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(54) Title: ANTIBODIES AGAINST PCSK9

(57) Abstract: Compositions and methods of inhibiting PCSK9 are provided herein, for example, using antibodies targeting the pro-domain of PCSK9.

ANTIBODIES AGAINST PCSK9

RELATED APPLICATIONS

The present application claims priority to U.S. Application Serial No. 61/749,672, filed January 7, 2013. The entire content of the foregoing application is hereby incorporated herein by reference.

FIELD OF THE INVENTION

The field of the invention relates to cholesterol metabolism. More particularly, the field relates to regulation of PCSK9.

BACKGROUND

Despite advances in treating high cholesterol, there is a significant unmet need for treatments for diseases associated with high cholesterol. Statins do not work for all patients. For example, some people are statin-intolerant, are not well controlled on statins, or have familial hypercholesterolemia. PCSK9 has been identified as a possible target for treating high cholesterol. However, molecules targeting PCSK9 have exhibited poor pharmacokinetics and therefore require frequent dosing and large volumes.

Proprotein convertase subtilisin-like/kexin type 9 (PCSK9, neural apoptosis-regulated convertase 1, NARC-1) is a member of the serine protease family that is primarily expressed in hepatocytes. PCSK9 is initially expressed as a proprotein of about 75 kD that undergoes autocatalytic cleavage to produce mature PCSK9. The mature PCSK9 is secreted and binds to LDL receptor at the cell surface creating a bound complex (PCSK9/LDLR). PCSK9/LDLR is directed to the lysosome where it is degraded. This mechanism plays a role in regulating serum LDL-c levels.

SUMMARY

The invention relates to the discovery of effective inhibitors of PCSK9, e.g., antibodies that target the prodomain of PCSK9. Accordingly, the invention relates to an antibody, antibody fragment, or derivative thereof that can specifically bind to the prodomain of a human PCSK9. In some embodiments, the antibody exhibits pH-dependent binding to PCSK9. Specific

examples of a PCSK9 inhibitor include polypeptide that includes at least one of SEQ ID NO:14, 15, or 16 and at least one of SEQ ID NO:17, 18, or 19 and can specifically bind to PCSK9. In certain embodiments, the PCSK9 inhibitor (e.g., antibody, fragment, or derivative) can bind to S127 and D129 of a human PCSK9 (SEQ ID NO:1)

In another embodiment, the invention relates to a method of inhibiting PCSK9. The method includes contacting a sample comprising a PCSK9 with a PCSK9 antibody, fragment, or derivative thereof that can specifically bind to the prodomain of human PCSK9.

A further embodiment includes a method of detecting a PCSK9 polypeptide in a sample. The method includes providing a sample (e.g., a biological sample), contacting the sample with a PCSK9 inhibitor described herein under conditions suitable for specific binding of a PCSK9 polypeptide, and detecting a complex comprising the PCSK9 inhibitor.

Another embodiment includes a method of treating a subject in need thereof with a therapeutically effective amount of a PCSK9 inhibitor (e.g., a PCSK9 antibody, fragment, or derivative thereof), wherein the PCSK9 inhibitor can specifically bind to the prodomain of a human PCSK9. In some aspects, the PCSK9 inhibitor is an antibody, fragment, or variant thereof that exhibits pH-dependent binding to a PCSK9. In certain aspects, the subject is also treated with a statin.

The invention also relates to a PCSK9 inhibitor (e.g., a PCSK9 antibody, fragment, or derivative thereof) for use in treating a PCSK9-associated disease in a subject, wherein the PCSK9 inhibitor can specifically bind to the prodomain of a human PCSK9. In some embodiments, the PCSK9 inhibitor is an antibody, fragment, or variant thereof that exhibits pH-dependent binding to a PCSK9. In some embodiments, the PCSK9 inhibitor is used in combination with a statin.

The invention further relates to use of a PCSK9 inhibitor (e.g., a PCSK9 antibody, fragment, or derivative thereof) in the manufacture of a medicament for the treatment of a PCSK9-associated disease in a subject, wherein the PCSK9 inhibitor can specifically bind to the prodomain of a human PCSK9. In some embodiments, the PCSK9 inhibitor is an antibody, fragment, or variant thereof that exhibits pH-dependent binding to a PCSK9. In some embodiments, the PCSK9 inhibitor is used in combination with a statin.

The entire disclosure of each patent document and scientific article referred to herein, and those patent documents and scientific articles cited thereby, is expressly incorporated by

reference herein for all purposes. Unless otherwise defined, technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the disclosed invention belongs.

Additional features and advantages of the invention are more particularly described below.

DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph depicting the results of an experiments in which cynomolgus monkeys were injected iv with 3 mg/kg chimeric antibody (ch2C6, catalytic domain targeted; ch6H7, C terminal domain targeted; or ch7F8, prodomain targeted) and LDL-c levels assayed.

