPQN LIKQNCLELFE

      430     440     450     460     470     480
QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV

45      490     500     510     520     530     540
LNQLCVLHEK TPVSDRVTKC CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL

      550     560     570     580     590     600
SEKERQIKKQ TALVELVKHK PKATKEQLKA VMDDFAAFVE KCKKADDKET CFAEEGKKLV
50

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609

AASQAALGL [SEQ ID NO:1]

5 An exemplary sequence of a human serum albumin (HSA) (in its mature form) is a 585 amino acid polypeptide as follows in SEQ ID NO:2. In some embodiments, the VSAs as described herein comprise variant domain III regions. Typically, such variant domain III regions retain the three-dimensional fold of domain III of serum albumin.

10 Exemplary variant domain III regions are at least 80% identical to domain III of a naturally occurring serum albumin, e.g., at least 90% identical to the domain III of a human serum albumin (e.g., amino acids 404 to 609 of SEQ ID NO:1 or amino acids 380 to 585 of SEQ ID NO:2 below).

A variant serum albumin polypeptide can have one or more (e.g., 1, 2, 3, 4, 5, or more) substitutions, insertions, or deletions relative to a corresponding native serum albumin. For example, the variant polypeptide can include a domain III with at least one, two, three, four or five substitutions relative to a domain III of HSA.

15 10 20 30 40 50 60
 DAHKSEVAHR FKDLGEEENFK ALVLIIFAQY LQQCPFEDHV KLVNEVTEFA KTCVADESAE

20 70 80 90 100 110 120
 NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE CFLQHKDDNP NLPRLVRPEV

 130 140 150 160 170 180
 DVMCTAFHDN EETFLKKYLY EIARRHPYFY APELLFFAKR YKAAFTECCQ AADKAACLLP

25 190 200 210 220 230 240
 KLDELRRDEGK ASSAKQRLKC ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK

 250 260 270 280 290 300
 VHTECCHGDL LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA

30 310 320 330 340 350 360
 DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLRLA KTYETTLEKC

 370 380 390 400 410 420
 CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE YKFQNALLRV YTKKVPQVST

35 430 440 450 460 470 480
 PTLVEVSRNL GKVGSKCKH PEAKRMPCAE DYLSVVLNQL CVLHEKTPVS DRVTKCTES

 490 500 510 520 530 540
 LVNRRPCFSA LEVDETYVPK EFNAETFTFH ADICTLSEKE RQIKKQTALV ELVKHKPKAT

 550 560 570 580
 KEQLKAVMDD FAAFVEKCK ADDKETCFAE EGKKLVAASQ AALGL [SEQ ID NO:2]

45 Typically, a VSA is a polypeptide that has a three-dimensional fold that is similar or identical to that of a native serum albumin, e.g., as described in PDB files 1AO6 (Sugio et al.(1999) Protein Eng. Jun;12(6):439-46); or 1E78 (Bhattacharya et al., (2000) J. Biol. Chem., 275:38731); or 1E7H (Bhattacharya et al., (2000) J. Mol. Biol., 303:721).

50 Exemplary variant serum albumin polypeptides are at least 70% identical to a naturally occurring serum albumin, e.g., at least 75%, 80%, 85%, 90%, or 95% identical to a human serum albumin (e.g., SEQ

ID NO:1 above). A variant serum albumin polypeptide can have one or more substitutions, insertions, or deletions. For example, the variant polypeptide can have at least one, two, three, four, or five substitutions relative to HSA.

The sequences of serum albumins from other species, particularly mammalian species are also known. Exemplary sequences include albumin sequences from *Bos taurus* (CAA76847, P02769, CAA41735, 229552, AAF28806, AAF28805, AAF28804, AAA51411); *Sus scrofa* (P08835, CAA30970, AAA30988); *Equus caballus* (AAG40944, P35747, CAA52194); *Ovis aries* (P14639, CAA34903); *Salmo salar* (CAA36643, CAA43187); *Gallus gallus* (P19121, CAA43098); *Felis catus* (P49064, S57632, CAA59279, JC4660); *Canis familiaris* (P49822, S29749, CAB64867). Variations, e.g., substitutions described herein in HSA, can also be introduced at corresponding positions into serum albumins from other species, e.g., into mammalian serum albumins, including, e.g., bovine serum albumin.

Serum albumin can be divided into at least three domains, termed Domain I, Domain II, and Domain III. A serum albumin protein useful in the present invention typically includes at least a domain III, and can also include domain I and domain II. Exemplary domains I, II, and III can have at least 60, 65, 70, 75, 80, 85, 90, 95% identity to respective domains from a mammalian serum albumin. In some embodiments, the variant serum albumin polypeptide of the present invention comprises domain III of human HSA, that is, amino acids 404 to 601 or 404 to 609 of SEQ ID NO:1, or amino acids 380 to 585 of SEQ ID NO:2, into which at least one, two, three, four, or five substitutions have been engineered relative to domain III of HSA.

In some embodiments, a residue that is within less than 7 angstroms of position H464 is substituted with another amino acid. For example, the residue is amino acid 415, 418, 458, 459, 460, 461, 462, 463, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, or 477. In some embodiments, a residue that is within less than 7 angstroms of position H510 is substituted with another amino acid. For example, the residue is amino acid 508, 509, 511, 512, 513, 514, 554, 555, 559, 564, 565, 568, or 569. In some embodiments, a residue that is within less than 7 angstroms of position H535 is substituted with another amino acid. For example, the residue is amino acid 412, 497, 498, 499, 500, 501, 502, 503, 507, 529, 530, 531, 532, 533, 534, 536, 537, 538, 580, or 583.

In some embodiments, residue V418 is altered, e.g., to a residue other than valine, e.g., to an hydrophobic residue such as methionine. In some embodiments, a residue that is within less than 7 angstroms of position V418 is substituted with another amino acid. For example, the residue is amino acid 411, 412, 415, 416, 417, 419, 420, 421, 422, 423, 424, 426, 460, 464, 469, 470, 473, 497, 530, or 534.

In some embodiments, residue T420 is altered, e.g., to a residue other than threonine, e.g., to an uncharged residue, e.g., a small uncharged residue such as alanine or glycine. In some embodiments, a residue that is within less than 7 angstroms of position T420 is substituted with another amino acid. For example, the residue is amino acid 408, 412, 416, 417, 418, 419, 421, 422, 423, 424, 425, 500, 526, 527, 528, 529, 530, 531, or 534.

In some embodiments, residue V424 is altered, e.g., to a residue other than valine, e.g., to an uncharged residue, e.g., to isoleucine. In some embodiments, a residue that is within less than 7 angstroms of position V424 is substituted with another amino acid. For example, the residue is amino acid 404, 408, 412, 418, 419, 420, 421, 422, 423, 425, 426, 427, 428, 429, 430, 522, 523, 524, 526, 527, 528, or 530.

5 In some embodiments, residue E505 is altered, e.g., to a residue other than glutamic acid, e.g., to an uncharged residue, e.g., a small uncharged residue such as alanine or glycine, or to a positively charged residue, e.g. lysine or arginine. In some embodiments, a residue that is within less than 7 angstroms of position E505 is substituted with another amino acid. For example, the residue is amino acid 502, 503, 504, 506, 507, 508, or 509.

10 In some embodiments, residue V547 is altered, e.g., to a residue other than valine, e.g., to an uncharged residue, e.g., a small uncharged residue such as alanine or glycine. In some embodiments, a residue that is within less than 7 angstroms of position V547 is substituted with another amino acid. For example, the residue is amino acid 529, 532, 542, 543, 544, 545, 546, 548, 549, 550, 551, or 552.

15 Variant serum albumin polypeptides can be incorporated into a fusion protein, with the N- or C-terminus thereof fused to the N- or C- terminus of a diagnostic or therapeutic protein, or variant thereof. In certain embodiments, the variant serum albumin polypeptide and the diagnostic or therapeutic protein are joined through a linker moiety, such as a peptide linker. Linkers useful in the present invention are described in greater detail further herein.

20 Therapeutic Uses of VSAs

A VSA can be used as a therapeutic, e.g., in uses for which albumin such as human albumin is typically used. For such uses, a VSA as described herein can have the advantage of extended PK, which can enable less frequent and/or reduced dosing for albumin replacement or supplementation. Such uses include, for example, hypovolemia, hypoalbuminemia, burns, adult respiratory distress syndrome, 25 nephrosis, and hemolytic disease of the newborn. Hypoalbuminemia can result from, for example, inadequate production of albumin (e.g., due to malnutrition, burns, major injury, or infection), excessive catabolism of albumin (e.g., due to burn, major injury such as cardio-pulmonary bypass surgery, or pancreatitis), loss through bodily fluids (e.g., hemorrhage, excessive renal excretion, or burn exudates), deleterious distribution of albumin within the body (e.g., after or during surgery or in certain inflammatory 30 conditions). Typically, for such uses, a VSA for such uses is administered by injection or iv in a solution that is from 5%-50% VSA (w/v), for example, 10%-40%, 15%-30%, 20%-25%, 20%, or 25%. Typically, administration is sufficient to produce a total albumin plus VSA concentration in a treated subject's serum that is 3.4 - 5.4 grams per deciliter (g/dL). Methods of assaying albumin concentration are well known in the art and can generally be used to assay total albumin plus VSA concentration.

35 Also provided herein is a VSA, or a pharmaceutical composition comprising a VSA, for use in therapy. Also included is a VSA, or a pharmaceutical composition comprising a VSA, for use in the treatment of a disease, e.g., a disease or condition for which albumin is indicated. Other embodiments

include the use of a VSA, or a pharmaceutical composition comprising a VSA, for the manufacture of a medicament for the treatment of a disease.

Uses of VSAs in Association with Other Agents

5 VSAs can also be used in association with other agents, e.g., therapeutic or diagnostic agents, to confer functional advantages, e.g., advantages of VSAs as described herein. The agent can be, e.g., any agent that is useful in the diagnosis or therapy of a disease or disorder, e.g., a disease or disorder that affects a human or a non-human animal.

Advantages of VSAs include, e.g., lack of Fc effector function, high solubility, potential for high
10 expression, low immunogenicity, and ability to be fused to another moiety at both termini to generate bivalent, bispecific, or bifunctional molecules.

Disclosed herein are VSAs with improved binding to an FcRn at endosomal pH, thereby potentiating the ability of the VSA and an agent associated with that VSA to have improved PK.

A VSA that has an extended PK can be associated with an agent (e.g., a therapeutic or diagnostic
15 agent) to extend the PK of the agent. The extended PK can have advantages; for example, the agent can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the agent can be achieved.

In some embodiments, associating a VSA with an agent improves the functional properties of the agent. In some embodiments, the dosage and/or frequency at which the agent is effective for producing a
20 particular effect (e.g., a desired therapeutic effect) is reduced when the agent is used in association with the VSA. In some embodiments, associating a VSA with an agent improves the pharmacokinetic properties of the agent (e.g., increases its half-life and/or reduces its clearance). Any relevant pharmacokinetic parameters that are known in the art can be used to assess pharmacokinetic properties. The pharmacokinetics of a VSA or a VSA associated with an agent can be measured in any relevant biological
25 sample, e.g., in blood, plasma, or serum.

In some embodiments, associating a VSA with an agent improves the half life (T_{1/2}) of the agent. In some embodiments, the T_{1/2} of the agent is increased by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%,
30 90%, or 100%. In some embodiments, the T_{1/2} is at least two times, three times, or four times as long as the T_{1/2} of the agent when it is not associated with the VSA. In some embodiments, the T_{1/2} of the VSA is at least twice as long as the T_{1/2} of the agent when it is not associated with the VSA.

In some embodiments, the dose at which the agent is effective for producing a particular effect (e.g., a desired therapeutic effect) is reduced when the agent is associated with the VSA. In some
embodiments, the effective dose is reduced to 80%, 70%, 60%, 50%, 40%, 30%, 20%, or 10% of the dose that is required when the agent is not associated with the VSA.

35 In some embodiments, the frequency of dosing of the agent that is effective for producing a particular effect (e.g., a desired therapeutic effect) is reduced when the agent is associated with the VSA. In some embodiments, the frequency of dosing at which the agent is effective when it is associated with the

VSA is decreased by 10%, 20%, 30%, 40%, 50%, or more compared with the frequency at which the agent is effective when it is not associated with the VSA.

In some embodiments, the frequency of dosing at which the agent is effective when it is associated with the VSA is decreased by at least 2 hours, 4 hours, 6 hours, 8 hours, 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 2 weeks, 3 weeks, or 4 weeks compared with the frequency at which the agent is effective when it is not associated with the VSA.

The improvement in the properties of the agent can be assessed relative to any appropriate control. For example, the improvement in the properties of an agent that is associated with a VSA can be assessed by comparing the properties of the agent that is associated with the VSA with the properties of the agent when it is not in an association with the VSA. Alternatively, the improvement in the properties of an agent that is associated with a VSA can be assessed by comparing the properties of the agent that is associated with the VSA with the properties of the agent when it is in an association with a corresponding native serum albumin polypeptide.

Also provided herein is a VSA, a VSA in association with another agent, a pharmaceutical composition comprising a VSA, or a pharmaceutical composition comprising a VSA in association with another agent, for use in therapy or for use in the treatment of a disease, e.g., a disease or condition described herein or described in U.S. Patent No 5,766,883, U.S. Patent No 5,875,969, U.S. Patent Publication No. US 2002/0151011, or International Publication No. WO 2011/103076, the entire contents of each of which are hereby incorporated herein by reference. Other embodiments include the use of a VSA, or a pharmaceutical composition comprising a VSA, a VSA in association with another agent, a pharmaceutical composition comprising a VSA, or a pharmaceutical composition comprising a VSA in association with another agent, for the manufacture of a medicament for the treatment of a disease.

The agent can be another protein, e.g., a heterologous protein. In some embodiments, the agent is a diagnostic agent. In some embodiments, the agent is a therapeutic agent. For example, a protein that comprises a VSA can be used to extend the PK of a systemically administered therapeutic agent. The heterologous protein can be, for example, a therapeutic protein or a diagnostic protein. The serum albumin polypeptide with altered FcRn binding properties or a domain thereof (e.g., domain III) can be associated with (e.g., attached covalently to) the therapeutic protein, or to an active fragment or variant of the therapeutic protein. The variant serum albumin or a domain thereof can be in the same polypeptide chain as is at least a component of the therapeutic protein.

A variant serum albumin (VSA) can be associated with another agent, e.g., a therapeutic agent or a diagnostic agent. The other agent (e.g., therapeutic agent) can be an entire protein (e.g., an entire therapeutic protein) or a biologically active fragment thereof. The activity of the agent (e.g., therapeutic agent) can be evaluated in an appropriate *in vitro* or *in vivo* assay for the agent's activity. In general, the activity of the agent fused to a VSA is not reduced, for example, by more than 50%, by more than 40%, by more than 30%, by more than 20%, by more than 10%, by more than 5%, or by more than 1% compared

with the activity of the agent when it is not in association with the agent. Examples of methods for assessing the activity of certain agents are provided herein.

In some embodiments, the VSA is attached to the agent by one or more covalent bonds to form a variant serum albumin fusion molecule. Any agent that can be linked to a VSA described herein can be used as the agent in a variant serum albumin fusion molecule. The agent can be a therapeutic or diagnostic agent. For example, the agent can be any polypeptide or drug known to one of skill in the art.

In some embodiments, an agent (e.g., therapeutic or diagnostic agent) is stably associated with a VSA, but the agent is not fused to the VSA. In such embodiments, the agent can be associated with the VSA by any means known in the art. For example, the agent can be conjugated to a moiety that is capable of binding the VSA. In some embodiments, the moiety is an albumin binding protein. In some embodiments the moiety is a fatty acid. In some embodiments, the agent is non-covalently bound to the VSA, generally via the affinity of the VSA for small lipophilic moieties. In embodiments wherein the agent is not fused to the VSA, the agent can be administered before, after, or concurrently with the VSA. In some embodiments, the agent is administered concurrently with the VSA. In some embodiments, the agent is administered at the same frequency as is the VSA. In some embodiments, the agent is administered more or less frequently than the VSA.

In some embodiments, the agent is a polypeptide consisting of at least 5, for example, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acid residues. The agent can be derived from any protein for which an improved property is desired, e.g., an increase in serum levels and/or serum half-life of the agent; or a modified tissue distribution and/or tissue-targeting of the agent.

In some embodiments, the agent is a cytokine or a variant thereof. Generally, a cytokine is a protein released by one cell population that acts on another cell as an intercellular mediator. Examples of such cytokines include lymphokines, monokines, and traditional polypeptide hormones. Specific examples include: interleukins (ILs) such as IL-1 (IL-1 α and IL1 β), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12; IL-15, IL-18, and IL-37; a tumor necrosis factor such as TNF-alpha or TNF-beta; growth hormone such as human growth hormone (HGH); somatotropin; somatrem; N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; insulin-like growth factors, such as insulin-like growth factors-1, -2, and -3 (IGF-1; IGF-2; IGF-3); proglucagon; glucagon and glucagon-like peptides, such as glucagon-like peptide-1 and -2 (GLP-1 and GLP-2); exendins, such as exendin-4; gastric inhibitory polypeptide (GIP); secretin; pancreatic polypeptide (PP); nicotinamide phosphoribosyltransferase (also known as visfatin); leptin; neuropeptide Y (NPY); interleukin IL-1Ra, including (N140Q); ghrelin; orexin; adiponectin; retinol-binding protein-4 (RBP-4); adiponin; relaxin; prorelaxin; neurogenic differentiation factor 1 (NeuroD1); glicentin and glicentin-related peptide; cholecystokinin (previously known as pancreozymin); glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factors (FGF) such as FGF-19, FGF-21 and FGF-23; prolactin; placental lactogen; tumor

necrosis factor-alpha and -beta; mullerian-inhibiting substance; gonadotropin-associated peptide; luteinizing-hormone-releasing hormone (LHRH); inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); growth factors (e.g., platelet-derived growth factor, PDGF and its receptor, EGF and its receptor, nerve growth factors, such as NGF-beta and its receptor, and KGF, such as
5 palifermin, and its receptor); platelet-growth factor (PGF); transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; osteoinductive and growth and differentiation factors, such as osteocalcin, BMP-2, BMP-4, BMP-6 and BMP-7; interferons such as interferon-alpha, beta, and -gamma, including interferon-alpha2B; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); erythropoietin (EPO); darbepoetin alfa;
10 tissue plasminogen activator (TPA) or alteplase; tenecteplase; dornase alfa; entanercept; calcitonin, oxyntomodulin; glucocerebrosidase; arginine deiminase, Arg-vasopressin, natriuretic peptides, including A-type natriuretic peptide; B-type natriuretic peptide, C-type natriuretic peptide and Dendroapsis natriuretic peptide (DNP); gonadotropin-releasing hormone (GnRH); endostatin; angiostatin, including (N211Q); Kiss-1; hepcidin; oxytocin; pancreatic polypeptide; calcitonin gene-related protein (CGRP);
15 parathyroid hormone (PTH); adrenomedullin; delta-opioids; kappa-opioids; mu-opioids; deltorphins; enkephalins; dynorphins; endorphins; CD276, including (B7-H3); ephrin-B1; tweak-R, cyanovirin, including cyanovirin-N; gp41 peptides; 5-helix protein; prosaptide; apolipoprotein A1; BDNF; brain-derived neural protein; CNTF (Axokine®); Antithrombin III; FVIII A1 domain; Kringle-5; Apo A-1 Milano; Kunitz domains; vWF A1 domain; Peptide YY, including PYY1-36 and PYY3-36; urate oxidase;
20 and other polypeptide factors including LIF and kit ligand (KL).

In one embodiment, the agent is BMP peptide analogue (e.g., THR-184, Thrasos Therapeutics, Inc.).

In one embodiment, the agent is GLP-2.

In some embodiments, the therapeutic protein is a monomeric protein (such as a monomeric
25 cytokine, e.g., an IL-1Ra, or an scFv). Albumin fusion proteins are known in the art; the variant serum albumin polypeptides with altered FcRn binding properties, described herein, can be used in place of serum albumin and linked to a therapeutic agent to form fusion proteins with improved properties, such as increased half-life.

Examples of other polypeptides useful as the active agent include, but are not limited to, various
30 types of antibodies, antibody fragments, such as antigen binding domains (e.g., scFv, Fv, Fab, F(ab)₂, domain antibodies, and the like); Surrobodyes, Adnectins™, anti-calix, affibody, or fragments of the foregoing. Also useful as active agents are receptor antagonists, such as IL-1Ra (e.g., anakinra), cell adhesion molecules (e.g., cadherins, such as cadherin-11, CTLA4, CD2, and CD28); anti-angiogenic factors such as endostatin; receptors, as well as soluble fragments of tissue-bound receptors.

35 The agent, e.g., therapeutic moiety included in a fusion protein as described herein, can also be a therapeutic moiety such as a cytotoxin (e.g., a cytostatic or cytotoxic agent), a non-peptide therapeutic agent or a radioactive element (e.g., alpha-emitters, gamma-emitters, etc.). Examples of cytostatic or

cytotoxic agents include, but are not limited to, paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Non-peptide therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 5-mercaptopyrimidine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC), and anti-mitotic agents (e.g., vincristine and vinblastine). The present invention also encompasses fusing the variant serum albumin (VSA) moiety to an active agent that is a diagnostic agent. The VSA-diagnostic molecule of the invention can be used diagnostically to, for example, monitor the development or progression of a disease, disorder or infection as part of a clinical testing procedure to, e.g., determine the efficacy of a given 5 treatment regimen. Detection can be facilitated by coupling the pharmacologic enhancing molecule to a detectable substance. Examples of detectable substances include enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals, and nonradioactive paramagnetic metal ions. The detectable substance can be coupled or conjugated either directly to the antibody or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Pat. No. 4,741,900 for metal ions that can be conjugated to antibodies for use as a diagnostic according to the methods and compositions described herein. Examples of suitable enzymes include, e.g., horseradish peroxidase, alkaline phosphatase, O-galactosidase, or acetylcholinesterase; examples of suitable prosthetic groups include, e.g., complexes including, e.g. streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include, e.g., ¹²⁵I, ¹³¹I, ¹¹¹In or ^{99m}Tc. See, for example, Plumridge et al., WO2011/051489; Lubman et al.; WO2010/141329.

The nucleotide and amino acid sequences and structures for the above molecules that are useful as, or in preparing the active moiety to be fused to the variant serum albumin moiety in the present invention are known, and all of the publications and websites described as providing such information, such as Genbank, the protein database and Uniprot (www.ncbi.nlm.nih.gov/genbank; www.pdb.org; and www.uniprot.org, respectively), are incorporated herein by reference for this purpose. Non-limiting examples of therapeutic proteins that can be used in preparing some of the embodiments of the present invention are described in further detail below.

Glucagon-Like Peptide 1 (GLP-1). In one embodiment, the therapeutic agent includes a GLP-1 or a biologically active variant or fragment thereof. GLP-1 is a 37 amino acid peptide that is formed from cleavage of glucagon, secreted by the L-cells of the intestine in response to food ingestion (Uniprot accession number: P01275). GLP-1 can stimulate insulin secretion, causing glucose uptake by cells and decreased serum glucose levels (e.g., Mojsov, *Int. J. Peptide Protein Research*, 40:333-343 (1992)). GLP-1 can be cleaved to produce a biologically active peptide GLP-1(7-37)OH. In addition, numerous GLP-1 analogs and derivatives are known and include, e.g., exendins which are peptides found in Gila monster venom. Exendins have sequence homology to native GLP-1 and can bind the GLP-1 receptor and initiate the signal transduction cascade of GLP-1(7-37)OH. GLP-1 compounds can have one or more of the following biological properties: ability to stimulate insulin release, lower glucagon secretion, inhibit gastric emptying, and enhance glucose utilization. (e.g., Nauck et al., *Diabetologia* 36:741-744 (1993); Gutniak et al., *New England J. of Med.* 326:1316-1322 (1992); Nauck et al., *J. Clin. Invest.* 91:301-307 (1993)). A therapeutic agent including GLP-1, GLP-1(7-37)OH, an exendin, or biologically active variants or fragments thereof can be used for treating a diabetic disorder, e.g., non-insulin dependent diabetes mellitus (NIDDM).

The amino acid sequence of human GLP-1 is as follows:

HDEFERHAEG TFTSDVSSYL EGQAAKEFIA WLVKGRG [SEQ ID NO:3]

Advantages of using a VSA in association with a GLP-1 (e.g., using a VSA-GLP-1 fusion protein) include, e.g., extending the PK of the GLP-1. The extended PK can have advantages; for example, the GLP-1 can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the GLP-1 can be achieved.

Insulin. In one embodiment, the therapeutic agent is an insulin. The insulin can be a full length insulin polypeptide or a biologically active variant or fragment thereof. For example, the insulin can be a glucose sensitive molecule that includes an insulin receptor agonist. An exemplary insulin polypeptide (110 amino acids) has the sequence: MALWMRLLPL LALLALWGPD PAAAFVNQHL CGSHLVEALY LVCGERGFFY TPKTRREAED LQVGQVELGG GPGAGSLQPL ALEGLSLQKR IVEQCCTSIC SLYQLENYCN [SEQ ID NO:4].

The B-chain (1-30 or FVNQHL CGSHLVEALY LVCGERGFFY TPKT) [SEQ ID NO:5] is present at amino acids 25-54. The A-chain (1-21 or G IVEQCCTSIC SLYQLENYCN) [SEQ ID NO:6] is present at amino acids 90-110. (See, Uniprot accession no. P01308). The C-peptide (RREAED LQVGQVELGG GPGAGSLQPL ALEGLSLQKR) [SEQ ID NO:7] which links the B-chain and A-chain is present at amino acids 55-89. Therapeutic proteins as disclosed herein can include the B-chain and A-chain of insulin, linked by the C-peptide of insulin or another natural or artificial sequence, such as a peptide linker. A number of adipose and muscle related cell lines can be used to test for glucose uptake/transport activity in the absence or presence of a combination of any one or more of the therapeutic drugs listed for the treatment of diabetes mellitus. In particular, the 3T3-L1 murine fibroblast cells and the L6 murine

skeletal muscle cells can be differentiated into 3T3-L1 adipocytes and into myotubes, respectively, to serve as appropriate *in vitro* models for the [³H]-2-deoxyglucose uptake assay (Urso et al., J Biol Chem, 274:30864-73 (1999); Wang et al., J Mol Endocrinol, 19:241-8 (1997); Haspel et al., J Membr Biol, 169:45-53 (1999); Tsakiridis et al., Endocrinology, 136:4315-22 (1995)). Female NOD (non-obese diabetic) mice are characterized by displaying IDDM with a course which is similar to that found in humans, although the disease is more pronounced in female than male NOD mice.

Advantages of using a VSA in association with an insulin (e.g., using a VSA-insulin fusion protein) include, e.g., extending the PK of the insulin. The extended PK can have advantages; for example, the insulin can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the insulin can be achieved.

Fibroblast Growth Factor 19 (FGF-19). In one embodiment, the therapeutic agent is an FGF-19 (e.g., human FGF-19) or a biologically active variant or fragment thereof. Human FGF-19 is a 216 amino acid protein, including a 24 amino acid N-terminal signal peptide. An exemplary FGF-19 peptide sequence is the sequence of human FGF-19 (Uniprot accession number: O95750):

MRSGCVVVHV WILAGLWLAV AGRPLAFSDA GPHVHYGWD PIRLRHLYTS GPHGLSSCFL
 RIRADGVVDC ARGQSAHSL EIKAVALRTV AIKGVHSVRY LCMGADGKMQ GLLQYSEEDC
 AFEEEEIRPDG YNVYRSEKHR LPVSLSSAKQ RQLYKNRGFL PLSHFPLPMLP MVPEEPEDLR
 GHLESDMFSS PLETDSMDPF GLVTGLEAVR SPSFEK [SEQ ID NO:8]

FGF-19 is active in the suppression of bile acid biosynthesis and stimulates glucose uptake in adipocytes. Holt et al., Genes Dev. 17:1581-91 (2003). FGF-19 can be used for treatment of dietary and leptin-deficient diabetes. Fu et al., Endocrinology, 145:2594-603 (2004).

Advantages of using a VSA in association with FGF-19 (e.g., using a VSA-FGF-19 fusion protein) include, e.g., extending the PK of the FGF-19. The extended PK can have advantages; for example, the FGF-19 can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the FGF-19 can be achieved.

Fibroblast Growth Factor 21 (FGF-21). In one embodiment, the therapeutic agent is an FGF-21 (e.g., human FGF-21) or a biologically active variant or fragment thereof. Human FGF-21 is a 209 amino acid protein, including a 28 amino acid N-terminal signal peptide. An exemplary FGF-21 peptide sequence is the sequence of human FGF-21 (Uniprot accession number: Q9NSA1):

MDSDETGFEB SGLWVSVLAG LLLGACQAH IPDSSPLLQF GGQVRQRYLY TDDAQQTEAH
 LEIREDGTVG GAADQSPESL LQLKALKPGV IQILGVKTSR FLCQRPDGAL YGSLHFDPEA
 CSFRELLED GYNVYQSEAH GLPLHLPGNK SPHRDPAPRG PARFLPLPGL PPALPEPPGI
 LAPQPPDVGS SDPLSMVGPS QGRSPSYAS [SEQ ID NO:9]

FGF-21 stimulates glucose uptake in differentiated adipocytes via the induction of the insulin-

independent glucose transporter GLUT1. In *ob/ob* mice, FGF-21 has been demonstrated to have durable glucose control and triglyceride lowering effects with minimal adverse side effects. Kharitononkov et al., J. Clin. Invest.115:1627-35 (2005). Accordingly, FGF-21 is useful in treatment of diabetes.

Advantages of using a VSA in association with FGF-21 (e.g., using a VSA-FGF-21 fusion protein) include, e.g., extending the PK of the FGF-21. The extended PK can have advantages; for example, the FGF-21 can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the FGF-21 can be achieved.

Fibroblast Growth Factor 23 (FGF-23). In one embodiment, the therapeutic agent is an FGF-23 (e.g., human FGF-23) or a biologically active variant or fragment thereof. Human FGF-23 is a 251 amino acid protein, including a 24 amino acid N-terminal signal peptide. FGF-23 is cleaved by protein convertases into an N-terminal peptide of approximately 155 amino acids (amino acid 25-179); and a C-terminal peptide of approximately 72 amino acids (amino acids 180-251). An exemplary FGF-23 peptide sequence is the sequence of human FGF-23 (Uniprot accession number: Q9GZV9):

MLGARLRLWV CALCSVCSMS VLRAYPNASP LLGSSWGGLI HLYTATARNS YHLQIHKNGH
VDGAPHQTIY SALMIRSEDA GFVVITGVMS RRYLCMDFRG NIFGSHYFDP ENCRFQHQTL
ENGYDVYHSP QYHFLVSLGR AKRAFLPGMN PPPYSQFLSR RNEIPLIHFN TPIPRRHTRS
AEDDSERDPL NVLKPRARMT PAPASCSQEL PSAEDNSPMA SDPLGVVRRG RVNTHAGGTG
PEGCRPFAKF I [SEQ ID NO:10]

FGF-23 is involved in regulating phosphate homeostasis. FGF-23 has been shown to inhibit renal tubule phosphate transport (Bowe et al., Biochem. Biophys. Res. Commun., 284:977-81 (2001)); to regulate vitamin D metabolism (Shimada et al., J. Bone Miner. Res., 19:429-35 (2004)); and to negatively regulate osteoblast differentiation and matrix mineralization (Wang et al., J. Bone Miner. Res., 23:939-48 (2008)). Accordingly, FGF-23 can be used in treatment of autosomal dominant hypophosphatemic rickets (ADHR) and tumor-induced osteomalacia (Riminucci et al., J. Clin. Invest., 112:683-92 (2003)).

Advantages of using a VSA in association with FGF-23 (e.g., using a VSA-FGF-23 fusion protein) include, e.g., extending the PK of the FGF-23. The extended PK can have advantages; for example, the FGF-23 can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the FGF-23 can be achieved.

Interleukin-2 (IL-2). In one embodiment, the therapeutic agent is an IL-2 (e.g., human IL-2) or a biologically active variant or fragment thereof. Human IL-2 is a 153 amino acid protein, including a 20 amino acid N-terminal signal peptide. An exemplary IL-2 peptide sequence is the sequence of human IL-2 (Uniprot accession number: P60568):

MYRMQLLS CI ALSLALVTNS APTSSSTKKT QLQLEHLLLD LQMILNGINN YKNPKLTRML
TFKFYMPKKA TELKHLQCLE EELKPLEEVL NLAQSKNFHL RPRDLISNIN VIVLELKGSE

TTFMCEYADE TATIVEFLNR WITFCQSIIS TLT [SEQ ID NO:11]

IL-2 can induce proliferation of antigen-activated T cells and stimulates natural killer (NK) cells, as well as stimulates proliferation of regulatory T cells (Tregs). The biological activity of IL-2 is mediated through a multi-subunit IL-2 receptor complex (IL-2R) of three polypeptide subunits that span the cell membrane: p55 (IL-2R α , the alpha subunit, also known as CD25 in humans), p75 (IL-2R β , the beta subunit, also known as CD122 in humans) and p64 (IL-2R γ , the gamma subunit, also known as CD132 in humans). IL-2-derived polypeptides can be used in cancer immunotherapies and to deliver therapeutic agents to CD25-positive cells in vivo or in cell culture. For example, Proleukin® is an approved biologic that contains IL-2. Exemplary agonists are described by Rao et al., Protein Engineering 16:1081-1087 (2003) and Rao et al., Biochemistry 44:10696-701 (2005), and in US Patent Application Publication 2005/0142106; see also US Patents 7,569,215 and 7,951,360. The therapeutic agent can also further include an IL-2 binding fragment of CD25, e.g., the sushi domain of IL-2R α . The IL-2 binding fragment of CD25 can be covalently linked to the biologically active portion of IL-2 or can be non-covalently linked. Exemplary IL-2 antagonists that bind CD25 but do not activate the IL-2 receptor are described in US 2011/0091412. The IL-2 antagonists can be used to specifically target T regulatory cells and/or specifically inhibit T regulatory cell function. The ability of low-dose IL-2 to reduce inflammation and/or immune response may be particularly surprising in light of previous publications advocating low doses of IL-2 for stimulation of immune response. See, US Patent 6,509,313. Low-dose IL-2 can be used to specifically induce Treg proliferation and promote an anti-inflammatory state. In particular embodiments, low-dose IL-2 can be administered subcutaneously, for example using a self-administered pen or cartridge device, such as is used with other self-administered peptide biotherapeutic drugs, such as long-acting insulin. Particular indications for which low-dose IL-2 can be appropriate include, for example, graft-vs-host-disease; for example, in connection with bone marrow transplantation. Administration of low-dose IL-2 can be advantageous in reducing the need for pre-graft conditioning and/or post-graft treatment with immunosuppressant drugs. Ferrara et al., Biology of Blood and Marrow Transplantation, 5:347-56 (1999). Additional advantages can include avoiding the adverse effects of short-term, high dose IL-2. See Sykes et al., Blood, 83:2560-69 (1994).

Advantages of using a VSA in association with IL-2 (e.g., using a VSA-IL-2 fusion protein) include, e.g., extending the PK of the IL-2. The extended PK can have advantages; for example, the IL-2 can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the IL-2 can be achieved.

Interleukin-15 (IL-15). In one embodiment, the therapeutic agent is an IL-15 (e.g., human IL-15) or a biologically active variant or fragment thereof. Human IL-15 is a 162 amino acid protein, including a 29 amino acid N-terminal signal peptide. An exemplary IL-15 peptide sequence is the sequence of human IL-15 (Uniprot accession number: P40933):

MRISKPHLRS ISIQCYLCLL LNSHFLTEAG IHVFILGCF S AGLPKTEANW VNVISDLKKI
EDLIQSMHID ATLYTESDVH PSCKVTAMKC FLELEQVISL ESGDASIHDT VENLIILANN

SLSSNGNVTE SGCKECEELE EKNIKEFLQS FVHIVQMFIN TS [SEQ ID NO:12]

IL-15 can induce proliferation of antigen-activated T cells and stimulate natural killer (NK) cells. The biological activity of IL-15 is mediated through a multi-subunit receptor complex (IL-15R) which shares the beta (CD122) and gamma (CD132) subunits of the IL-2 receptor complex, but has a unique
 5 alpha subunit (IL-15R α , also known as CD215 in humans). IL-15 derived polypeptides can be used in cancer immunotherapies. The therapeutic agent can also further include an IL-15 binding fragment of the IL-15Ra receptor, e.g., the sushi domain of IL-2Ra. The IL-15 binding fragment of IL-15Ra can be covalently linked to the biologically active portion of IL-15 or can be non-covalently linked.

Advantages of using a VSA in association with IL-15 (e.g., using a VSA-IL-15 fusion protein)
 10 include, e.g., extending the PK of the IL-15. The extended PK can have advantages; for example, the IL-15 can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the IL-15 can be achieved.

Hepcidin. In certain embodiments, the therapeutic agent is a hepcidin (e.g., human hepcidin) , or
 15 a biologically active variant of hepcidin. Human hepcidin is an 84 amino acid protein (Uniprot Accession number P81172), including a 24 amino acid N-terminal signal peptide, a thirty amino acid propeptide, and a 25 amino acid mature peptide (amino acids 60 to 84); hepcidin can be alternately cleaved to form a 20 amino acid mature peptide (amino acids 65 to 84).