Fig. 2 is a graph depicting the results of testing antibody PK in monkeys from the experiment described in Fig. 1.

Fig. 3 is a graph depicting the results of testing total PCSK9 levels in monkeys from the experiment described in Fig. 1.

Fig. 4 is a graph depicting the results of testing for free (unbound) PCSK9 levels in monkeys from the experiment described in Fig. 1.

DETAILED DESCRIPTION

It has been found that a PCSK9 inhibitor (e.g., an antibody) can be targeted to, for example, the prodomain of PCSK9 and is effective in inhibiting at least one PCSK9 activity. In addition, embodiments include PCSK9 inhibitors (e.g., antibodies) that exhibit pH-dependent binding. Such inhibitors can be used to improve PK and improve dosing, for example, by requiring less frequent dosing and/or delivery of relatively small amounts of inhibitor. Inhibitors described herein can also be targeted to the catalytic domain or the C terminal domain. Protein molecules described herein with any of these features are termed herein PCSK9 binding proteins (PCSK9BPs).

pH-dependent PCSK9 inhibitors can reduce the amount of receptor-mediated clearance and may not reduce soluble target clearance to the same extent as a corresponding non-pH dependent antibody, i.e., an antibody that recycles bound antigen. Without committing to any particular theory of action, it is believed that in the case of a PCSK9 inhibitor (e.g., a PCSK9BP)

that can bind to an FcRn, circulating PCSK9 inhibitor can bind to circulating PCSK9 and the bound complex is taken up by cells via an endosomal mechanism. For a PCSK9 inhibitor as described herein, once bound in an endosome, the PCSK9 inhibitor and PCSK9 dissociate. The PCSK9 inhibitor can be recycled into the circulation while the PCSK9 remains in the endosome and is metabolized. This is in contrast to a PCSK9 binding molecule that does not dissociate from PCSK9 at endosomal pH, in which case the PCSK9 inhibitor/PCSK9 complex, or at least a significant portion of both components of such complexes, recycle back into circulation.

PCSK9 Inhibitors

Affinity

In general, a molecule that is a PCSK9 inhibitor (e.g., an antibody, fragment, or derivative thereof) can specifically bind to a PCSK9 and does not significantly bind to molecules other than PCSK9, unless the molecule also contains a distinct binding portion that binds to a molecule other than a PCSK9, (for example as in some bispecific or bifunctional molecules that have multiple functions, at least one of which is to specifically bind PCSK9). A molecule that can specifically bind a PCSK9 generally has a dissociation constant (Kd) of $\leq$1 μM, for example, $\leq$100 nM, $\leq$10 nM, $\leq$1 nM, $\leq$0.1 nM, $\leq$0.01 nM, or $\leq$0.001 nM. For example, the Kd is 10^{-8}M or less, from 10^{-8} M to 10^{-13} M, or from 10^{-9} M to 10^{-13} M. In some embodiments, the Kd is between about 0.1 nM and 100 nM, between about 0.1 nM and 50 nM, between about 0.1 and 20 nM, between about 0.2 nM and 10 nM, between about 0.2 nM and 1 nM, between about 1 nM and 10 nM for binding to a PCSK9, e.g., a human PCSK9. In some embodiments, the Kd is between 0.1 nM and 100 nM, between 0.1 nM and 50 nM, between 0.1 and 20 nM, between 0.2 nM and 10 nM, between 0.2 and 1 nM, between 1 nM and 10 nM for binding to a PCSK9, e.g., a human PCSK9. In some cases, a PCSK9 antibody can specifically bind to an epitope, e.g., an epitope in the prodomain, of PCSK9 that is conserved among PCSK9 from different species, for example, at least between human and one or more of a primate (e.g., rhesus monkey, African green monkey, or cynomologous monkey), rodent (e.g., rat, mouse, guinea pig), or other mammal (e.g., canine, bovine, ovine, equine, or camelid). An antibody that can specifically bind to a PCSK9 or fragment thereof is referred to herein as a "PCSK9 antibody." In some embodiments, a PCSK9 inhibitor (e.g., an antibody) is an inhibitor (e.g., an antibody) that can specifically bind to a PCSK9 and can inhibit at least one activity of PCSK9. "Inhibition" includes reduction of a

PCSK9 activity. In some embodiments, the PCSK9 inhibitor inhibits a PCSK9 activity, e.g., with a Ki of less than 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, or 10^{-10} M. In some embodiments, the PCSK9 inhibitor has an IC50 of less than 100 nM, 10 nM, or 1 nM. Exemplary PCSK9 inhibitors include, but are not limited to, antagonists which bind a PCSK9 (e.g., an antibody, a polypeptide, a mutant or variant of a PCSK9, a small molecular weight organic molecule, and a competitive inhibitor), and substances which inhibit one or more PCSK9 functions without binding thereto (e.g., an anti-idiotypic antibody).

Affinity of a PCSK9 inhibitor can be determined using methods known in the art. For example, Kd can be measured using a radiolabeled antigen binding assay (RIA) performed with the Fab derived from a test PCSK9 antibody and PCSK9. In one such as assay, solution binding affinity of a Fab for PCSK9 is measured by equilibrating the Fab with a minimal concentration of (^{125}I)-labeled PCSK9 in the presence of a titration series of unlabeled PCSK9, then capturing bound antigen with an anti-Fab antibody-coated plate. Methods of performing RIAs are known in the art.

In some cases, Kd is determined using a surface plasmon resonance assay with a BIACORE®-2000 or a BIACORE®-3000 (BIAcore, Inc., Piscataway, N.J.) with PCSK9 immobilized on carboxymethylated dextran biosensor chips. Methods are generally those recommended by the manufacturer. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE® Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (Kd) is calculated as the ratio k_{off}/k_{on} (e.g., Chen et al., J Mol Biol 293:865-881, 1999). In some cases, the on-rate can be determined by using a fluorescent quenching technique that measures a change in fluorescence emission intensity (excitation=295 nm; emission=340 nm, 16 nm band-pass). Such measurements are made using a spectrometer, such as a stop-flow equipped spectrophotometer (Aviv Instruments, Lakewood Township, NJ).

Antibodies

In some embodiments, the invention is an antibody that can specifically bind a PCSK9. The term "antibody" as used herein refers to antibody molecules including, but not limited to monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific

antibodies), and antibody fragments that exhibit specific binding to PCSK9. An "antibody fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include without limitation Fv, Fab, Fab', Fab'-SH, $F(ab')_2$; diabodies; triabodies, tetrabodies, linear antibodies; single-chain antibody molecules (e.g., scFv); and multispecific antibodies formed from antibody fragments. Such fragments are further discussed *infra*. In general, throughout this disclosure, the term "antibody" should be read as "antibody, fragment, or variant thereof" except when, as will be apparent to those in the art, the term exclusively refers to an antibody. Examples of CDRs from PCSK9 antibodies that are PCSK9 inhibitors are in Table 1.

Table 1

Designation	Heavy chain CDRs	Light chain CDRs
2C6	GYTFTDYYMN (SEQ ID NO:2; CDR1), DINPNNGGPS (SEQ ID NO:3; CDR2), GGPIYYGNSAWFAY (SEQ ID NO:4; CDR3)	SVSSSISSSNLH (SEQ ID NO:5; CDR1) GTSNLAS (SEQ ID NO:6; CDR2), QQWSGYPLT, (SEQ ID NO:7; CDR3)
6H7	GYTFTSYWIT (SEQ ID NO:8; CDR1), DIYPGSGSTD (SEQ ID NO:9; CDR2), WAYGEDY (SEQ ID NO:10; CDR3)	KASQNVGTNVA (SEQ ID NO:11; CDR1), SASYRYS (SEQ ID NO:12; CDR2), QQYNSYPYT (SEQ ID NO:13; CDR3)
7F8	GYTFTTYPID (SEQ ID NO:14; CDR1), NFHPYNDDTN (SEQ ID NO:15; CDR2), RGELAWFAY (SEQ ID NO:16; CDR3)	RTSENVYSYLT (SEQ ID NO:17; CDR1), NAKTLAE (SEQ ID NO:18; CDR2), QHHYGTPYT (SEQ ID NO:19; CDR3)

The invention described herein also encompasses antibodies with a shared epitope with an antibody described herein. Methods of determining whether a first antibody has a shared epitope with a second antibody are known in the art. For example, an antibody with a shared epitope can be identified by the ability of the antibody to block binding of a reference antibody (e.g., a PCSK9 antibody described herein) in a competition assay by at least 50%. Another method for identifying such an antibody is by determining whether a reference antibody described herein can block binding of the antibody to PCSK9 by at least 50%. Another method for determining competition between two antibodies is a bridging assay in which a first antibody is captured on an ELISA plate or SPR chip, then PCSK9 is bound to the first antibody followed by incubation with the second antibody to permit binding. If the second antibody is captured on the plate or chip through the bound PCSK9 then it is non-competitive with the first antibody.

Antibodies having a shared epitope and therefore encompassed by the invention include antibodies that have a shared epitope with an antibody described herein as determined using mutation studies, crystallography, or other methods known in the art.

In some embodiments, a PCSK9 inhibitor increases the availability of LDL receptors when administered to an animal, e.g., a mammal such as a human, non-human primate, mouse, rat, guinea pig, pig, sheep, cow, dog, cat, or horse.