MALSSQIWAA CLLLLLLLAS LTSGSVFPQQ TGQLAELQPQ DRAGARASWM PMFQRRRRRD
 20 THFPICIFCC GCCHRSKCGM CCKT [SEQ ID NO:13]

Hepcidin is known to be involved in iron homeostasis (e.g., Laftah et al., Blood, 103:3940-44 (2004); US Patent 7,169,758). Accordingly, hepcidin can be used an agent for regulation of iron absorption and homeostasis. It can be used to treat abnormal iron absorption, e.g., in individuals with β -thalassemia and related disorders.

The therapeutic agent can comprise a hepcidin peptide, or a variant of such peptide containing one
 25 or more amino acid variations from the mature hepcidin peptide. In a particular embodiment, two mature hepcidin peptides are fused or joined to each end of an albumin moiety, such as a VSA, such that the hepcidin molecules are able to interact with the natural binding partner for hepcidin, ferroportin. Ferroportin is known to exist in dimers, and both monomers must be capable of binding hepcidin for Jak2 to
 30 bind to ferroportin.

Advantages of using a VSA in association with a hepcidin (e.g., using a VSA-hepcidin fusion
 protein) include, e.g., extending the PK of the hepcidin. The extended PK can have advantages; for example, the hepcidin can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the hepcidin can be achieved.

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Coagulation Factor VII (FVII). In certain embodiments, the therapeutic agent is an FVII (e.g., a human FVII) or a biologically active variant or fragment thereof. Human FVII is a 466 amino acid protein, including a 20 amino acid N-terminal signal peptide and a 40 amino acid propeptide, a 152 amino acid light

chain and a 254 amino acid heavy chain. Thim et al., *Biochemistry*, 27:7785-93 (1998); Hagen et al., *PNAS USA* 83:2412-16 (1986); O'Hara et al., *PNAS USA* 84:5158-62 (1987) and Sabater-Lleal et al., *Hum Genet* 118:741-51 (2006); Hagen, US Patent 4,784,950; Uniprot Accession No. P08709. Thus, the mature FVII is a single chain glycoprotein (mol. wt. 50,000) of 406 amino acids that is secreted into the blood where it circulates in a zymogen form. FVII comprises a 45 amino acid Gla domain (amino acids 61 through 105); a potentially calcium-binding 37 amino acid EGF-like domain (amino acids 106 through 142); a 42 amino acid EGF-like domain (amino acids 147 through 188) and a 240 amino acid serine peptidase domain (amino acids 213 through 452). *In vitro*, FVII can be proteolytically cleaved to form activated FVII, or FVIIa, by the action of activated coagulation factors Factor X (FXa), Factor IX (FIXa), Factor XII (FXIIa) or Factor II (FIIa). FVIIa does not promote coagulation by itself, but can complex with tissue factor (TF) exposed at the site of injury. The FVIIa/TF complex can convert FX to FXa, and FIX to FIXa, thereby inducing local hemostasis at the site of injury. Activation of FVII to FVIIa involves proteolytic cleavage at a single peptide bond between Arg-152 and Ile-153, resulting in a two-chain molecule consisting of a light chain of 152 amino acid residues and a heavy chain of 254 amino acid residues held together by a single disulfide bond. Persons with hemophilia may have normal levels of FVII. However, they suffer from a relative deficiency in FVIIa and other activated clotting factors.

It is known that basal levels of Factor VIIa in plasma are greatly reduced in subjects with hemophilia B (Factor IX deficiency) and, to a lesser extent, subjects with hemophilia A (Factor VIII deficiency). Wildgoose et al., *Blood* 1:25-28 (1992). In the absence of activated FVIIa, the intrinsic blood clotting pathway involving FVII and FIX, is severely limited in effective coagulation. Recently, recombinant activated Factor VII (rFVIIa, NovoSeven[®], Novo, Nordisk) has been shown to have therapeutic value to bypass or correct the coagulation defects in hemophilia A and B subjects with inhibitors, especially in subjects with inhibitors who were undergoing surgical procedures. NovoSeven[®] is a 406 amino acid glycoprotein that is structurally similar to plasma-derived FVIIa. However, recombinant FVIIa is expensive to manufacture. Another critical problem is the short half-life (2 hours) of recombinant FVIIa. Therefore, recombinant FVIIa therapy requires an intravenous infusion of high doses of the protein every 2 hours. Accordingly, sequences useful as the therapeutic agent in the present invention include sequences encoding FVII and FVIIa.

In particular embodiments, the agent used herein can be a Factor VII or Factor VIIa peptide. In particular embodiments, the Factor VII peptide can contain one or more mutations to provide an enzymatic cleavage site, such as an enzymatic cleavage site susceptible to cleavage by SKI-1 or furin (e.g., US Patent 7,615,537).

Advantages of using a VSA in association with a FVII (e.g., using a VSA-FVII fusion protein) include, e.g., extending the PK of the FVII. The extended PK can have advantages; for example, the FVII can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the FVII can be achieved.

Coagulation Factor VIII (FVIII). In certain embodiments, the therapeutic agent is an FVIII (e.g., human FVIII) or a biologically active variant or fragment thereof. For example, Toole, US 4,757,006 discloses the amino acid sequence of the full-length wild-type human FVIII, and for example, Toole, US 4,868,112 discloses the amino acid sequence of human FVIII, wherein the B-domain has been deleted.

5 FVIII, also commonly referred to as antihemophilic factor (AHF) is a large, 2351 amino acid protein that is processed into multiple chains in a complex manner, including a 19 amino acid signal peptide (amino acids 1 to 19); a 1313 amino acid (amino acids 20 through 1332) or 740 amino acid (amino acids 20 through 759) light chain; a 573 amino acid B chain (amino acids 760 through 1332); and a 684 amino acid light chain (amino acids 1668 through 2351). Wood et al., Nature 312:330-37 (1984); Toole et al., Nature, 312:342-47
10 (1984); UniProt Accession No. P00451. AHF molecules have been approved as therapeutic treatments for subjects with hemophilia A (Recombinate[®], Baxter Healthcare/Wyeth BioPharma; Advate[®], Baxter Healthcare; Kogenate[®], Bayer Healthcare; Xyntha[®] Wyeth Pharmaceuticals; Monoclate-P[®], CSL Behring LLC). It has been found that the B-domain is not essential to activity, Pittman et al., Blood, 81:2925-35 (1993), and therefore, B-domain-deleted Factor VIII molecules have also been approved as a therapeutic
15 treatment for hemophilia A (ReFacto[®]; Genetics Institute/Wyeth Pharmaceuticals). See, also, US Patent 7,572,619. Accordingly, sequences useful as the therapeutic agent in the present invention include sequences encoding FVIII and B-domain deleted FVIII.

FVIII and B-domain-deleted FVIII may be expressed and/or administered in conjunction with von Willebrand's Factor (VWF). VWF is a large (2,813 amino acid) protein comprising multiple repetitive
20 domains which form multimers and acts as a chaperone protein for FVIII. Accession number P04275. VWF/FVIII complex has been approved as a therapeutic treatment for spontaneous or trauma-induced bleeding episodes in patients with moderate or severe von Willebrand Disease (Wilate[®], Octapharma).

Advantages of using a VSA in association with a FVIII (e.g., using a VSA-FVIII fusion protein) include, e.g., extending the PK of the FVIII. The extended PK can have advantages; for example, the FVIII
25 can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the FVIII can be achieved.

Coagulation Factor IX (FIX). In certain embodiments, the therapeutic agent is an FIX (e.g., a human FIX) or a biologically active variant or fragment thereof. Human FIX is a 461 amino acid secreted
30 protein, including a 28 amino acid N-terminal signal peptide and an 18 amino acid propeptide. The protein comprises a 415 amino acid chain [amino acids 47 through 461]; or can be processed by human Factor XIa to form a 145 amino acid Factor IXa light chain [amino acids 47 through 191]; a 35 amino acid propeptide/activation peptide [amino acids 192 through 226]; and a 235 amino acid Factor IXa heavy chain [amino acids 227 through 461]. Kurachi and Davie, PNAS USA 79:6461-64 (1982); Anson et al., Nucleic
35 Acids Res. 11:2325-35 (1983); UniProt Accession No. P00740. Coagulation Factor IX therapy has been approved as therapeutic treatment for subjects suffering from hemophilia B, including BeneFIX[®] (Wyeth);

Mononine[®] (Behring); and Alphanine[®] (Grifols Biologics). Accordingly, sequences useful as the therapeutic agent in the present invention include sequences encoding FIX (below) and FIXa.

MQRVNMIMAE SPGLITICLL GYLLSAECTV FLDHENANKI LNRPKRYNSG KLEEFVQGNL
 ERECMEEKCS FEEAREVFEN TERTTEFWKQ YVDGDQCESN PCLNGGSKD DINSYECWCP
 5 FGFEGKNCEL DVTCNIKNGR CEQFCKNSAD NKVVCSTEG YRLAENQKSC EPAVPPFCGR
 VSVSQTSKLT RAETVFPDVD YVNSTEAETI LDNITQSTQS FNDFTRVVG EDAPGQFPW
 QVVLNGKVDA FCGGSIVNEK WIVTAAHCVE TGVKITVVAG EHNIEETEHT EQKRNVIIRII
 PHHNYNAAIN KYNHDIALLE LDEPLVLNSY VTPICIAKKE YTNIFLKFVS GYVSGWGRVF
 HKGRSALVLQ YLRVPLVDRA TCLRSTKFTI YNNMFCAGFH EGGRDSCQGD SGGPHVTEVE
 10 GTSFLTGIIS WGEECAMK GK YGIYTKVSRV VNWIKKTKL T [SEQ ID NO:14]

Advantages of using a VSA in association with a FIX (e.g., using a VSA-FIX fusion protein) include, e.g., extending the PK of the FIX. The extended PK can have advantages; for example, the FIX can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the FIX can be achieved.

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Erythropoietin (EPO). In one embodiment, the therapeutic agent is an EPO (e.g., a human EPO) or a biologically active variant or fragment thereof. Human EPO is a 193 amino acid protein, including a 27 amino acid N-terminal signal peptide. (Uniprot Accession Number: P01588). For example, the therapeutic agent can have the ability to increase red blood cell production. EPO is an approved therapeutic for
 20 treatment of anemia, and is marketed as Epogen[®] (Amgen, Thousand Oaks, CA); Recormon[®] (Roche, South San Francisco, CA) and under a number of other marketed names. An exemplary amino acid sequence encoding human EPO is:

MGVHECPAWL WLLLSLLSLP LGLPVLGAPP RLI CDSRVLE RYLLEAKEAE NIT TGCAEHC
 25 SLNENITVPD TKVNFYAWKR MEVGQQAQVEV WQGLALLSEA VLRGQALLVN SSQPWEPLQL
 HVDKAVSGLR SLTTLRLALG AQKEAISPPD AASAAPLRTI TADTFRKLFV VYSNFLRGKL
 30 KLYTGEACRT GDR [SEQ ID NO:15]

Advantages of using a VSA in association with an EPO (e.g., using a VSA-EPO fusion protein) include, e.g., extending the PK of the EPO. The extended PK can have advantages; for example, the EPO can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the EPO can be achieved.

35

Granulocyte Colony Stimulating Factor (G-CSF). In one embodiment, the therapeutic agent is a G-CSF (e.g., human G-CSF) or a biologically active variant or fragment thereof. Human G-CSF is a 207 amino acid protein, including a 29 amino acid N-terminal signal peptide. (Uniprot Accession No. P09919). For example, the therapeutic agent can have the ability to increase the level of neutrophils in the blood. G-
 40 CSF is an approved therapeutic for the treatment of neutropenia, that is low neutrophil levels, and is marketed as Neupogen[®] (Amgen); and as Granocyte[®] (Chugai, Turnham Green, UK). An exemplary amino acid sequence encoding human G-CSF is:

MAGPATQSPM KLMALQLLLW HSALWTVQEA TPLGPASSLP QSFLLKCLEQ VRKIQGDGAA
 LQEKLVSECA TYKLCHPEEL VLLGHSLGIP WAPLSSCPSQ ALQLAGCLSQ LHSGLFLYQG
 LLQALEGISP ELGPTLDTLQ LDVADFATTI WQOMEELGMA PALOPTQGAM PAFASAFQRR
 AGGVLVASHL QSFLEVSYRV LRHLAQP [SEQ ID NO: 16]

5 Advantages of using a VSA in association with an G-CSF (e.g., using a VSA- G-CSF fusion protein) include, e.g., extending the PK of the G-CSF. The extended PK can have advantages; for example, the G-CSF can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the G-CSF can be achieved.

Interferon. In one embodiment, the therapeutic agent is an interferon (e.g., a human interferon) or
 10 a biologically active variant or fragment thereof. In some embodiments, the interferon is a human alpha interferon, beta interferon or gamma interferon. For example, the therapeutic can have antiviral, antibacterial or anticancer activities, and important immunoregulatory activity, such as activation of macrophages.

Alpha interferons are a family of closely related proteins. Alpha interferon-2 is a 188 amino acid
 15 protein, including a 23 amino acid N-terminal signal peptide. (Uniprot Accession No. P01563). Alpha interferon-2 is an approved therapeutic for the treatment of various cancers, and is marketed as Roferon-A[®] (Roche); and Intron-A[®] (Schering-Plough). An exemplary amino acid sequence of human alpha interferon-2 is:

20 MALTFALLVA LLVLSCKSSC SVGCDLPQTH SLGSRRTLML LAQMRKISLF SCLKDRHDFG
 FPQEEFGNQF QKAETIPVLH EMIQQIFNLF STKDSSAAWD ETLLEDKIFYTE LYQQLNDDLEA
 CVIQGVGVTE TPLMKEDSIL AVRKYFQRIT LYLKEKKYSP CAWEVVRAEI MRSFSLSTNL
 25 QESLRSKE [SEQ ID NO:17]

Beta interferon is a 187 amino acid protein, including a 21 N-terminal amino acid signal peptide. (Uniprot Accession No. P01574). Beta interferon is an approved therapeutic for the treatment of multiple sclerosis, and is marketed as Avonex[®] (Biogen Idec, Cambridge, MA); Betaseron[®] (Berlex) and Rebif[®] (Ares-Serono). See also, Houghton et al., Nucleic Acids Res., 8:2885-94 (1980). An exemplary amino
 30 acid sequence of human beta interferon is:

35 MTNKLLQIA LLLCFSTTAL SMSYNLLGFL QRSSNFQCQK LLWQLNGRLE YCLKDRMNF
 IPEEIKQLQQ FQKEDAALTI YEMLQNIFAI FRQDSSSTGW NETIVENLLA NVYHQINHLK
 TVLEEKLEKE DFTRGKLMSS LHLKRYYGRI LHYLKAKEYS HCAWTIVRVE ILRNFYFINR
 LTGYLRN [SEQ ID NO:18]

Gamma interferon is a 166 amino acid protein, including a 23 amino acid N-terminal signal peptide, and a 5 amino acid propeptide [residues 162-166]. (Uniprot Accession No. P01579). Gamma
 40 interferon is an approved therapeutic for the treatment of serious infections, such as those associated with chronic granulomatous disease, and is marketed as Actimmune[®] (Genentech). See also, Rinderknecht et al., J. Biol. Chem., 259:6790-97 (1984). An exemplary amino acid sequence of human gamma interferon is:

MKYTSYILAF QLCIVLGLSLG CYCQDPYVKE AENLKKYFNA GHSDVADNGT LFLGILKNWK
 EESDRKIMQS QIVSFYFKLF KNFKDDQSIQ KSVETIKEDM NVKFFNSNKK KRDDFEKLTN

5 YSVTDLNVQR KAIHELIOVM AELSPAAGTG KRKRSQMLFR GRRASQ [SEQ ID NO:19]

Advantages of using a VSA in association with an interferon (e.g., using a VSA-interferon fusion protein) include, e.g., extending the PK of the interferon. The extended PK can have advantages; for example, the interferon can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the interferon can be achieved.

10

Cytokine antagonists. In some embodiments, the therapeutic agent is an antagonist of cytokine signaling, e.g., an antagonist of interleukin or interferon signaling. For example, the therapeutic agent is an IL-1 receptor antagonist (IL-1ra) such as Kineret® (Amgen) or a variant thereof (see, e.g., US Patent 6,599,873). Human IL-1Ra is a 177 amino acid protein, including a 25 amino acid N-terminal signal peptide (Uniprot Accession No. P018510). IL-1Ra is an approved therapeutic for rheumatoid arthritis. An exemplary IL-1 receptor antagonist (IL-1ra) includes the following human amino acid sequence:

MEICRGLRSH LITLLLFLFH SETICRPSGR KSSKMQAFRI WDVNQKTFYL RNNQLVAGYL
 QGPNVNLEEK IDVVPPIEPA LFLGIHGGKM CLSCVKSGDE TRLQLEAVNI TDLSENKQD

20 KRFAFIRSDS GPTTSFESAA CPGWFLCTAM EADQPVSLTN MPDEGVMVTK FYFQEDE

[SEQ ID NO:20]

In some embodiments, the antagonist is a variant cytokine can be a cytokine with altered function, e.g., a dominant negative cytokine. Exemplary variant cytokines include IL-17 molecules described in WO2011/044563 and IL-23 molecules described in WO2011/011797.

25

Advantages of using a VSA in association with a cytokine antagonist (e.g., using a VSA-cytokine antagonist fusion protein) include, e.g., extending the PK of the cytokine antagonist. The extended PK can have advantages; for example, the cytokine antagonist can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the cytokine antagonist can be achieved.

30

Urate oxidase In one embodiment, the therapeutic agent is a urate oxidase, also known as uricase, or a biologically active variant or fragment thereof. An exemplary urate oxidase sequence is the sequence from *Aspergillus flavus*:

MSAVKAARYG KDNVRVYKVH KDEKTGVQTV YEMTVCVLE GEIETSYTKA DNSVIVATDS
 IKNTIYITAK QNPVTPPELF GSILGTHFIE KYNHIHAAHV NIVCHRWTRM DIDGKPHPHS
 35 FIRDSEKRN VQVDVVEGKG IDIKSSLSGL TVLKSTNSQF WGFLRDEYTT LKETWDRILLS
 TDVDATWQWK NFSGLQEVRS HVPKFDATWA TAREVTLKTF AEDNSASVQA TMYKMAEQIL
 ARQQLIETVE YSLPNKHIFE IDLSWHKGLQ NTGKNAEVFA PQSDPNGLIK CTVGRSSLKS
 KL [SEQ ID NO:21]

40

Both humans and certain other primates lack a naturally occurring urate oxidase protein, presumably due to an adaptive evolution. (Wu et al., PNAS USA, 86:9412-16 (1989)). However, many mammalian species of urate oxidase are known, including pig (Uniprot Accession No. P16164); cynomolgus monkey (Uniprot Accession No. Q8MHW6); baboon (Uniprot Accession No. P25689); rabbit (Uniprot Accession No. P011645); rat (Uniprot Accession No. P09118); and mouse (Uniprot Accession No. P25688). Urate oxidase catalyzes the oxidation of uric acid to 5-hydroxyisourate, leading to increased

solubility and renal excretion, which can prevent symptoms of gout. Urate oxidase is an approved therapeutic for the treatment of hyperuricaemia, and is marketed as Rasburicase[®] (recombinant uricase from *aspergillus flavus* produced in *saccharomyces cerevisiae*; Sanofi-Aventis) or Krystexxa[®] (recombinant pegylated chimeric uricase, sequence from pig/baboon, produced in *saccharomyces cerevisiae*, Savient Pharmaceuticals, Inc.). These products have been reported to be immunogenic, which can limit the ability to treat patients repeatedly. For this reason, the approaches provided herein can have additional advantages in that the VSA can serve to prevent or impede the native immune system from readily accessing the biologically active uricase moiety, and thereby can reduce or prevent the formation of antibodies to the biologically active uricase moiety after administration.