In some embodiments, the PCSK9 inhibitor is an antibody that can specifically bind to an epitope within a fragment of PCSK9 as described herein. In some cases, the antibody binds to an epitope in a fragment of a human PCSK9 polypeptide, the amino acid sequence of which is depicted in SEQ ID NO:1.

1 mgtvssrrsw wplpllllll lllgpagara qededgdyee lvlalrseed glaeapehgt
61 tatfhrcakd pwrlpgtyvv vlkeethlsq sertarrlqa qaarrgyltk ilhvfhgllp
121 gflvkmsgdl lelalklphv dyieedssvf aqsipwnler itppryrade yqppdggslv
181 evylldtsiq sdhreiegrv mvtdfenvpe edgtrfhrqa skcdshgthl agvvsgrdag
241 vakgasmrsl rvlncqgkgt vsgtliglef irksqlvqpv gplvvllpla ggysrvlnaa
301 cqrlaragvv lvtaagnfrd daclyspasa pevitvgatn aqdqpvtlgt lgtnfgrcvd
361 lfapgediig assdcstcfv sqsgtsqaaa hvagiaamml saepeltlae lrqrlihfsa
421 kdvineawfp edqrvltpnl vaalppsthg agwqlfcrtv wsahsgptrm atavarcapd
481 eellscssfs rsgkrrgerm eaqggklvcr ahnafggegv yaiarccllp qancsvhtap

541 paeasmgtrv hchqqghvlt gcsshweved lgthkppvlr prgqpnqcvg hreasihasc
601 chapgleckv kehgipapqe qvtvaceegw tltgcsalpg tshvlgayav dntcvvrsrd
661 vsttgstseg avtavaiccr srhlaqasqe lq (SEQ ID NO:1)

In certain embodiments, the functional and/or structural epitope of an antibody according to this invention is between residues 31 and 152 (SEQ ID NO:24), inclusive (prodomain) of human PCSK9 (as depicted in SEQ ID NO:1).

qededgdyee lvlalrseed glaeapehgt tatfhrcakd pwrlpgtyvv vlkeethlsq
sertarrlqa qaarrgyltk ilhvfhgllp gflvkmsgdl lelalklphv dyieedssvf aq (SEQ ID NO:24)

In some embodiments, the epitope is between residues 61 and 152, inclusive of human PCSK9 (e.g., of SEQ ID NO:1), for example,

tatfhrcakd pwrlpgtyvv vlkeethlsq sertarrlqa qaarrgyltk ilhvfhgllp
gflvkmsgdl lelalklphv dyieedssvf aq (SEQ ID NO:20)

In some embodiments, the epitope includes at least residues 31 to 151, inclusive of SEQ ID NO:1 (SEQ ID NO:21)

qededgdyee lvlalrseed glaeapehgt tatfhrcakd pwrlpgtyvv vlkeethlsq
sertarrlqa qaarrgyltk ilhvfhgllp gflvkmsgdl lelalklphv dyieedssvf a (SEQ ID NO:21)

In some cases, the epitope includes at least residue of human PCSK9 selected from the group consisting of S127 and D129.

In certain embodiments, the functional and/or structural epitope of an antibody according to this invention is in the catalytic domain of PCSK9, for example between residues 153 and 447, inclusive, of human PCSK9 (between the residues of SEQ ID NO:22, inclusive).

sipwnler itppryrade yqppdggslv evylldtsiq sdhreiegrv mvtdfenvpe
edgtrfhrqa skcdshgthl agvvsgrdag vakgasmrsl rvlncqgkgt vsgtliglef

irksqlvqpv gplvvllpla ggysrvlnaa cqrlaragvv lvtaagnfrd daclyspasa
pevitvgatn aqdqpvtlgt lgtnfgrcvd lfapgediig assdcstcfv sqsgtsqaaa
hvagiaamml saepeltlae lrqrlihfsa kdvineawfp edqrvltpnl vaalpps (SEQ ID NO:22)

In some cases, the epitope includes at least one residue of human PCSK9 selected from the group consisting of D192, R194, D238, D374, S376. In some embodiments, the epitope includes at least two, three, four, or five of these residues.

In certain embodiments, the functional and/or structural epitope of an antibody according to this invention is in the C-terminal domain of PCSK9, for example the epitope is within residues 453 to 662 of SEQ ID NO:1, inclusive, e.g., within SEQ ID NO: 23, inclusive.

wqlfcrtv wsahsgptrm atavarcapd eellscssfs rsgkrrgerm eaqggklvcr
ahnafggegv yaiarccllp qancsvhtap paeasmgtrv hchqqghvlt gcsshweved
lgthkppvlr prgqpnqcvg hreasihasc chapgleckv kehgipapqe qvtvaceegw
tltgcsalpg tshvlgayav dntcvvrsrd vs (SEQ ID NO:23)

In some embodiments, the epitope of a PCSK9 antibody does not include one or more of residues R194, E195, D238, A239, A341, Q342, E366, D367, I369, S376, T377, C378, F379, S381, H391 of SEQ ID NO:1, e.g., the epitope does not include two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or fifteen of the residues. In some cases, the epitope of a PCSK9 antibody does not extend from residues 153 to 205 of SEQ ID NO:1, e.g., residues 194-199 of SEQ ID NO:1.

pH-Dependent PCSK9 Antibodies

PCSK9 antibodies that are PCSK9 inhibitors that are described herein include antibodies for which binding to PCSK9 is pH dependent.

Non-limiting exemplary methods of generating and selecting pH dependent antibodies directed against PCSK9 are described in the Examples.

Chimeric and Humanized Antibodies

Antibodies described herein can include chimeric antibodies. Chimeric antibodies are known in the art, for example, see U.S. Pat. No. 4,816,567; and Morrison et al. (Proc Natl Acad Sci USA, 81:6851-6855, 1984). In some cases, a chimeric antibody comprises a non-human variable region (e.g., a variable region derived from a mouse, rat, hamster, rabbit, or non-human primate, such as a monkey) and a human constant region. In a further example, a chimeric antibody is a class switched antibody in which the class or subclass has been changed to differ from that of the parent antibody. Chimeric antibodies include antigen-binding fragments of a parent antibody or variants of such fragments.

A chimeric antibody can be a humanized antibody. Generally, a humanized antibody comprises one or more variable domains in which CDRs or portions of CDRs are derived from a non-human antibody, and framework regions or portions of framework regions are derived from human antibody sequences. Optionally, a humanized antibody also comprises at least a portion of a human constant region. In some embodiments, selected framework residues are substituted with corresponding residues from a non-human antibody (e.g., the antibody from which the CDRs are derived), e.g., to restore or improve antibody specificity, affinity, or pH dependence.

Humanized antibodies and methods of making them are known in the art, for example, Konterman and Dübel (Antibody Engineering, 2010, Springer), Riechmann et al.(Nature 332:323-329,1988); Queen et al.(Proc Nat Acad Sci USA 86:10029-10033, 1989); U.S. Pat. Nos. 5,821,337, 7,527,791, 6,982,321, and 7,087,409; Kashmiri et al. (Methods 36:25-34, 2005, specificity determining residue (SDR) grafting); Padlan (Mol Immunol 28:489-498, 1991, resurfacing); Dall'Acqua et al. (Methods 36:43-60, 2005, framework shuffling); and Osbourn et al. (Methods 36:61-68, 2005) and Klimka et al. (Br J Cancer, 83:252-260, 2000, guided selection approach to framework shuffling).

Human framework regions that can be used for humanization include but are not limited to framework regions selected using a best-fit method (e.g., Sims et al. J Immunol 151:2296, 1993); framework regions derived from the consensus sequence of human antibodies of a particular subgroup of light or heavy chain variable regions (e.g., Carter et al. Proc Natl Acad Sci USA, 89:4285, 1992; and Presta et al., J Immunol, 151:2623, 1993); human mature (somatically mutated) framework regions or human germline framework regions (e.g., Almagro and Fransson, Front Biosci 13:1619-1633, 2008); and framework regions derived from screening framework

libraries (e.g., Baca et al., J Biol Chem 272:10678-10684, 1997; and Rosok et al., J Biol Chem 271:22611-22618, 1996)).

A PCSK9 antibody as described herein can be a human antibody. Such antibodies can be generated using methods known in the art, for example, in Lonberg (Curr Opin Immunol 20:450-459, 2008). In some techniques, a human antibody is generated by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions. Examples of such methods are found in Lonberg, (Nat Biotech 23:1117-1125, 2005); U.S. Pat. Nos. 6,075,181 and 6,150,584; U.S. Pat. No. 5,770,429; U.S. Pat. No. 7,041,870, and U.S. Patent Application Publication No. 2007/0061900. Human variable regions from intact antibodies generated using such animals can be further modified, for example, via combination with other human constant regions.

Human antibodies can also be made by hybridoma-based methods. Such methods are known in the art, and are described, for example, in Kozbor (J Immunol, 133:3001,1984); Brodeur et al. (Monoclonal Antibody Production Techniques and Applications, pp. 51-63, Marcel Dekker, Inc., New York, 1987); and Boerner et al., J Immunol, 147: 86, 1991). Human antibodies generated using human B-cell hybridoma technology can be used (for example, see Li et al., Proc Natl Acad Sci USA, 103:3557-3562, 2006).