Advantages of using a VSA in association with a urate oxidase (e.g., using a VSA-urate oxidase fusion protein) include, e.g., extending the PK of the urate oxidase. The extended PK can have advantages; for example, the urate oxidase can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the urate oxidase can be achieved.

C-Natriuretic Peptide (CNP). In one embodiment, the therapeutic agent is a CNP (e.g., human CNP) or a biologically active variant or fragment thereof. Human CNP is a 126 amino acid protein, including a 23 amino acid N-terminal signal peptide; a 50 amino acid propeptide (amino acids 24-73); and a 53 amino acid CNP-53 peptide (amino acids 74-126). The 53 amino acid CNP-53 peptide may be further processed to a 29 amino acid CNP-29 peptide (amino acids 98-126) or a 22 amino acid CNP-22 peptide (amino acids 105-126) (Uniprot Accession No. P23582). An exemplary amino acid sequence encoding human CNP is:

MHLSQLLACA LLLTLLSLRP SEAKPGAPPK VPRTPPAEEL AEPQAAGGGQ KKGDKAPGGG
GANLKGDRSR LLRDLRVDTK SRAAWARLLQ EHPNARKYKG ANKKGLSKGC FGLKLDRIQS
MSG LGC [SEQ ID NO:22]

CNP has been implicated in a number of physiological processes, including vasorelaxant with powerful venodilator effects, exhibiting the ability to reduce mean arterial pressure, atrial pressure and cardiac output in mammals; dose-dependent relaxant effects on bronchial smooth muscle and pulmonary arterial relaxation; and can also have involvement in neurotransmission. See, Barr et al., *Peptides* 17:1243-51 (1996).

Advantages of using a VSA in association with a CNP (e.g., using a VSA-CNP fusion protein) include, e.g., extending the PK of the CNP. The extended PK can have advantages; for example, the CNP can be administered less frequently and/or at reduced concentrations and/or more consistent delivery levels of the CNP can be achieved.

Production

A variety of molecular biology techniques can be used to design nucleic acid constructs encoding a protein that includes a serum albumin or a domain thereof. The nucleic acid sequences can be any sequences that code for the VSA of interest. For example, a nucleic acid sequence can be based on a known sequence that encodes a corresponding native (e.g., wild type) serum albumin. For example, a

sequence that encodes a wild type albumin can be that corresponding to UniProt DB Accession No. P02768, e.g., ENI database sequence V00494.1 (www.ebi.ac.uk/ena/data/view/V00494).

The coding sequence can include, e.g., a sequence encoding a protein described herein, a variant of such sequence, or a sequence that hybridizes to such sequences. An exemplary coding sequence for mammalian expression can further include an intron. Coding sequences can be obtained, e.g., by a variety of methods including direct cloning, PCR, and the construction of synthetic genes. Various methods are available to construct useful synthetic genes, see, e.g., the GeneArt® GeneOptimizer® from Life Technologies, Inc. (Carlsbad, CA), Sandhu et al. (2008) *In Silico Biology* 8: 0016; Gao et al. (2004) *Biotechnol Prog*, 20: 443–8.; Cai et al. (2010) *J Bioinformatics Sequence Analysis* 2: 25–29; and Graf et al (2000), *J Virol* 74: 10822–10826.

The coding sequence generally employs one or more codons according to the codon tables for eukaryotic or prokaryotic expression. A coding sequence can be generated with specific codons (e.g., preferred codons) and/or one or more degenerate codons. A possible set of degenerate codons is set forth in the table below.

TABLE 1

Amino acid	Code	Codons	Degenerate
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAV
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG

Stop	.	TAA TAG TGA	TRR
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The degenerate codon can be representative of all possible codons encoding each amino acid, but may not always be unambiguous. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequences disclosed herein. Variant sequences can be readily tested for functionality as described herein.

In some embodiments, the coding sequence includes one or more preferred codons for the cell in which it is to be expressed. Generally preferred codons are those that are translated most efficiently and can include the codons that are most frequently used by cells of the species in question. Each species can exhibit its own codon preferences. See, e.g. Gustafsson et al. (2004) *Trends in Biotechnol.* 22:346-353; Grantham et al. (1980) *Nuc. Acids Res.* 8:1893-912; Haas et al. (1996) *Curr. Biol.* 6:315-24; Wain-Hobson et al. (1981) *Gene* 13:355-64; Grosjean and Fiers (1982) *Gene* 18:199-209; Holm (1986) *Nuc. Acids Res.* 14:3075-87; Ikemura (1982) *J. Mol. Biol.* 158:573-97. For example, the amino acid threonine (Thr) can be encoded by ACA, ACC, ACG, or ACT. In mammalian cells, ACC is the most commonly used Thr codon, whereas different Thr codons may be preferred in other species. Preferred codons for a particular species can be introduced into coding sequences by a variety of methods, including direct cloning, PCR mutagenesis, and the construction of synthetic genes. Introduction of preferred codon sequences into recombinant DNA can increase translational efficiency. In some embodiments, at least 10, 20, 30, 50, 60, 70, or 80% of the codons in a coding sequence are preferred codons. Sequences containing preferred codons can be constructed, and tested for expression in various species.

A protein described herein, such as a protein containing a serum albumin domain described herein, can be expressed in bacterial, yeast, plant, insect, or mammalian cells.

Exemplary mammalian host cells for recombinant expression include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, e.g., as described in Kaufman and Sharp (1982) *Mol. Biol.* 159:601-621), lymphocytic cell lines, e.g., NS0 myeloma cells and SP2 cells, COS cells, K562, and a cell from a transgenic animal, e.g., a transgenic mammal. For example, the cell can be a mammary epithelial cell.

Coding nucleic acid sequences can be maintained in recombinant expression vectors that include additional nucleic acid sequences, such as a sequence that regulate replications of the vector in host cells (e.g., origins of replication) and a selectable marker gene. The selectable marker gene facilitates selection of host cells into which the vector has been introduced. Exemplary selectable marker genes appropriate for

mammalian cells include the dihydrofolate reductase (DHFR) gene (for use in *dhfr* host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

Within the recombinant expression vector, the coding nucleic acid sequences can be operatively linked to transcriptional control sequences (e.g., enhancer/promoter regulatory elements) to drive high levels of transcription of the genes. Examples of eukaryotic transcriptional control sequences include the metallothionein gene promoter, promoters and enhancers derived from eukaryotic viruses, such as SV40, CMV, adenovirus and the like. Specific examples include sequences including a CMV enhancer/AdMLP promoter regulatory element or an SV40 enhancer/AdMLP promoter regulatory element.

An exemplary recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the protein.

An adenovirus system can also be used for protein production. By culturing adenovirus-infected non-293 cells under conditions where the cells are not rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are grown to confluence in cell factories, and exposed to the adenoviral vector encoding the secreted protein of interest. The cells are then grown under serum-free conditions, which allows infected cells to survive for several weeks without significant cell division. In another method, adenovirus vector-infected 293 cells can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein (See Garnier et al., (1994) Cytotechnol. 15:145-55, and Liu et al., (2009) J. Bioscience and Bioengineering, 107:524-529). The expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant, lysate, or membrane fractions depending on the disposition of the expressed protein in the cell. Within the infected 293 cell production protocol, non-secreted proteins can also be effectively obtained.

Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica* nuclear polyhedrosis virus (AcNPV) according to methods known in the art. Recombinant baculovirus can be produced through the use of a transposon-based system described by Luckow et al. (J. Virol. 67:4566-4579, 1993). This system, which utilizes transfer vectors, is commercially available in kit form (Bac-to-Bac™ kit; Life Technologies, Rockville, Md.). An exemplary transfer vector (e.g., pFastBac1™ Life Technologies) contains a Tn7 transposon to transfer the DNA encoding the protein of interest into a baculovirus genome maintained in *E. coli* as a bacmid. See, Condreay et al., (2007) Current Drug Targets 8:1126-1131. In addition, transfer vectors can include an in-frame fusion with DNA encoding a polypeptide extension or affinity tag as disclosed above. Using techniques known in the art, a transfer vector containing nucleic acid sequence encoding a variant serum albumin fusion is transformed into *E. coli* host cells, and the cells are screened for bacmids which contain an interrupted *lacZ* gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, such as Sf9 cells.

Recombinant virus that expresses a protein containing a serum albumin domain is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.

For protein production, the recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda* (e.g., Sf9 or Sf21 cells) or *Trichoplusia ni* (e.g., High Five™ cells; Invitrogen, Carlsbad, Calif.). See, for example, U.S. Pat. No. 5,300,435. Serum-free media are used to grow and maintain the cells. Suitable media formulations are known in the art and can be obtained from commercial suppliers. The cells are grown up from an inoculation density of approximately $2\text{-}5 \times 10^5$ cells to a density of $1\text{-}2 \times 10^6$ cells, at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally known in the art.

Other higher eukaryotic cells can also be used as hosts, including plant cells and avian cells. *Agrobacterium rhizogenes* can be used as a vector for expressing genes in plant cells. See e.g., O'Neill et al. (2008) *Biotechnol. Prog.* 24:372-376.

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*, and *Pichia methanolica*. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). Production of recombinant proteins in *Pichia methanolica* is described, e.g., in US 5,716,808, US 5,736,383, US 5,854,039, and US 5,888,768.

The binding protein is recovered from the culture medium and can be purified. Various methods of protein purification can be employed and such methods are known in the art and described for example in Deutscher, *Methods in Enzymology*, 182 (1990); and Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag, New York (2010) (ISBN: 1441928332). Purified variant serum albumin fusion proteins can be concentrated using standard protein concentration techniques.

Exemplary of purification procedures include: ion exchange chromatography, size exclusion chromatography, and affinity chromatography as appropriate. For example, variant serum albumin fusion proteins can be purified with a HSA affinity matrix.

To prepare the pharmaceutical composition a variant serum albumin fusion protein is typically at least 10, 20, 50, 70, 80, 90, 95, 98, 99, or 99.99% pure and typically free of other proteins including undesired human proteins and proteins of the cell from which it is produced. It can be the only protein in the composition or the only active protein in the composition or one of a selected set of purified proteins. Purified preparations of a variant serum albumin fusion protein described herein can include at least 50, 100, 200, or 500 micrograms, or at least 5, 50, 100, 200, or 500 milligrams, or at least 1, 2, or 3 grams of the binding protein. Accordingly, also featured herein are such purified and isolated forms of the binding proteins described herein. The term "isolated" refers to material that is removed from its original environment (e.g., the cells or materials from which the binding protein is produced).

Linkers

In some embodiments described herein, a VSA is associated with an agent (e.g., a diagnostic or therapeutic agent), e.g., for the purpose of improving a functional property (e.g., extending the PK) of the agent. In some embodiments, the VSA is physically attached to the agent. The VSA can be directly
5 attached to the agent or it can be attached to the agent via a linker.

In some embodiments, a heterologous protein that comprises a VSA and an additional agent (e.g., a diagnostic or therapeutic agent) is made using recombinant DNA techniques. In some embodiments, the VSA is produced (e.g., using recombinant DNA techniques) and subsequently linked to the agent, e.g., by
10 chemical means.

A variety of linkers can be used to join a polypeptide component of an agent to domain III or a variant serum albumin. The linker can be a molecule or group of molecules (such as a monomer or polymer) that connects two molecules and optionally to place the two molecules in a particular configuration. Exemplary linkers include polypeptide linkages between N- and C-termini of proteins or protein domains, linkage via disulfide bonds, and linkage via chemical cross-linking reagents.
15

In some embodiments, the linker includes one or more peptide bonds, e.g., generated by recombinant techniques or peptide synthesis. The linker can contain one or more amino acid residues that provide flexibility. In some embodiments, the linker peptide predominantly includes the following amino acid residues: Gly, Ser, Ala, and/or Thr. The linker peptide should have a length that is adequate to link two molecules in such a way that they assume the correct conformation relative to one another so that they
20 retain the desired activity. Suitable lengths for this purpose include at least one and not more than 30 amino acid residues. For example, the linker is from about 1 to 30 amino acids in length. A linker can also be, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 amino acids in length.

Exemplary linkers include glycine-serine polymers (including, for example, (GS)_n, (GSGGS)_n, (SEQ ID NO: 23), (GGGGS)_n (SEQ ID NO: 24) and (GGGS)_n (SEQ ID NO: 25), where n is an integer of
25 at least one, e.g., one, two, three, or four), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers. Glycine-serine polymers can serve as a neutral tether between components. Secondly, serine is hydrophilic and therefore able to solubilize what could be a globular glycine chain. Third, similar chains have been shown to be effective in joining subunits of recombinant proteins such as single chain antibodies. Suitable linkers can also be identified from three-dimensional structures in structure databases
30 for natural linkers that bridge the gap between two polypeptide chains. In some embodiments, the linker is from a human protein and/or is not immunogenic in a human. Thus linkers can be chosen such that they have low immunogenicity or are thought to have low immunogenicity. For example, a linker can be chosen that exists naturally in a human. In certain embodiments the linker has the sequence of the hinge region of an antibody, that is the sequence that links the antibody Fab and Fc regions; alternatively the linker has a
35 sequence that comprises part of the hinge region, or a sequence that is substantially similar to the hinge region of an antibody. Another way of obtaining a suitable linker is by optimizing a simple linker, e.g., (Gly₄Ser)_n (SEQ ID NO: 24), through random mutagenesis. Alternatively, once a suitable polypeptide

linker is defined, additional linker polypeptides can be created to select amino acids that more optimally interact with the domains being linked. Other types of linkers include artificial polypeptide linkers and inteins. In another embodiment, disulfide bonds are designed to link the two molecules. Other examples include peptide linkers described in US Patent 5,073,627, the disclosure of which is hereby incorporated by
5 reference. In certain cases, the diagnostic or therapeutic protein itself can be a linker by fusing tandem copies of the peptide to a variant serum albumin polypeptide. In certain embodiments, charged residues including arginine, lysine, aspartic acid, or glutamic acid can be incorporated into the linker sequence in order to form a charged linker.

In another embodiment, linkers are formed by bonds from chemical cross-linking agents. For
10 example, a variety of bifunctional protein coupling agents can be used, including but not limited to N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido
15 compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). Chemical linkers can enable chelation of an isotope. For example, C¹⁴ 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (see PCT WO
94/11026).

The linker can be cleavable, facilitating release of a payload, e.g., in the cell or a particular milieu.
20 For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Research 52:127-131(1992)) can be used. In some embodiments, the linker includes a non-proteinaceous polymer, e.g., polyethylene glycol (PEG), polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol.

In one embodiment, the variant serum albumin fusion of the present invention is conjugated or
25 operably linked to another therapeutic compound, referred to herein as a conjugate. The conjugate can be a cytotoxic agent, a chemotherapeutic agent, a cytokine, an anti-angiogenic agent, a tyrosine kinase inhibitor, a toxin, a radioisotope, or other therapeutically active agent. Chemotherapeutic agents, cytokines, anti-angiogenic agents, tyrosine kinase inhibitors, and other therapeutic agents have been described above, and
30 all of these aforementioned therapeutic agents can find use as variant serum albumin fusion conjugates. In an alternate embodiment, the variant serum albumin fusion is conjugated or operably linked to a toxin, including but not limited to small molecule toxins and enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof. Small molecule toxins include but are not
35 limited to calicheamicin, maytansine (U.S. Pat. No. 5,208,020), trichothene, and CC1065. In one embodiment of the invention, the variant serum albumin fusion is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antibody molecule). Maytansine can, for example, be converted to May-SS-Me which can be reduced to May-SH3 and reacted with a variant serum

albumin fusion (Chari et al., 1992, Cancer Research 52: 127-131) to generate a maytansinoid-antibody or maytansinoid-Fc fusion conjugate. Another conjugate of interest comprises a variant serum albumin fusion conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double-stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin that can be used include but are not limited to γ_1^1 , α_2^1 , α_3 , N-acetyl- γ_1^1 , PSAG, and θ_1^1 , (Hinman et al., 1993, Cancer Research 53:3336-3342; Lode et al., 1998, Cancer Research 58:2925-2928) (US 5,714,586; US 5,712,374; US 5,264,586; US 5,773,001). Dolastatin 10 analogs such as auristatin E (AE) and monomethylauristatin E (MMAE) can find use as conjugates for the variant serum albumin fusions of the present invention (Doronina et al., 2003, Nat Biotechnol 21:778-84; Francisco et al., 2003 Blood 102:1458-65). Useful enzymatically active toxins include but are not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, PCT WO 93/21232. The present invention further contemplates a conjugate or fusion formed between a variant serum albumin fusion of the present invention and a compound with nucleolytic activity, for example a ribonuclease or DNA endonuclease such as a deoxyribonuclease (DNase).

In an alternate embodiment, a variant serum albumin fusion of the present invention can be conjugated or operably linked to a radioisotope to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugate variant serum albumin fusions. Examples include, but are not limited to, At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹², P³², and radioactive isotopes of Lu.

Screening Methods

Assays

Binding of a serum albumin or a domain thereof to FcRn can be evaluated *in vitro*, e.g., by surface plasmon resonance, (see, e.g., Example 2), ELISA (see, e.g., Example 4) or other binding assay known in the art.

FcRn can be produced as a single chain molecule, e.g., in CHO cells. An exemplary method for producing single chain FcRn is described in Feng et al., Protein Expression and Purification, 79:66-71 (2011).

The half-life of a protein that includes serum albumin or a domain thereof *in vivo* can be evaluated in a mammal, e.g., a murine model that includes a human FcRn. See e.g., Example 3. For example, the protein that is evaluated can be a protein that includes serum albumin or a domain thereof and a therapeutic agent.

Activity Assays

To assess the activity of an agent (e.g., a therapeutic agent) that is associated with a VSA as described herein, methods known in the art for testing the activity of the agent can be used. The following description provides examples of such methods.