Human antibodies can also be generated by isolating Fv clone variable domain sequences selected from human-derived phage display libraries. The selected variable domain sequences can be combined with a selected human constant domain.

PCSK9 antibodies may be isolated by screening combinatorial libraries for antibodies with the desired activity or activities. For example, a variety of methods are known in the art for generating phage display libraries and screening such libraries for antibodies possessing the desired binding characteristics (e.g., Hoogenboom et al. In: Methods in Molecular Biology 178:1-37, O'Brien et al., ed., Human Press, Totowa, NJ, 2001; McCafferty et al., Nature 348:552-554; Clackson et al., Nature 352: 624-628, 1991; Marks et al., J Mol Biol 222: 581-597, 1992; Marks and Bradbury, In: Methods in Molecular Biology 248:161-175, Lo, ed., Human Press, Totowa, N.J., 2003); Sidhu et al., J Mol Biol 338:299-310, 2004; Lee et al., J Mol Biol 3405:1073-1093, 2004); Fellouse, Proc Nat Acad Sci USA 10:12467-12472, 2004; and Lee et al., J Immunol Methods 284:119-132, 2004). In some embodiments, methods of identifying PCSK9 inhibitors (e.g., antibodies) are those described in the Examples.

In certain phage display methods, repertoires of VH and VL genes are separately cloned by polymerase chain reaction (PCR) and recombined randomly in phage libraries, which can then be screened for antigen-binding phage as described in Winter et al. (Ann Rev Immunol 12: 433-455, 1994). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments. Libraries from immunized sources provide high-affinity antibodies to the immunogen without the requirement of constructing hybridomas. Alternatively, the naive repertoire can be cloned (e.g., from human) to provide a single source of antibodies to a wide range of non-self and also self antigens without any immunization as described by Griffiths et al., EMBO J, 12: 725-734 (1993). Naive libraries can be made synthetically by cloning unrearranged V-gene segments from stem cells, and using PCR primers containing random sequence to encode the highly variable CDR3 regions and to accomplish rearrangement *in vitro*, as described by Hoogenboom and Winter (J Mol Biol, 227: 381-388, 1992). Patent publications describing human antibody phage libraries include, for example: U.S. Pat. No. 5,750,373, and US Patent Publication Nos. 2005/0079574, 2005/0119455, 2005/0266000, 2007/0117126, 2007/0160598, 2007/0237764, 2007/0292936, and 2009/0002360.

In some embodiments, yeast display methods such as those known in the art can be used to identify a PCSK9 inhibitor, e.g., antibody, for example, see Feldhaus et al. (Nature Biotechnology, 21:163-170, 2003) and Chao et al. (Nature Protocols, 1:755-768, 2006).

A human antibody or fragment that is isolated from a human antibody library is a human antibody or fragment.

Antibody variants

As used herein, the term "antibody variants" refers to variants and derivatives of an antibody described herein. In certain embodiments, amino acid sequence variants of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. Amino acid sequence variants of an antibody may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the antibody, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of residues within the amino acid sequences of the antibody.

Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding. In certain embodiments, antibody variants having one or more amino acid substitutions are provided. Sites of interest for substitution mutagenesis include the HVRs and framework sequences. Examples of such substitutions are provided in Table 2. Amino acid substitutions may be introduced into an antibody of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC. Substitutions may be conservative or non-conservative.

Starting residue	Substitution (examples)
Ala (A)	Val; Leu; Ile
Arg (R)	Lys; Gln; Asn
Asn (N)	Gln; His; Asp, Lys; Arg
Asp (D)	Glu; Asn
Cys (C)	Ser; Ala
Gln (Q)	Asn; Glu
Glu (E)	Asp; Gln
Gly (G)	Ala
His (H)	Asn; Gln; Lys; Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe
Lys (K)	Arg; Gln; Asn
Met (M)	Leu; Phe; Ile
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr
Pro (P)	Ala
Ser (S)	Thr
Thr (T)	Val; Ser
Trp (W)	Tyr; Phe
Tyr (Y)	Trp; Phe; Thr; Ser
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine

In another example of a substitution to create a variant PCSK9 antibody, one or more hypervariable region residues of a parent antibody are substituted. In general, variants are then selected based on improvements in desired properties compared to a parent antibody, for example, increased affinity, reduced affinity, reduced immunogenicity, increased pH dependence of binding. For example, an affinity matured variant antibody can be generated, e.g., using phage display-based affinity maturation techniques such as those described herein and known in the art.

Substitutions can be made in hypervariable regions (HVR) of a parent PCSK9 antibody to generate variants having desirable properties (e.g., as in Chowdhury, Methods Mol Biol 207:179-196, 2008), and/or in SDRs. The variants generated using such methods are generally selected by testing binding affinity. Affinity maturation by constructing and reselecting from secondary libraries has been described, e.g., in Hoogenboom et al. in Methods in Molecular Biology 178:1-37 (O'Brien et al., ed., Humana Press, Totowa, N.J., 2001). In some embodiments of affinity maturation, diversity is introduced into the variable genes chosen for maturation by any of a variety of methods (e.g., error-prone PCR, chain shuffling, or oligonucleotide-directed mutagenesis). A secondary library is then created. The library is then screened to identify any antibody variants with the desired affinity. Another method to introduce diversity involves HVR-directed approaches, in which several HVR residues (e.g., 4-6 residues at a time) are randomized. HVR residues involved in antigen binding may be specifically identified, e.g., using alanine scanning mutagenesis or modeling. Substitutions can be in one, two, three, four, or more sites within a parent antibody sequence.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue. Other insertional variants of the antibody molecule include the fusion to the N- or C-terminus of the antibody to an enzyme (e.g., for ADEPT) or a polypeptide which increases the serum half-life of the antibody.

In some cases, a PCSK9 antibody is modified such that its glycosylation pattern is altered compared to a parent antibody. Addition or deletion of glycosylation sites to an antibody can be accomplished by altering the amino acid sequence such that one or more glycosylation sites is created or removed. Methods of engineering antibodies to modify glycosylation sites are known in the art.

In certain embodiments, one or more amino acid modifications may be introduced into the Fc region of an antibody provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions.

In certain embodiments, the invention relates to an antibody variant that possesses some but not all effector functions, which make it a desirable candidate for applications in which the half-life of the antibody *in vivo* is important yet certain effector functions (such as complement and ADCC) are unnecessary or deleterious. *In vitro* and/or *in vivo* cytotoxicity assays can be conducted to confirm the reduction/depletion of CDC and/or ADCC activities. For example, Fc receptor (FcR) binding assays can be conducted to ensure that the antibody lacks FcγR binding (hence likely lacking ADCC activity), but retains FcRn binding ability. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet (Annu Rev Immunol 9:457-492, 1991). Non-limiting examples of in vitro assays to assess ADCC activity of a molecule of interest is described in U.S. Pat. No. 5,500,362 (see, e.g. Hellstrom et al. Proc. Nat'l Acad. Sci. USA 83:7059-7063, 1986) and Hellstrom et al., Proc Nat'l Acad Sci USA 82:1499-1502, 1985; U.S. Pat. No. 5,821,337 (see Bruggemann et al., J. Exp. Med. 166:1351-1361, 1987). Alternatively, non-radioactive assays methods may be employed (see, for example, CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and natural killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al. (Proc Nat Acad Sci USA 95:652-656, 1998). Clq binding assays may also be carried out to confirm that the antibody is unable to bind Clq and hence lacks CDC activity (e.g., WO 2006/029879 and WO 2005/100402). A CDC assay can be performed to evaluate complement activity (for example, Gazzano-Santoro et al., J Immunol Meth 202:163, 1996; Cragg et al., Blood 101:1045-1052, 2003; and Cragg and Glennie, Blood 103:2738-2743, 2004). FcRn binding and *in vivo* clearance/half-life determinations can be performed using methods known in the art (e.g., Petkova et al., Int Immunol 18:1759-1769, 2006).

Antibodies with increased half-lives and improved binding to the neonatal Fc receptor (FcRn) are described in, for example, US2005/0014934A1. Such antibodies comprise an Fc region with one or more substitutions therein which improve binding of the Fc region to FcRn. The Fc variants include those with substitutions at one or more of Fc region residues: 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434, e.g., substitution of Fc region residue 434 (U.S. Pat. No. 7,371,826). Other examples of Fc region variants are known in the art, for example, Duncan and Winter (Nature 322:738-740, 1988); U.S. Pat. No. 5,648,260; U.S. Pat. No. 5,624,821; and WO 94/29351.

Derivatives

In certain embodiments, a PCSK9 antibody provided herein is further modified to contain one of more non-protein moieties that are known in the art. The moieties include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary. In embodiments in which more than one polymer is attached, the polymers can be the same or different molecules.

Expression of recombinant PCSK9 antibodies

PCSK9 antibodies described herein can be produced using recombinant methods and compositions as are known in the art, e.g., as described in U.S. Pat. No. 4,816,567. In one embodiment, isolated nucleic acid encoding a PCSK9 antibody described herein is provided. Such nucleic acid can encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In another embodiment, a host cell comprising a nucleic acid encoding all or a

fragment of a PCSK9 antibody is provided. In one such embodiment, a host cell comprises a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In some embodiments, the host cell comprises nucleic acid sequences encoding a VH and a VL of a PCSK9 antibody and, optionally a vector or portion of a vector. In some embodiments, the VH and VL encoding sequence is integrated into a chromosome of the host cell.