5 **Assays for testing the activity of a FVII**

Examples of assays that can be used to test the activity of a FVII entity are known in the art. The following exemplary coagulation assays are adapted from Wildgoose et al., *Blood*, 80:25-28 (1992). All plasma factor VIIa levels are measured in a one-stage clotting assay using an ACL300R automated coagulation instrument which can be purchased from Instrumentation Laboratories (Ascoli Piceno, Italy).
10 Test samples are diluted fivefold in 0.1 mol/L NaCl/0.05 mol/L Tris-HCl/0.1% BSA pH 7.4 (TBS/BSA) and mixed with an equal volume of hereditary factor VII deficient plasma to yield a total volume of 100 μ L. Each aliquot is subsequently incubated for 5 minutes at 37°C with 50 μ L of bovine phospholipids (Thrombofax). Coagulation is then initiated by the addition of a 100- μ L aliquot of 10 nmol/L TF1.218 diluted in 12.5 mmol/L CaCl₂/0.1 mol/L NaCl/0.05 mol/L Tris/1% BSA pH 7.4. Coagulation times are
15 subsequently converted to factor VIIa concentration (nanograms per milliliter) by comparison to a standard curve constructed with varying concentrations (0.05 to 50 ng/mL) of purified recombinant factor VIIa diluted in TBS/BSA.

Venipunctures are performed atraumatically and blood samples drawn into citrated vacutainers. The citrated samples are centrifuged for 15 minutes at 1,200 X g after which time the plasma is removed
20 with a plastic pipette and stored at -80°C. Normal plasma samples are collected from 10 fasting and 10 nonfasting individuals who have a negative history for bleeding as well as thrombosis and are not taking any medications at the time of sample collection. Samples are obtained from 13 severe hemophilia A and seven hemophilia B subjects (< 1% FVIII:C and < 1% FIXC). Subjects are excluded from the study if they have received factor concentrates, cryoprecipitate, and/or antifibrinolytics within the previous 48 hours.

The following assay is adapted from Silveira et al., *Arteriosclerosis and Thrombosis*, 14:60-69 (1994). Coagulation factor VII is assessed in plasma samples drawn before ingestion of the test meal and 3, 6, and 12 hours thereafter. Factor VIIc is determined as described in van Deijk et al., *Haemostasis*, 13:192-97 (1983) in an LODE coagulometer (Groningen, the Netherlands). Briefly, 100 μ L of diluted plasma sample, 100 μ L of factor VII-deficient plasma (Helena Laboratories, Beaumont, Tex), and 100 μ L
30 of human brain thromboplastin (prepared according to Owren and Aas, *Scand J. Clin Lab Invest* 3:201-18 (1951)) are incubated together at 37°C for 30 seconds. Calcium, 100 μ L of a 33 mmol/L solution, is added and the clotting time recorded. Factor VIIa is determined with either Thrombotest (a bovine brain thromboplastin preparation that also contains adsorbed bovine plasma; Nyegaard & Co, Oslo, Norway) (VIIa:A) or bovine brain thromboplastin (Diagnostic Reagents Ltd, Thames, Oxon, UK) (VIIa:B) in
35 clotting assays that are otherwise essentially as described above. Factor VII amidolytic activity (VIIam) is determined with a commercially available kit (Coa-Set FVII; Chromogenix, M61ndal, Sweden). Factor VII antigen (VII:Ag) concentration is determined with an enzyme immunoassay kit (Novoclone Factor VII

EIA kit, Novo Nordic A/S, Bagsvaerd, Denmark). Results are expressed in units per milliliter, one unit being the amount or activity of factor VII present in 1 mL of a standard pooled plasma. Ratios of VIIa:B to VIIc and of VIIa:B to VII:Ag are also calculated for evaluation of the activity state of factor VII in plasma. Control experiments are performed to rule out the possibility of a direct effect of high plasma concentrations of TG-rich lipoproteins on the factor VII levels obtained in the different assays. An Sf 20 to 400 lipoprotein fraction is isolated from blood that had been drawn from a healthy control subject 3 hours after intake of a fat load. Addition of this lipoprotein fraction to control plasma to a final plasma level 500% of normal does not influence factor VII measurements.

Additional examples of clotting assays that can be adapted for use to assess the activity of a FVII in association with a VSA are described in the following publications: Broze and Majerus, *J. Biol. Chem.* 255:1242-47 (1980); Morrissey et al., *Blood*, 81:734-44 (1993); Herzog et al., *Nature Medicine*, 5:56-63 (1999); and Sorensen and Ingerslav, *J. Thrombosis and Haemostasis*, 2:102-110 (2004).

Assays for assessing the activity of a hepcidin

Several significant factors can be assayed to evaluate a subject for iron overload and/or risk of iron overload. First, serum iron levels can be used as an indicator of iron overload. Tests include the serum ferritin test. A subject can be considered to be suffering from iron overload if their serum iron levels are in excess of about 350 µg/dL (mild iron toxicity); generally in excess of about 500 µg/dL (serious iron toxicity). A subject is considered to be at risk of iron overload if their serum iron levels are high normal or above normal ranges. Normal iron range is considered to be from about 40 to about 220 µg/dL; for example, from about 50 to about 160 µg/dL for adult males. Normal iron ranges for adult females are approximately 5 to 10 percent lower than that for adult males. 'High normal' iron concentration is considered to be in the upper quarter (25%) of the normal range; generally in the upper tenth (10%) of the normal range. See, Jacobs & DeMott, *Laboratory Test Handbook*, 5th ed., (LexiComp Inc, Hudson, OH)(2001) at p. 203-205). As is known to those skilled in the art, 'normal ranges' of iron and iron binding capacity can vary depending upon the specific laboratory and test. Other parameters that can be used to evaluate a patient for iron overload and/or risk of overload include: measurement of hepcidin levels (see US Patent 7,998,691); genetic testing for the presence of mutations in one or more genes related to hemochromatosis, such as juvenile hemochromatosis (HFE2A and HFE2B genes) (see US Patent 8,080,651)

Assays for assessing the activity of a GLP-1

Culture of BRIN-BD11 cells. BRIN-BD11 cells are cultured in RPMI-1640 tissue culture medium containing 10% (v/v) foetal calf serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin), and 11.1 mM glucose. BRIN-BD11 cells are produced by electrofusion of a New England Deaconess Hospital (NEDH) rat pancreatic β-cell with RINm5F cell to produce and immortal, glucose sensitive cell line which is described in detail elsewhere. McClenaghan et al., *Diabetes* (1996) 45:1132-40. All cells are maintained in sterile tissue culture flasks (Corning Glass Works, Sunderland, UK) at 37 °C in an atmosphere of 5% CO₂ and 95% air using a LEEC incubator (Laboratory Technical Engineering,

Nottingham, UK). [Green et al., *Id.*]

Stimulation of adenylate cyclase. BRIN-BD11 cells are seeded into 24-well plates (3×10^5 /well) and cultured for 48 h before being pre-incubated in media supplemented with tritiated adenine (2 mCi) for 16 h. The cells are washed twice with cold Hanks' buffered saline (HBS) and test solution (400 μ l; 37 °C) is added. The cells are then exposed to varying concentrations (10^{-10} – 10^{-5} M) of GLP-1 glycopeptides in HBS buffer, in the presence of 1 mM IBMX and 5.6 mM glucose (20 min; 37 °C). Following incubation, test solutions are removed and 300 μ l of lysis solution (5% TFA, 3% SDS, 5 mM of unlabelled ATP, and 300 μ M of unlabelled cAMP) is added. Dowex and alumina exchange resins are used to separate tritiated cAMP from tritiated adenine and ATP in the cell lysate, as described previously. [Miguel et al., *Biochem Pharm.* (2003) 65:283-92]. The highest concentration of GLP-1 (10^{-5} M) is used as a maximum. Each peptide is tested by single experiment ($n=3$) which incorporated an internal control incubation of GLP-1 (60 nM) to ensure consistency and accuracy. [Green et al., *Id.*]

Insulin secretory responses in the pancreatic β -cell. BRIN-BD11 cells are seeded into 24-multiwell plates at a density of 1×10^5 /well, and allowed to attach during overnight culture. Acute studies of insulin release are preceded by a 40 minute pre-incubation at 37 °C in 1.0 ml Krebs–Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.2 mM KH_2PO_4 , 1.2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM NaHCO_3 , and 5 g/L bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose. Test incubations are performed at 37 °C in the presence of 5.6 mM glucose with a range of concentrations of GLP-1 glycopeptides (10^{-12} – 10^{-6} M). After a 20 minute incubation, the buffer is removed from each well and aliquots are stored at -20 °C for measurement of insulin. [Green et al., *Id.*]

Glucose-lowering and insulin secretory activity in obese diabetic (ob/ob) mice. The *in vivo* biological activity of variant serum albumin/GLP-1 fusion proteins can be assessed in 12–16 week old obese diabetic (*ob/ob*) mice. The animals are housed individually in an air-conditioned room at 22 ± 2 °C with a 12 hour light:12 hour dark cycle. Animals are allowed drinking water *ad libitum* and continuous access to standard rodent maintenance diet (Trouw Nutrition, Cheshire, UK). Mice are fasted for 18 hours and intraperitoneally administered 8 ml/kg body weight with saline (9 g/L NaCl), glucose alone (18 mM/kg body weight), or in combination with GLP-1 or a variant serum albumin/GLP-1 fusion protein (25 nM/kg body weight). Blood samples are collected into chilled fluoride/heparin microcentrifuge tubes (Sarstedt, Numbrecht, Germany) immediately prior to injection and at 15, 30, and 60 minutes post injection, and the plasma obtained is stored at -20 °C. All animal studies are carried out in accord with the UK Animals (Scientific Procedures) Act 1986 [Green et al., *Id.*] or other applicable laws.

Analyses and statistics. Plasma glucose levels are determined using an Analox glucose analyser (Hammersmith, London, UK), which employs the glucose oxidase method. Insulin levels are assayed by dextran-coated charcoal radioimmunoassay. Incremental areas under plasma glucose and insulin curves (AUC) are calculated using GraphPad PRISM version 3.0 (Graphpad Software, San Diego, CA, USA), which employs the trapezoidal rule. Results are expressed as means \pm SEM and data are compared as appropriate using Student's *t* test, repeated measures ANOVA or one-way ANOVA, followed by the

Student–Newman–Keuls post hoc test. Groups of data are considered significantly different if $P < 0.05$.

Measurement of Binding Affinity and cAMP Production. Binding affinity is assessed by measuring the inhibition of radiolabeled GLP-1 binding to human GLP-1 receptor-expressing chinese hamster ovary (CHO) cell membrane. Cell membrane fractions (5 μg) are incubated with 62 pM [125I]GLP-1 and variant serum albumin/GLP-1 fusion protein (final conc. 10^{-11} to 10^{-6} M) in 25 mM HEPES (pH 7.4) containing 5 mM MgCl₂, 1 mM CaCl₂, 0.25 mg/mL bacitracin, and 0.1% bovine serum albumin (BSA) at room temperature for 2 hours (100 μL). Membranes are filtered onto a 96-well GF/C plate (PerkinElmer, Inc.) that had been presoaked in 1% polyethylenimine containing 0.5% BSA, and then washed with 25 mM HEPES buffer containing 0.5% BSA (pH 7.4). Radioactivity associated with the lysates is determined using a gamma counter. Nonspecific binding is determined by the amount of binding in the presence of 1 μM unlabeled GLP-1. Dose-response curves are plotted for the individual compounds. IC₅₀ values are calculated using XLfit software (IDBS Inc.). For measurement of cAMP production, human GLP-1 receptor expressing CHO cells are passaged into multiwell plates (4000 cells/well) and cultured for an additional 48 h. The cells are washed with assay buffer (Hanks balanced salt solution containing 20 mM HEPES, 0.1% BSA, pH 7.4) and then exposed to variant serum albumin/GLP-1 fusion proteins (final conc. 10^{-12} to 10^{-6} M) in assay buffer containing 0.33 mM isobutylmethylxanthine and 0.67 mM RO20-1724 at room temperature for 1 h. The cells are lysed with 1% Triton X-100, and the cAMP formed is measured using a cAMP femtomolar kit (Cis Bio international). Dose-response curves are plotted for the individual compounds. EC₅₀ values are calculated using XLfit software. [Ueda et al., J. ACS, 2009 131:6237-45]

Characterization of Stability against Recombinant Human DPP-IV. GLP-1 or variant serum albumin/GLP-1 fusion protein (20-500 μM) is incubated at 37 °C in 100 mM HEPES buffer containing 0.05% Tween80 and 1 mM EDTA-2Na (pH 7.5) with 0.33 $\mu\text{g}/\text{mL}$, 0.66 $\mu\text{g}/\text{mL}$ (**19**), or 1.32 $\mu\text{g}/\text{mL}$ recombinant human DPP-IV (60 μL). At 5 or 10 min intervals, 7 μL is removed from the reaction mixture, and the reaction is terminated by the addition of 28 μL of 8 M GuHCl solution. The reaction products are subjected to RP-HPLC on a Develosil RPAQUEOUS- AR-3 2.0 μm 100 mm at 30 °C, and the C-terminal degradation product is quantified by using UV absorption at 210 nm. The initial rate of the degradation reaction is determined from the slope of the linear part obtained by plotting product concentration versus time. The resulting initial rates are plotted versus peptide concentration, and kinetic parameters (K_M and K_M/k_{cat}) are determined using XLfit software based on the Michaelis-Menten kinetic equation. [Ueda et al., *Id.*]

Characterization of Stability against Recombinant Human NEP 24.11. The 125 μM GLP-1 or variant serum albumin/GLP-1 fusion protein is incubated at 37 °C in 50 mM HEPES buffer containing 50 mM NaCl, and 0.05% Tween 80 with 4 $\mu\text{g}/\text{mL}$ recombinant human NEP 24.11 (pH 7.4, 84 μL). After 0.5, 1, 2, 3.5, and 5 h, 8 μL is removed from the reaction mixture, and the reaction is terminated by addition of 32 μL of 8 M GuHCl solution. The reaction products are subjected to RP-HPLC on a Develosil ODSHG-54.6 μm 150 mm at 30 °C, and the area of intact variant serum albumin/GLP-1 fusion protein is measured

using UV absorption at 210 nm. [Ueda et al., *Id.*]

Blood Glucose-Lowering Activity in Obese Diabetic db/db Mice. Male BKS.Cg-+ Leprdb/+ Leprdb mice (13-15 weeks of age) are allowed *ad libitum* access to food and water until the start of the experiment. At t -2 h, access to food is restricted, and the tip of the tail is cut. At t) 0 min, a 1 μ L blood sample is collected. Immediately thereafter, each mouse is injected subcutaneously with test sample (100 nmol/kg) or vehicle, and additional blood samples are collected. The vehicle is saline containing 1% BSA. Blood glucose levels are measured with a glucose oxidase biosensor (DIAMETERR; Arkray, Inc.). The effects of the test samples on blood glucose are expressed as % change relative to the respective pretreatment (t) 0 min) level. The number of mice tested is 6-7 for each group. Data are presented as means (SEM). Statistical differences are analyzed using the Dunnett's multiple comparison test, and P values less than 0.05 are regarded as significant. [Ueda et al., *Id.*]

Assays for assessing uric acid/gout

Methods for assessing uric acid levels and/or function as well as gout are known in the art. Methods for measurements of uric acid, e.g., in urine can be employed, as disclosed, e.g., in Ballesta-Claver et al., *Analytica Chimica Acta*, 702:254-61 (2011) and WO 2000/08207.

Therapeutic Administration of a VSA Composition

In certain embodiments, a VSA composition (a composition comprising a VSA or a VSA associated therapeutic agent) is administered in a therapeutically effective amount to a subject to treat a disease or condition, or ameliorate one or more symptoms of a disease or condition. Methods for delivering a therapeutic composition are known in the art and can be used to administer a VSA composition e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells that can expressing the VSA compound, receptor-mediated endocytosis (e.g., Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432). Methods of introduction can be enteral or parenteral, including but not limited to, intradermal, transdermal, intramuscular, intraperitoneal, intravenous, subcutaneous, pulmonary, intranasal, intraocular, epidural, topical, intramuscular, subcutaneous, intravenous, intravascular, and intrapericardial administration and oral routes. A VSA composition can also be administered, for example, by infusion or bolus injection, by absorption through epithelial or mucosa (e.g., oral mucosa, rectal, or intestinal mucosa) and can be administered together with other biologically active agents. Administration can be systemic or local. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. In certain aspects, the disclosure provides a composition comprising the HSA variant or the chimeric polypeptide of the disclosure, and a pharmaceutically acceptable carrier. In certain embodiments, VSA composition is delivered locally to an area in need of treatment (e.g., muscle); this may be achieved, for example, by local infusion, topical application, by injection, by catheter, or by implant (e.g., an implant of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, fibers, or commercial skin substitutes). In some embodiments, a VSA composition is delivered in a vesicle such as a liposome (see Langer, 1990, *Science*

249: 1527-1533), a controlled release system, or with a pump (see Langer, 1990, *supra*), or using polymeric materials can be used (see Howard et al, 1989, J. Neurosurg. 71 : 105).

Further illustration of the invention is provided by the following non-limiting examples.

5

EXAMPLES

Example 1

cDNA encoding mature human serum albumin was cloned into a modified version of the pYC2/CT yeast expression vector (Invitrogen) containing a Trp marker, app8 leader peptide (see Rakestraw et al., Biotechnology and Bioengineering, 103:1192-1201 (2009) for leader sequence), and N-terminal His₆ tag (SEQ ID NO: 26) and Factor Xa cleavage site. Point mutations, either alone or in combination, were introduced by Quikchange® mutagenesis (Agilent). The vector was transformed into BJ5α *S. cerevisiae* cells using the EZ-yeast kit (Zymo Research) and transformants selected on SDCAA +ura plates (2% glucose, 0.67% yeast nitrogen base, 0.5% casamino acids, 0.54% Na₂PO₄, 0.86% NaH₂PO₄·H₂O, 18.2% sorbitol, 1.5% agar, and 40 mg/L uracil). Selected colonies were grown in 5 mL liquid SDCAA + ura overnight at 30°C with shaking at 250 RPM. The 5 mL overnight culture was diluted into 50 mL SDCAA + ura in a 250 mL baffled flask and grown at 30°C/250 RPM to an OD₆₀₀ ~ 5. Cells were then pelleted at 3000 RPM and resuspended in 50 mL YPG media (2% galactose, 2% peptone, 1% yeast extract, 0.54% Na₂PO₄, 0.86% NaH₂PO₄·H₂O) to induce albumin expression. After 48 hours in YPG at 20°C/250 RPM, the cells were pelleted at 3000 RPM and the cleared supernatant filter sterilized. The secreted human serum albumin was purified by affinity chromatography using either Ni-NTA resin (Invitrogen), CaptureSelect HSA affinity resin (BAC), or Vivapure anti-HSA kit (Sartorius-Stedin). Eluted protein was buffer exchanged into PBS by several rounds of concentration and dilution using Amicon Ultra-15 spin concentrators with a 10 kDa cutoff (Millipore). Protein purity was assessed by SDS-PAGE and concentration determined by absorbance at 280.

For single-chain FcRn production, DNA encoding human beta-2 microglobulin fused to the extracellular domain of human FcRn heavy chain through a (G₄S)₃ linker (SEQ ID NO: 27) was synthesized by DNA2.0. The scFcRn DNA was cloned into a modified version of the pcDNA3.1(+) vector (Invitrogen) containing an IL-2 leader sequence and C-terminal FLAG tag by standard digestion and ligation. 80 mL of Freestyle-CHO-S cells (Invitrogen) at 1x10⁶ cells/mL were transiently transfected with scFcRn-FLAG using the Freestyle®-MAX reagent (Invitrogen) according to manufacturer's instructions. Transfected cells were incubated at 37°C, 8% CO₂, with shaking at 130 rpm for 6 days. The supernatant was clarified by centrifugation and the scFcRn-FLAG protein purified on a 0.5 mL M2 anti-FLAG agarose gravity-flow column (Sigma). Bound protein was eluted with 100 mM glycine-HCl, pH 3.5 and exchanged into PBS, pH 7.4 by several rounds of concentration and dilution using Amicon Ultra-15 spin concentrators with a 10 kDa cutoff (Millipore). Protein purity was assessed by SDS-PAGE and concentration determined by absorbance at 280.

Example 1A: Methods of Identifying Mutations Modulating FcRn Binding to an Albumin Moiety

A library of albumin variants with random mutations in domain III was generated and displayed on the surface of yeast. FACS selections were performed to enrich for variants with improved binding to soluble FcRn at pH 5.6. After three to four rounds of selection, a population was identified with significantly increased binding compared to a wild type human serum albumin (HSA). Twelve clones from the population after sort three and eight clones from the population after sort four were cloned and sequenced. The sequence alignments revealed mutations appearing in multiple clones after sort four: K402E (2/8), V424I (2/8), P447L/S (3/8), E492G (3/8), E505G (5/8) and V547 (6/8).

Populations after sorts three, four, five and six were also sequenced to identify other enriched mutations and the following mutations were identified: V418M, T420A, V424I, N429D, M446V, A449V, T467M, E505G/K/R, A552T, V547A.

After round seven, the library had enriched to a single clone with the following mutations: V418M, T420A, E505R, V424I, and N429D.

These data demonstrate a method for identifying mutations that can be useful for modulating FcRn binding. Further selection can be carried out by identifying mutations located near residues involved in FcRn binding, e.g., near His residues, for example H510 and H535. These data also demonstrate specific sites in an HSA useful for modulating the PK of an albumin molecule.

Example 2: Characterization of Albumin Variants

SPR was used to characterize binding of the selected variants to human FcRn at pH values from 5.5 to 7.5. An exemplary apparatus that can be used is a Reichert SR7000C® machine. FLAG-tagged, single-chain human FcRn was immobilized on a 500,000 Da carboxymethyl dextran chip by NHS/EDC chemistry. Unconjugated sites were blocked with 1 M ethanolamine. A reference channel was generated in parallel with no FcRn. HSA variants at concentrations of 1 nM – 100 µM were injected at a flow rate of 50 µL / min and the difference in signal between the FcRn channel and reference channel was recorded over time to assess association. Wash buffer (PBS + 0.01% Tween-20) was flowed through the channels to assess dissociation. Experiments were repeated at pH 5.5, 6.0, 6.5, and 7.4 to assess the pH dependency of binding. The results are shown in FIG. 1. The VSAs that were used are described in Example 5. These results demonstrate that affinity of certain VSAs to FcRn at pH 5.5 had increased, as did the affinity of some of these to FcRn at pH 7.4. In particular, HSA-5 and HSA-7 bound FcRn at pH 5.5 (a typical endosomal pH), but not at pH 7.4 (a typical pH of blood). In each case, the pH dependence of FcRn binding as known for native albumin was preserved.

These data demonstrate that VSAs can be generated that have increased affinity for FcRn at endosomal pH without significantly altering the affinity for FcRn at a neutral pH (e.g., a pH associated with blood).

Example 3

PK studies were performed for selected variants in human FcRn mice to determine the effect of FcRn affinity on plasma clearance. The mouse strains 4919 and 14565 from Jackson Laboratories are homozygous for mouse FcRn knockout and either hemi- or homozygous for human FcRn knock-in.

5 Selected HSA variants were injected intravenously into such mice and bleeds were collected at various time intervals. The plasma concentration of the HSA at each time point was assessed using a non-mouse cross-reactive HSA ELISA and plotted to calculate clearance rates and plasma AUC.

Example 4

ELISA studies were performed to characterize the binding of selected HSA variants to human
10 FcRn at pH values of 5.5 – 7.4. Purified HSA variants at 1 µg/mL in PBS were immobilized in a 96 well flat bottom EIA plate (Costar 9018) at 4°C overnight. Coated wells were then blocked with 200 µL PBS + 2% fish gelatin, pH 7.4 for 2 hours at room temperature. After blocking, wells were washed 3x with 200 µL PBS + 0.1% Tween-20 at the appropriate pH (5.5 – 7.4). 100 µL of FLAG-tagged single-chain FcRn diluted to concentrations of 50 pM – 200 nM in PBS + 0.1% fish gelatin at pH 5.5 – 7.4 was added to each
15 well and incubated for 2 hours at room temperature. Wells were then washed 3x with 200 µL PBS + 0.1% Tween-20 at the appropriate pH. 100 µL of anti-FLAG-HRP (Sigma) diluted 1:1000 in PBS + 0.1% fish gelatin at pH 5.5 – 7.4 was added to each well and incubated for 60 minutes at room temperature. Wells were washed as above and 100 µL TMB substrate (Pierce) added to each well. Color development was stopped after two minutes with 2 M sulfuric acid and the signal read at absorbance 450 – 550 on a
20 Spectramax M5 plate reader.

Example 4A: pH Dependent Binding Using Enzyme Linked Immunosorbent Assay (ELISA)

Experiments were carried out in which the binding of various VSAs and HSA to FLAG-tagged FcRn was assessed at different pHs. Results demonstrated a range of affinities for the VSAs, which were tested at pH 5.5, pH 6.0, and pH 7.4 for binding to 0.2 nM to 200 nM FcRn-FLAG. A table summarizing
25 the results of such an experiment is shown in FIG. 2. The VSAs had K_{D} s from 3 nM to >100 nM at pH 5.5. Wild type HSA is known to have a K_{D} of about 1- 2 µM at pH 5.5. Many of the VSAs, including HSA-15, HSA-13, HSA-12, HSA-7, HSA-21, HSA-11, HSA-2, HSA-14, HSA-5, HSA-10, HSA-6, HSA-9, and HSA-18 had improved affinity for FcRn compared with wild type HSA. Additional related information, including the mutations present in the VSAs, is provided in Example 5, *infra*. The data of FIG. 3
30 demonstrate that the binding of these VSAs to FcRn, as assessed by ELISA, preserved the pH dependence of native albumin (greater binding at pH 5.5 than at pH 7.4).

These data demonstrate that VSAs with modified affinity compared to wild type HSA can be generated and provide guidance for generation of additional VSAs with increased affinity of FcRn at selected pHs.

Example 5

The amino acid sequence of Domain 3 of human serum albumin has the following sequence:

LVEEPQNLIKQNCLEFQELGEYKFNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKHPEAKRMPCAE
 DYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKER
 QIKKQ TALVELVKHKPKATKEQLKAVMDDFAAFVEKCKADDKETCFAEEGKKLVAAASQAALGL [SEQ
 ID NO:28]

5 Exemplary human serum albumin variants that were prepared include the sequences in the following table:

TABLE 2: Human Serum Albumin Variants and FcRn Binding

VARIANT NUMBER	AMINO ACID VARIATIONS FROM DOMAIN 3 OF WILD-TYPE HUMAN SERUM ALBUMIN	FcRn BINDING [Kd@pH 5.5] Compared to WT-HSA
hsa-1	WT - Full Length HSA	±
hsa-2	K573Y	+++
hsa-3	WT - Domain 3 of HSA	±
hsa-4	E492G	±
hsa-5	V547A	++
hsa-6	E505G	+
hsa-7	E505G; V547A	++++
hsa-8	V418M	±
hsa-9	T420A	+
hsa-10	V418M; T420A	++
hsa-11	V418M; T420A; E505G	+++
hsa-12	V418M; T420A; V547A	++++
hsa-13	V418M; T420A; E505G; V547A	+++++
hsa-14	V418M; T420A; E505G; M446V; A449V; T467M; A552T	+++
hsa-15	V418M; T420A; V424I; N429D E505R	+++++
hsa-16	E505R	±
hsa-17	E505K	±
hsa-18	V24II	+
hsa-19	N429D	±
hsa-20	M446V; A449V	±
hsa-21	V418M; T420A; E505R	++++
hsa-22	A552T	±
hsa-23	PBS [Negative Control]	±

10 All of the variants described in Table 2, with the exception of HSA-3, are full length HSA (sequence corresponding to SEQ ID NO:2) with the indicated mutations (numbering of the mutations is based on SEQ ID NO:2). As indicated in Table 2, HSA-3 is Domain 3 only of HSA with the sequence corresponding to SEQ ID NO:22. In subsequent experiments, domain 3 only versions of HSA-5 and HSA-13 were also made; these versions also demonstrated improved affinity.

Example 5A

15 The experiments described in this example illustrate experimental difficulties that were involved in accurately identifying serum albumin mutants that exhibit improved pharmacokinetics (e.g., increased half-life and reduced clearance) and show how these difficulties were effectively overcome.

The anti-HSA sandwich ELISA utilized in the mouse PK studies is unable to detect HSA spiked into cynomolgus monkey plasma due to the high homology of human and monkey albumin. Therefore, we

developed a novel assay utilizing an epitope tag genetically fused to albumin and an anti-tag capture antibody. HSA variants with a FLAG tag, His tag, HA tag, or c-myc tag were expressed in yeast, purified, and titrated in their respective anti-tag ELISA assays in 10% monkey plasma. Both the FLAG tag and HA tag were detected in their respective assays with high sensitivity (EC₅₀ = 10 ng/mL for HA, 50 ng/mL for FLAG).

In a first cynomolgus monkey PK study, FLAG-tagged HSA-wt, HSA-5, HSA-7, HSA-11, and HSA-13 were administered into two monkeys each as a 5 mg/kg iv bolus dose. To control for monkey to monkey variability and directly test for tag-specific effects on PK, a 1 mg/kg dose of HA-tagged HSA-wt was co-dosed into each animal. Bleeds were drawn at 15 minutes, 2 hours, 6 hours, 1 day, 2 days, 5 days, 9 days, 12 days, 16 days, and 21 days, and HSA concentrations were measured by both anti-FLAG and anti-HA ELISAs. A comparison of the PK traces for the FLAG-tagged HSA's (both wild-type and mutant) with the HA-tagged HSA-wt internal control indicated that the FLAG-tagged proteins were cleared at a significantly faster rate, particularly in the alpha phase. To confirm the more rapid clearance of the FLAG-tagged protein, a study was performed in which HA- and FLAG-tagged HSA-wt and HSA-7 were injected into mice. Bleeds were drawn as before and at each time point, the concentration of tagged HSA was measured with both an anti-HSA ELISA and anti-tag ELISA. For HA-HSA-wt and HA-HSA-7, the anti-HSA and anti-HA ELISA's measured HSA concentrations consistent with each other and with previous measurement of untagged HSA in mice suggesting that the HA tag accurately reflected the concentration of plasma HSA. In contrast, the anti-FLAG ELISA measured a significantly lower concentration of FLAG-HSA compared to the anti-HSA ELISA confirming that the tag was cleaved or obscured in a way that distorted the HSA measurement.

Example 6

HSA variant binding to mouse FcRn was measured in an ELISA in which recombinant mouse FcRn (R&D Systems, cat# 6775-FC-050) was directly immobilized on a Costar Maxisorp® plate. After blocking for 3 hours with PBS + 4% fish gelatin, HSA variants at concentrations of 500 nM to 685 pM in PBS + 0.1% Tween + 0.1% fish gelatin, pH 5.5 were added to each well and incubated for 2.5 hours at RT. Bound HSA was detected with a HRP conjugated goat anti-HSA antibody. The rank order of binding affinity was HSA-13 > HSA-7 > HSA-11 > HSA-5 > HSA-wt.

To test whether the *in vitro* quality of increased affinity corresponds to improved pharmacokinetics (PK) *in vivo*, selected examples of VSAs, e.g., HSA-5, HSA-11, and HSA-13 and wild type HSA were injected into wild type mice and the plasma concentration monitored over time (to about 170 hours after administration).

In wild type mice, increased affinity in a serum albumin extended its pharmacokinetics (see FIG. 4). The VSAs exhibited a longer half-life and a reduced clearance compared with wild type HSA. The observed improvement in pharmacokinetic properties was correlated with the affinities observed in ELISA experiments that tested the affinities of these VSAs for mouse FcRn. In particular, greater affinity for

mouse FcRn was associated with longer half-life and reduced clearance. The highest affinity variant HSA-13 had the most extended PK.

The results obtained in the human-FcRn transgenic mice are shown in FIG. 5. In these mice, the relationship between affinity for FcRn and PK was more complex; the VSAs with the highest affinity for human FcRn did not show the greatest improvement in pharmacokinetics.

Taken in combination, the data from experiments in mice with VSAs indicate that the optimal FcRn affinity for PK extension is determined by a tradeoff between improved endosomal recycling at pH 5.5 and increased surface binding at pH 7.4. In wild type mice, in which wild type HSA binds FcRn weakly ($K_d \sim 80 \mu\text{M}$), the highest affinity variant HSA-13 had the most extended PK. In human-FcRn transgenic mice, in which wild type HSA binds FcRn with higher affinity ($K_d \sim 2 \mu\text{M}$), HSA-13 and HSA-11 bound FcRn too tightly at pH 7.4 (see also FIG. 1), driving rapid clearance and a robust antibody response (see FIG. 5). Medium affinity variants HSA-5 and HSA-7 which have minimal pH 7.4 binding (see also FIG. 1) have extended PK in the FcRn transgenic mouse, as shown in FIG. 5. It should be noted that mouse albumin has a high affinity for human FcRn so it is reasonable to expect that a murine model under predicts the improvement that would be observed in humans. Therefore, selection of VSAs suitable for use in humans should not be tested solely in a murine model.

Example 7: VSAs in a Primate Model

To evaluate the PK of a VSA in a primate model, cynomolgus monkeys were injected with an iv bolus dose of 1 mg/kg or 5 mg/kg dose of HSA-7 or wild type HSA (HSA-wt). Two monkeys were analyzed per group by assaying plasma HSA concentration over time using an anti-epitope tag ELISA.

FIG. 6 provides the PK parameters of this experiment, demonstrating that the VSA (HSA-7) has a longer plasma half-life and reduced beta phase clearance in a primate model.

Example 8: Fusion Proteins

When fused to a heterologous polypeptide, protein moieties that extend half-life often do not extend half-life to the same absolute degree as seen with the unfused moiety. This is usually due to active clearance mediated by the heterologous partner. Because it is not clear whether active clearance provides an absolute maximum to the extension seen, or whether the half-life of a fusion is a combination in some way of the half-lives of the two components, we compared wild type HSA fused to IL-2 with a VSA fused to IL-2. Fusion proteins in which human IL-2 is genetically fused to the N- or C-terminus of a selected HSA variant were produced as follows. For N-terminal fusions, cDNA encoding mature human IL-2 was PCR amplified with a 5' primer that introduces 20-40 bp of homology with the app8 yeast leader sequence and a 3' primer that introduces a $(G_4S)_3$ linker (SEQ ID NO: 27) followed by 20-40 bp of homology with the N-terminus of mature HSA. For C-terminal fusions, the human IL-2 cDNA was PCR amplified with a 5' primer that introduces 20-40 bp of homology with the C-terminus of HSA followed by a $(G_4S)_3$ linker (SEQ ID NO: 27) and a 3' primer that introduces 20-40 bp of homology with the stop codon and

downstream sequence of the pYC2/CT vector. In both cases, a Quikchange® mutagenesis reaction (Agilent) was then performed in which a selected HSA variant was used as the template and the IL-2 PCR product was used in place of the primers. DpnI treated reactions were transformed into XL-1 Blue E. coli cells (Agilent) and selected on LB + Amp plates. Selected colonies were minipreped and sequenced. In place of wild type human IL-2 cDNA, DNA encoding a variant of IL-2, such as those described in United States Patents 7,569,215 and 7,951,360, can be fused to the selected HSA variant.

Plasmids encoding the desired fusion sequence were transformed into BJ5α *S. cerevisiae* cells using the EZ-yeast kit (Zymo Research) and transformants selected on SDCAA + ura plates. Selected colonies were grown in liquid SDCAA + ura media at 30°C with shaking at 250 RPM to an OD600 ~ 5. Cells were then pelleted at 3000 RPM and resuspended in YPG media to induce albumin expression. After 48 hours in YPG at 20°C/250 RPM, the cells were pelleted at 3000 RPM and the cleared supernatant filter sterilized. The secreted fusion protein was purified by affinity chromatography using CaptureSelect® HSA affinity resin (BAC).

Both mouse and human IL-2 fused to the N-terminus of HSA-wt or HSA-13 through a (G4S)₂ linker were also cloned into the pLVX-Puro mammalian expression plasmid. Fusion sequences were amplified by PCR using primers that introduced an N-terminal XhoI site and C-terminal MluI site. The PCR product and pLVX-Puro plasmid were both double digested with XhoI/MluI in NEBuffer 3 + BSA and ligated with the Quick Ligation kit (New England Biolabs) according to manufacturer's instructions. Plasmids were transiently transfected in Hek-293 cells using PEI as a transfection reagent and cultured for 4-7 days at 37°C/8% CO₂.

Example 9: Fusion Expression

Transient transfections were performed in Hek-293 cells to express HSA, IL-2-GS10-HSA, IL-2-GS25-HSA, hepcidin-GS10-HSA, or factor VII-GS10-HSA. Expressed proteins were evaluated using non-reducing SDS-PAGE. All of the tested proteins were expressed and run on the gel in a manner consistent with their expected molecular weights. Subsequently, products of such transient transfections were purified using an anti-HSA resin. Purified products (murine IL-2-HSA wild type and IL-2-HSA-13) were injected iv into mice at a dose of 0.5 mg/kg. The VSA fusion (i.e., HSA-13 fusion) extended the PK of IL-2 to a greater degree than did the fusion to wild type HSA (shown in FIG. 7). The IL2-HSA-13 fusion protein had increased half-life, greater AUC, and reduced clearance.

These data demonstrate that a VSA as described herein can be used to improve the PK of another agent, e.g., a therapeutic protein, e.g., IL-2.

The skilled artisan, having read the above disclosure, will recognize that numerous modifications, alterations of the above, and additional optimization of the above, may be conducted while remaining

within the scope of the invention. These include but are not limited to the embodiments that are within the scope of the following claims.

What is claimed is:

1. An isolated, recombinant protein comprising a variant serum albumin polypeptide (VSA) sequence that is a variant of domain III of a naturally-occurring serum albumin sequence, wherein the
5 variant comprises a mutation at one or more of the positions corresponding to V418, T420, V424, E505 and V547.

2. The protein of claim 1 wherein the VSA or the recombinant protein binds to FcRn at a pH in the range of 5.5 to 6.0, e.g., at a pH of 5.5 or 6.0, with higher affinity than a corresponding native serum
10 albumin.

3. The protein of claim 1 or 2, wherein the ratio of the binding affinity of a serum albumin comprising the VSA sequence at a pH of 5.5 to 6.0 to that at a pH of 7.0 to 7.4 is greater than or equal to the ratio for a corresponding native human albumin.
15

4. The protein of any one of claims claim 1-3, wherein the ratio of the binding affinity of a serum albumin comprising the VSA sequence at a pH of 5.5 to 6.0 to that at a pH of 7.0 to 7.4 is 5; 10; 50; 100; 1000; 10,000; 100,000; or 1 million times that of a corresponding native human albumin.

20 5. The protein of claim any one of claims 1-4, wherein a serum albumin comprising the VSA sequence binds to FcRn at a pH in the range of 7.0 to 7.4 with an affinity not greater than a corresponding native human albumin.

25 6. The protein of claim any one of claims 1-5 wherein the VSA sequence comprises a substitution of V418 with a methionine.

7. The protein of any one of claims 1-6, wherein the VSA sequence comprises a substitution of T420 with an uncharged amino acid.

30 8. The protein of claim 7 wherein the VSA sequence comprises a substitution of T420 with alanine.

9. The protein of any one of claims 1-8, wherein the VSA sequence comprises a substitution of V424 with an uncharged amino acid.
35

10. The protein of claim 9, wherein the VSA sequence comprises a substitution of V424 with isoleucine.

11. The protein of any one of claims 1-10, wherein the VSA sequence comprises a substitution of E505 with an uncharged amino acid or positively charged amino acid.

5 12. The protein of claim 11, wherein the VSA sequence comprises a substitution of E505 with arginine, lysine or glycine.

13. The protein of any one of claims 1-12, wherein the VSA sequence comprises a substitution of V547 with an uncharged amino acid.

10

14. The protein of claim 13, wherein the VSA sequence comprises a substitution of V547 with alanine.

15 15. The protein of any one of claims 1-14, wherein the VSA sequence comprises mutations at two or more of the positions selected from V418, T420, E505, and V547.

15

16. The protein of claim 15, wherein the VSA sequence comprises two or more mutations selected from V418M, T420A, E505(R/K/G) and V547A.

20 17. The protein of claim 15, wherein the VSA sequence comprises mutations at three or more positions selected from V418M, T420A, E505(R/K/G) and V547A.

20

18. The protein of claim 15, wherein the VSA sequence comprises mutations V418M, T420A, E505(R/K/G) and V547A.

25

19. The protein of any one of claims 1-18, wherein the VSA sequence comprises at least one mutation selected from V424I, N429D, M446V; A449V; T467M and A552T.

20. The protein of any one of claims 1-19, wherein the VSA sequence is at least 80% identical but less than 100% identical to domain III of a naturally-occurring serum albumin.

30

21. The protein of claim 20 wherein the VSA sequence is at least 80% identical but less than 100% identical to the corresponding sequence of human serum albumin.

35 22. The protein of any one of claims 1-21, wherein the protein comprises a heterologous sequence.

35

23. The protein of claim 22, wherein the protein comprises a first and a second heterologous sequence.

5 24. The protein of claim 24 wherein the first and the second heterologous sequence are identical and are positioned in tandem.

25. The protein of claim 23 wherein the first heterologous sequence is located N-terminal to the variant sequence and the second heterologous sequence is located C-terminal to the variant sequence.

10 26. The protein of claim 22 wherein the heterologous sequence comprises a cytokine domain.

27. The protein of claim 26 wherein the cytokine is interleukin-2.

15 28. The protein of claim 22, wherein the heterologous sequence comprises an immunoglobulin variable domain.

29. The protein of claim 22, wherein the heterologous sequence comprises an Adnectin™, a DARPin, or an anti-calin, or a fragment of an Adnectin™, a DARPin, or an anti-calin,

20 30. The protein of claim 22, wherein the heterologous sequence comprises a soluble fragment of a cell surface receptor.

31. The protein of claim 22, wherein the heterologous sequence comprises an enzyme.

25 32. The protein of claim 22, wherein the heterologous sequence comprises a functional fragment of a coagulation protein.

33. The protein of claim 32, wherein the heterologous sequence comprises a functional fragment of FVII.

30

34. The protein of claim 32, wherein the heterologous sequence comprises a functional fragment of FVIII.

35 35. An isolated, recombinant protein comprising a VSA that has altered binding properties for human FcRn relative to a wild type human serum albumin and binds to FcRn with a K_D of less than 50 nM at pH 5.5 and optionally an affinity for FcRn at pH 7.4 that is less than or equal to the affinity for FcRn of a wild type albumin at pH 7.4.

36. A method of treating a subject , the method comprising administering to the subject an effective amount of a therapeutic agent in association with the protein of any one of claims 1-21 or 35, such that the dosage and/or frequency of administration at which the agent produces a therapeutic effect is reduced relative to the dosage and/or frequency of administration at which the agent produces a therapeutic effect when it is not in association with the albumin protein.

37. The method of claim 36, wherein the agent comprises a polypeptide component that is fused to the albumin protein.

38. The method of claim 37, wherein the polypeptide component and the albumin protein are separated by a linker sequence.

39. The method of claim 37, wherein the polypeptide component and the albumin protein are covalently linked by a non-peptide bond.

40. The method of claim 37, wherein the polypeptide component and the albumin protein are non-covalently and stably associated.

41. A method of engineering a VSA associated therapeutic agent , the method comprising: providing a biologically or pharmaceutically active agent; and associating the agent with the protein of any one of claims 1-21 or 35 to provide a VSA associated therapeutic agent.

42. The method of claim 41, further comprising formulating the VSA associated therapeutic agent for administration to a subject.

43. A method of engineering a VSA associated diagnostic agent, the method comprising: providing a diagnostic agent; and associating the agent with the protein of any one of claims 1-21 or 35 to provide a VSA associated diagnostic agent.

44. The method of claim 43 further comprising formulating the VSA associated diagnostic agent for administration to a subject.

45. The method of claim 44 further comprising administering the VSA associated diagnostic agent to a subject and detecting the VSA associated diagnostic agent.

46. The method of claim 45, wherein the subject is imaged.

47. A method of treating a subject with a VSA fusion protein, the fusion protein comprising a
5 therapeutic agent linked to a VSA, wherein the VSA comprises a sequence that is a variant of domain III of
a naturally-occurring serum albumin that comprises a mutation at one or more of the positions
corresponding to V418, T420, V424, E505 and V547;

the method comprising administering to the subject a therapeutically effective amount of the VSA
fusion protein, such that the dosage and/or frequency of administration at which the agent produces a
10 therapeutic effect is reduced relative to the dosage and/or frequency of administration at which the agent
produces a therapeutic effect when it is not in association with the VSA.

48. The method of claim 47, wherein the therapeutic agent comprises a sequence encoding human
IL-2 or an active variant of IL-2.

15

49. The method of claim 48, wherein the subject suffers from, or is at risk of suffering from, an
immune disorder.

50. The method of claim 49, wherein the subject has undergone, or plans to undergo, a procedure
20 selected from the group consisting of an organ transplant, or blood transfusion, or bone marrow
transplantation.

51. The method of claim 47, wherein the therapeutic agent comprises a sequence encoding a urate
oxidase, or an active variant of a urate oxidase.

25

52. The method of claim 51, wherein the subject suffers from gout.

FcRn/HSA SPR

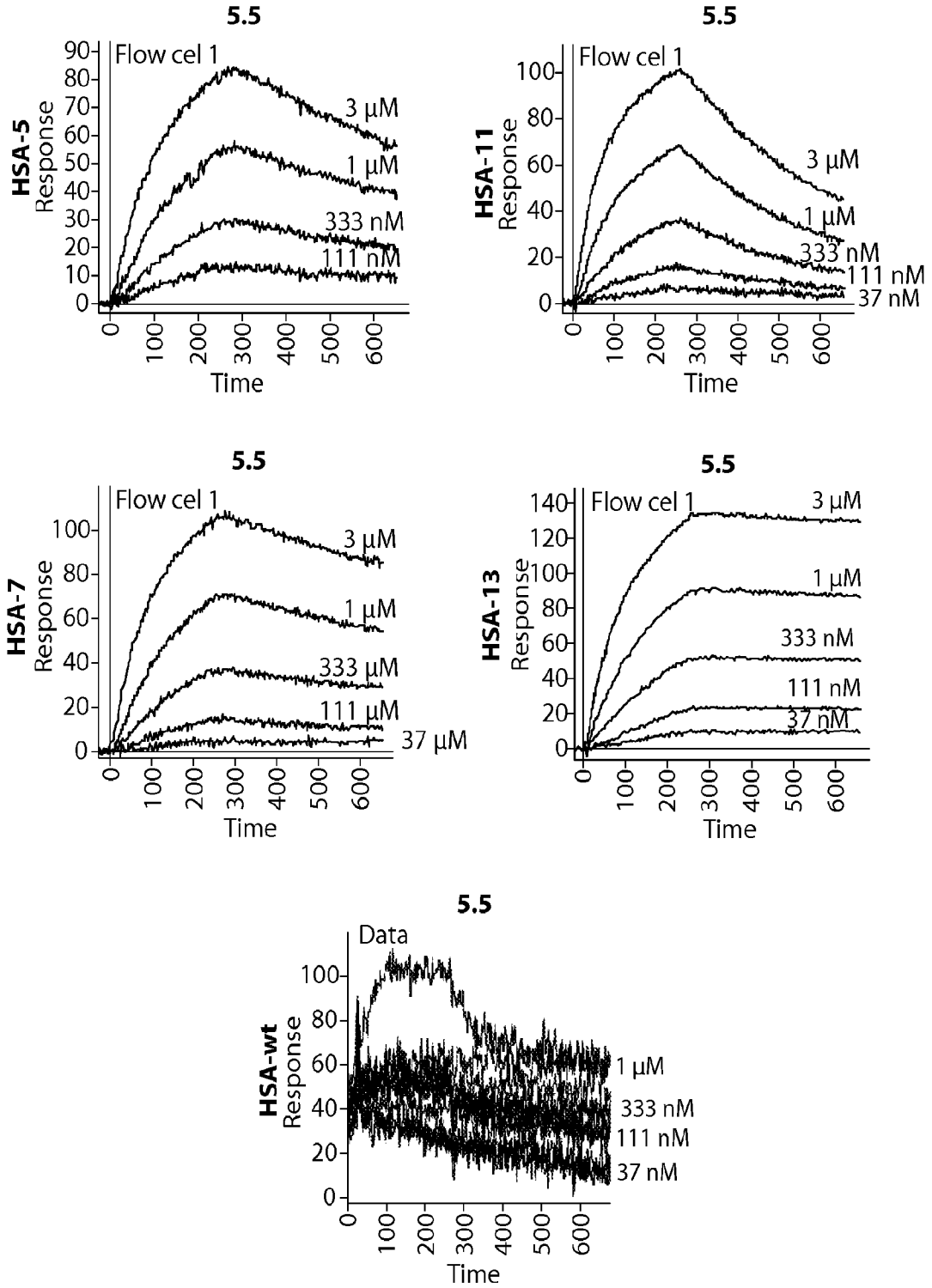


Fig. 1-1

FcRn/HSA SPR

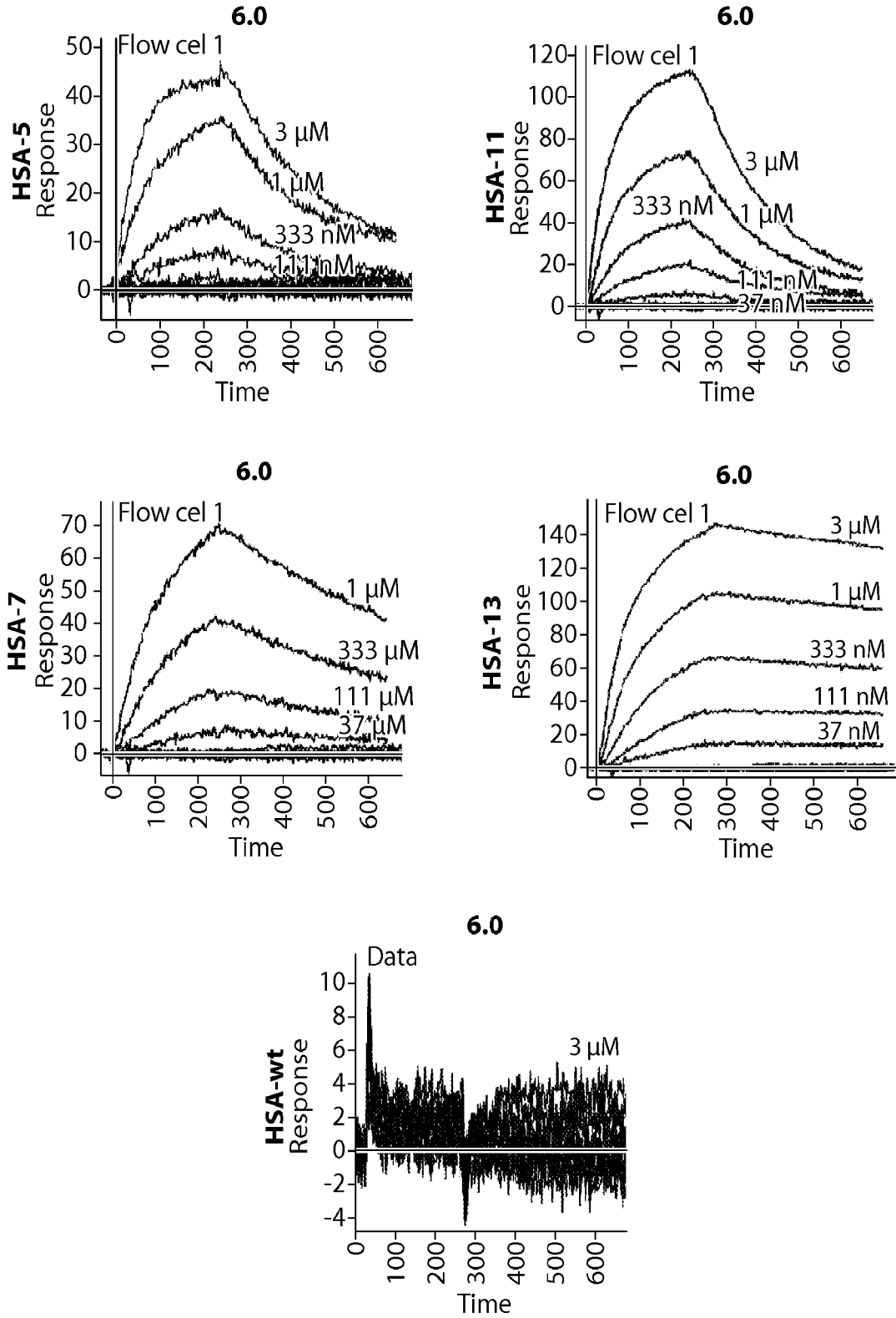
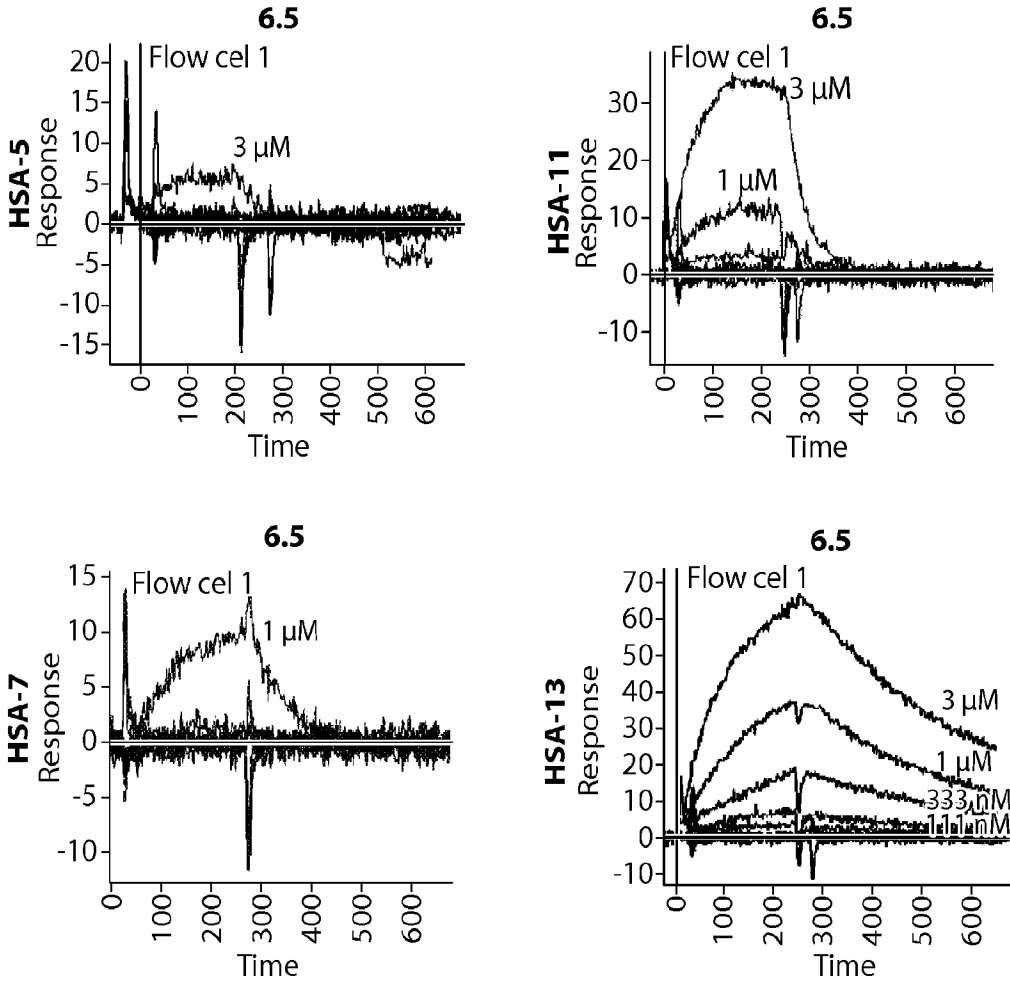


Fig. 1-2

FcRn/HSA SPR



6.5

HSA-wt

Not tested

Fig. 1-3

FcRn/HSA SPR

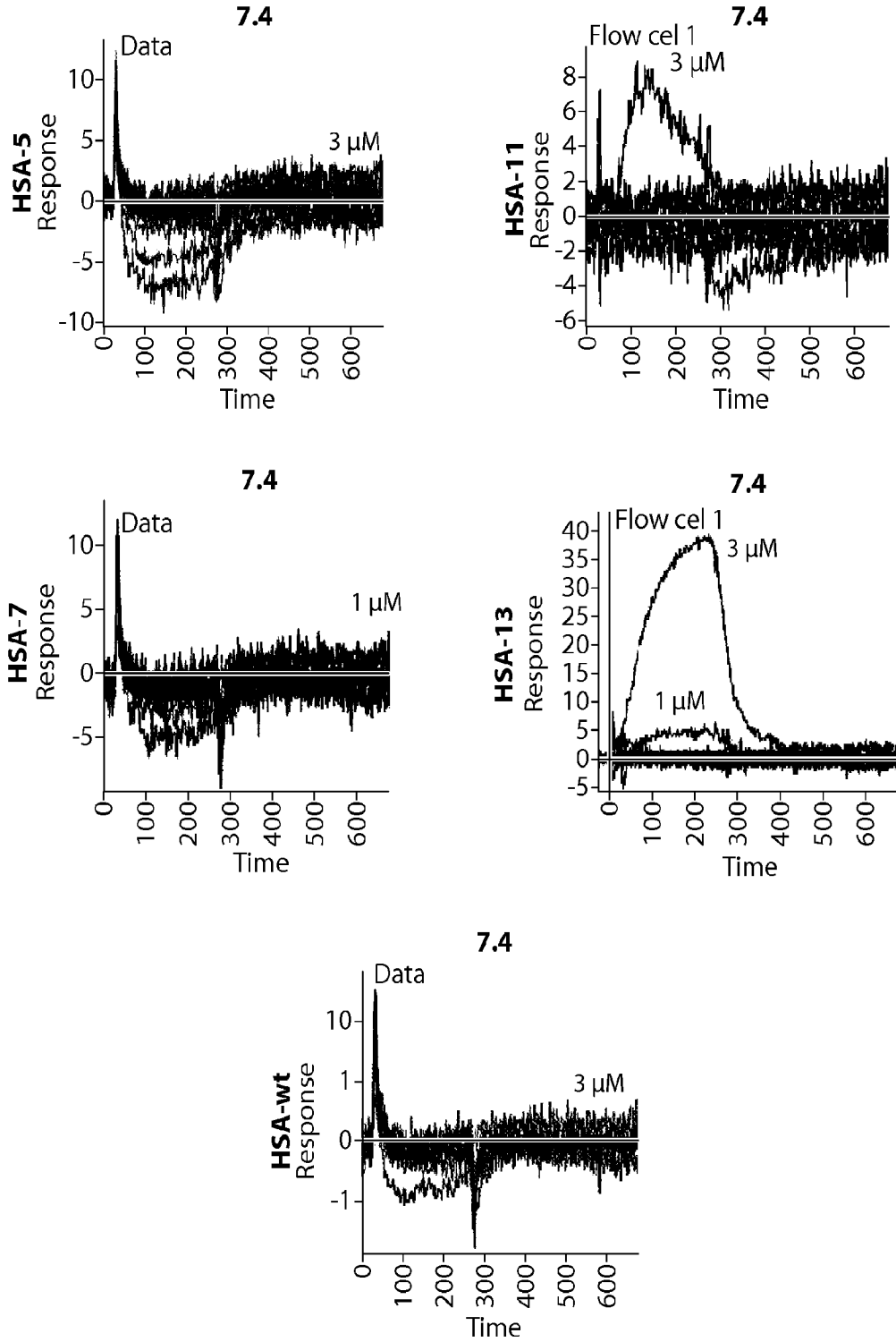


Fig. 1-4

Rank Order of Binding		~Kd at pH 5.5	V418	T420	E505	V547	Other
1	HSA-15	3 nM	M	A	R		V424I, N429D
2	HSA-13	3 nM	M	A	G	A	
3	HSA-12	6 nM	M	A		A	
4	HSA-7	10 nM			G	A	
5	HSA-21	12 nM	M	A	R		
6	HSA-11	25 nM	M	A	G		
7	HSA-2	25 nM					K573Y
8	HSA-14	50 nM	M	A	G		M44GV, A449V, T467M, A552T
9	HSA-5	50 nM				A	
10	HSA-10	75 nM	M	A			
11	HSA-6	>100 nM			G		
12	HSA-9	>100 nM		A			
13	HSA-18	>100 nM					V424I
14	HSA-3						WT-D3
15	HSA-1						WT
16	HSA-4						E492G
17	HSA-8		M				
18	HSA-16				R		
19	HSA-17				K		
20	HSA-19						N429D
21	HSA-20						M446V, A449V
22	HSA-22						T467M
23	HSA-23						A552T
24	HSA-23						PBS

↑ Increasing affinity } ~wt binding

Fig. 2

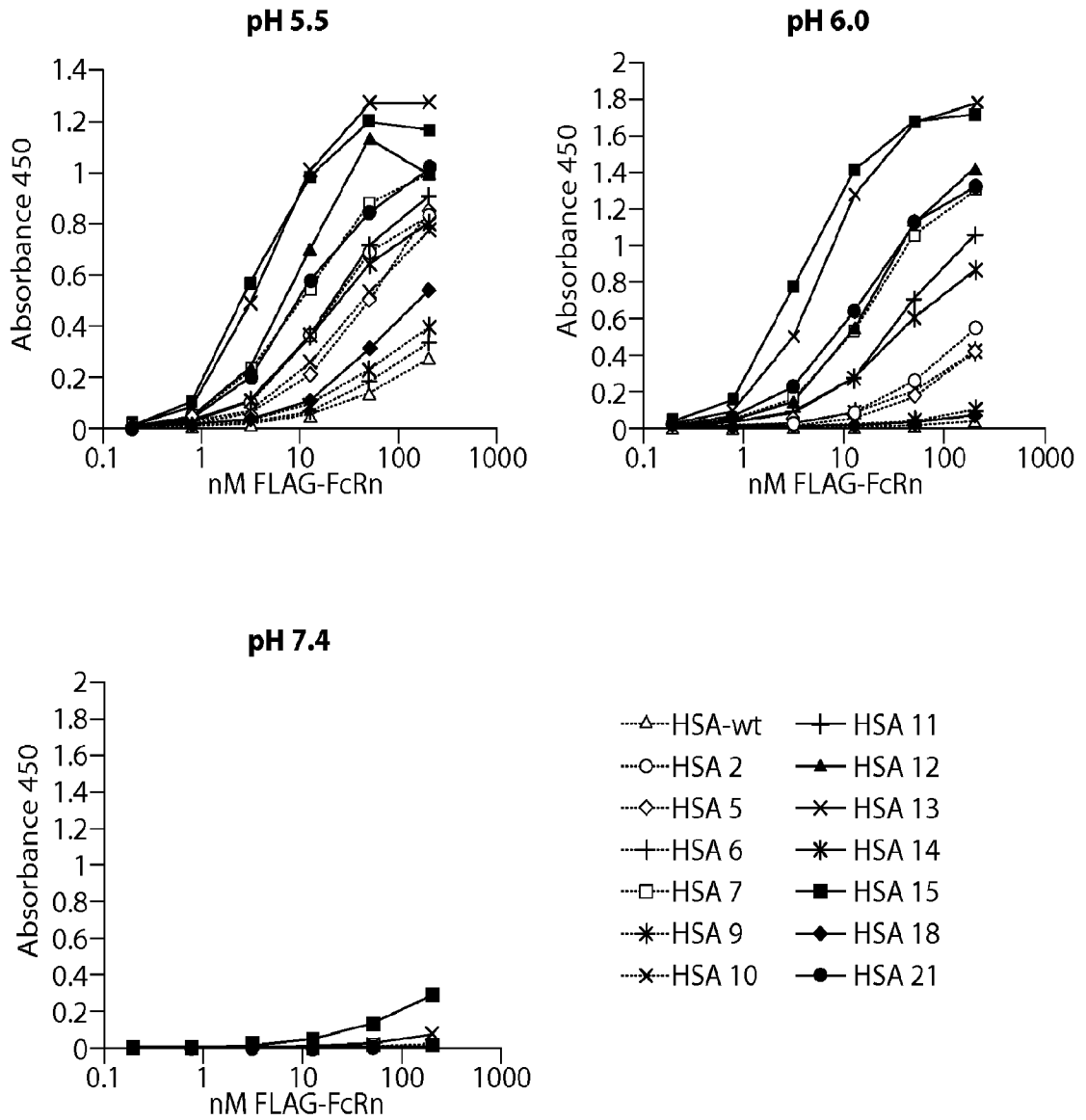


Fig. 3

7/10

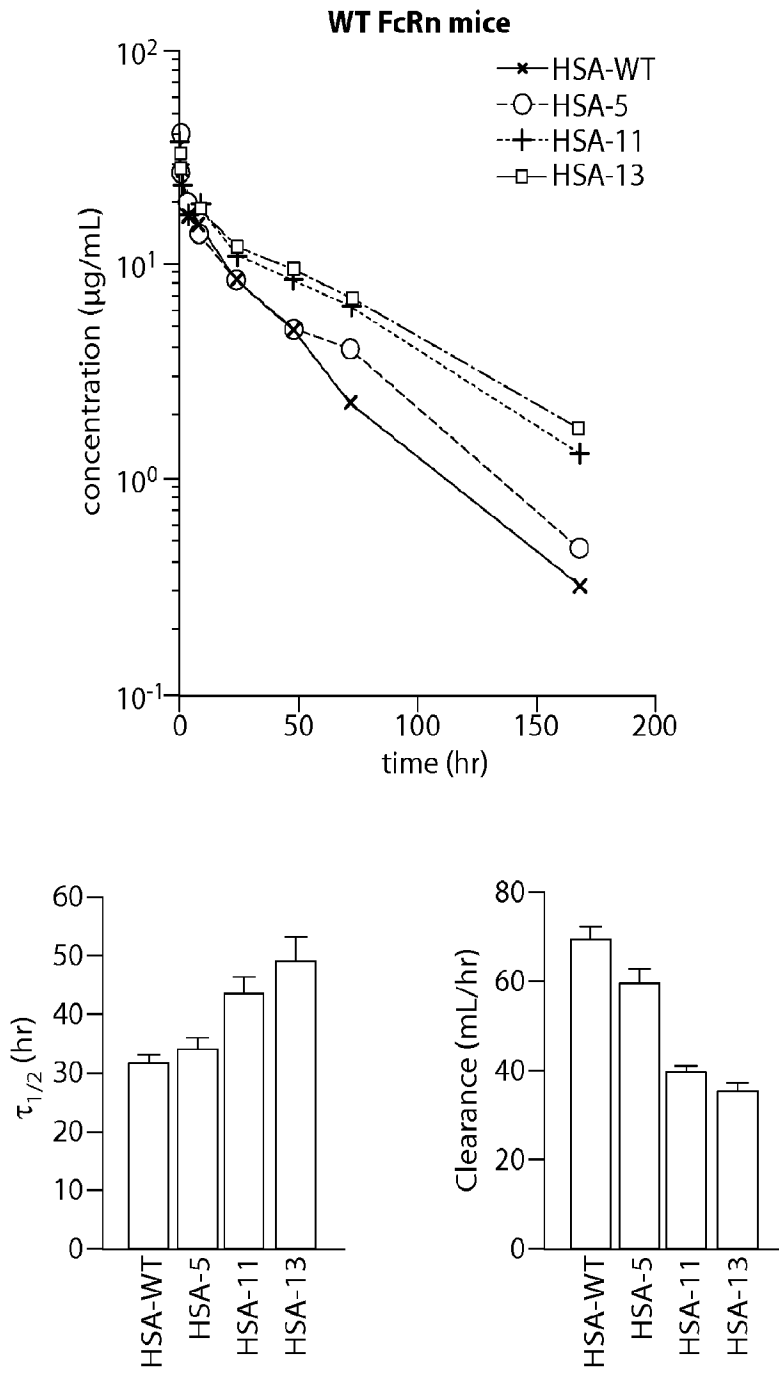


Fig. 4

8/10

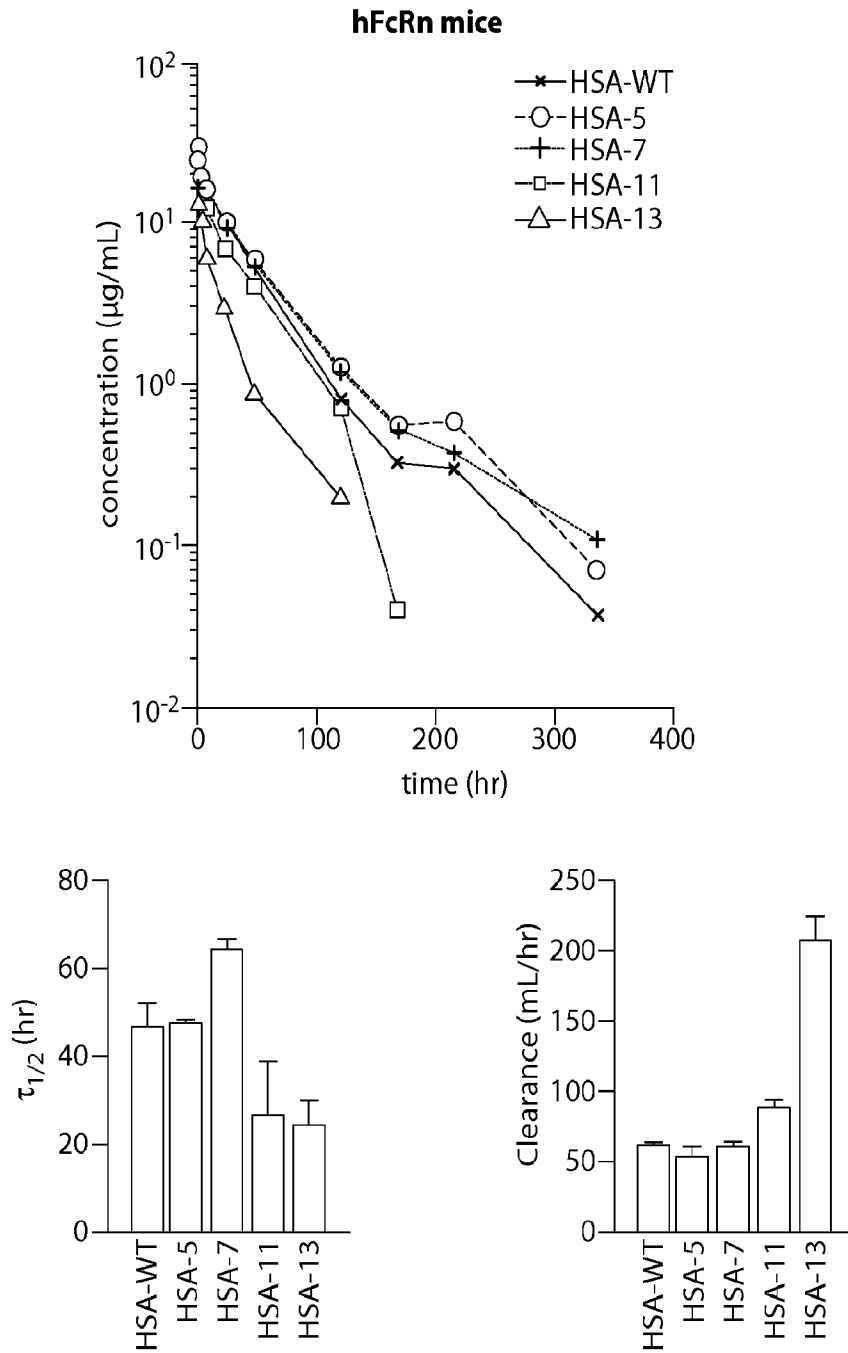


Fig. 5

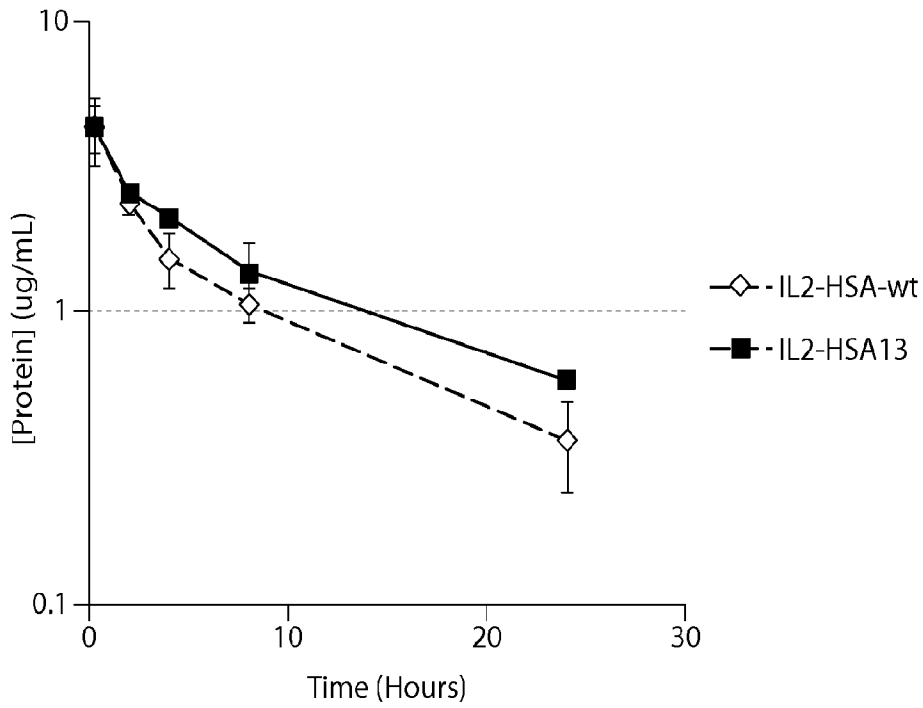
Molecule	Dose	N (animals)	C _{max} (µg/mL)	AUC (µg*hr/mL)	Cl (ml/min/kg)	V _{dist} (mL/kg)	T1/2 (Hrs)	T1/2 _α (Hrs)	T1/2 _β (Hrs)
HSA-wt	1 mg/kg	6	18.2	1,808	0.010	165	182	4.4	152
HSA-7	1 mg/kg	2	38.1	2,824	0.0059	115	225	5.1	239
HSA-wt	5 mg/kg	2	239	15,338	0.0059	120	251	9.0	218
HSA-7	5 mg/kg	2	196	17,888	0.0047	125	302	11.0	279
HSA-wt	All	8			0.0090	153	199	5.6	168
HSA-7	All	4			0.0053	120	264	8.0	259

Non-compartmental fit

Biexponential fit

Fig. 6

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Molecule	Dose	Cmax (µg/mL)	AUC (µg*hr/mL)	Cl (ml/min/kg)	V_dist (mL/kg)	T1/2 (Hrs)
IL2-HSA-wt	0.5 mg/kg	4.3	32.8	0.26	217	9.9
IL2-HSA-13	0.5 mg/kg	4.3	43.7	0.19	188	11.4

Fig. 7