In some embodiments, a method of making a PCSK9 antibody is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody or variant thereof, and optionally recovering the antibody from the host cell or from host cell culture medium.

For recombinant production of a PCSK9 antibody or variant thereof, nucleic acid encoding an antibody or variant thereof, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such a nucleic acid can be isolated and sequenced using methods known in the art.

Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies or variants thereof may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed or are undesirable. Non-limiting examples of such methods are described in, e.g., U.S. Pat. Nos. 5,648,237, 5,789,199, and 5,840,523; and Charlton (Methods in Molecular Biology, Vol. 248 (B. K. C. Lo, ed., Humana Press, Totowa, N.J., 2003, pp. 245-254). After expression, the antibody can be isolated from the bacteria and further purified.

Eukaryotes such as filamentous fungi or yeast can also be used as cloning and/or expression hosts for vectors comprising nucleic acid sequences encoding PCSK9 antibodies or fragments, including fungi and yeast strains whose glycosylation pathways have been humanized, and therefore can express an antibody with a partially or fully human glycosylation pattern (e.g., Gerngross, Nat Biotech 22:1409-1414, 2004), and Li et al., Nat Biotech 24:210-215, 2006).

Suitable host cells for the expression of an antibody include those of invertebrate origin. Baculovirus strains are known in the art and can be used as vectors in conjunction with insect cells.

Plant cells can be utilized as hosts (e.g., U.S. Pat. Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429, and plantibody technology for producing antibodies in transgenic plants).

Cells derived from a vertebrate, e.g., a mammal can be used as hosts. Examples of mammalian host cell lines include monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293 cells); baby hamster kidney cells (BHK); mouse sertoli cells (TM4 cells as described, e.g., in Mather, Biol Reprod 23:243-251, 1980)); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK; buffalo rat liver cells (BRL 3A); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); TR1 cells (e.g., in Mather et al., Annals N.Y. Acad Sci 383:44-68, 1982); MRC 5 cells; FS4 cells; Chinese hamster ovary (CHO) cells, including $DHFR^-$ CHO cells (Urlaub et al., Proc Nat Acad Sci USA 77:4216, 1980)); and myeloma cell lines such as YO, NSO and Sp20. Methods for using such cells are known in the art, for example, Yazaki and Wu (Methods in Molecular Biology, Vol. 248, B.K.C. Lo, ed., Humana Press, Totowa, NJ, pp. 255-268, 2003).

Assays

PCSK9 antibodies as described herein can be identified, screened for, or characterized for their physical/chemical properties and/or biological activities by various assays known in the art. Non-limiting methods are known in the art and some are described *infra* and in the Examples.

In some embodiments the PCSK9 binding activity of a PCSK9 antibody is assayed. Methods for conducting such assays are known in the art, for example, using ELISA, Western blot, or related methods. A competitive binding assay can be used to determine whether a selected PCSK9 antibody competes with another antibody. In an example, competition can be assayed using solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see, e.g., Stahli et al., 1983, Methods in Enzymology 9:242-253); solid phase direct biotin-avidin EIA (see, e.g., Kirkland et al., 1986, J Immunol 137:3614-3619) solid phase direct labeled assay, solid phase direct labeled

sandwich assay (see, e.g., Harlow and Lane, 1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Press); solid phase direct label RIA using I^{125} label (see, e.g., Morel et al., Molec Immunol 25:7-15, 1988); solid phase direct biotin-avidin EIA (see, e.g., Cheung, et al., Virology 176:546-552, 1990); and direct labeled RIA (Moldenhauer et al., Scand J Immunol. 32:77-82, 1990). Typically in such assays, purified antigen is bound to a solid surface or cells bearing an unlabeled test antigen binding protein and a labeled reference antigen binding protein. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test antigen binding protein. In general, the test antigen binding protein is present in excess. Antigen binding proteins identified by competition assay (competing antigen binding proteins) include antigen binding proteins binding to the same epitope as the reference antigen binding proteins and antigen binding proteins binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference antigen binding protein for steric hindrance to occur. Additional details regarding methods for determining competitive binding are provided in the examples herein. In general, when a competing antigen binding protein is present in excess, it will inhibit (e.g., reduce) specific binding of a reference antigen binding protein to a common antigen by at least 40-45%, 45-50%, 50-55%, 55-60%, 60-65%, 65-70%, 70-75% or 75% or more. In certain embodiments, binding is inhibited by at least 80-85%, 85-90%, 90-95%, 95-97%, or 97% or more.

In certain embodiments, a competition assay is used to identify an antibody that can compete with a selected PCSK9 antibody described herein. In some cases, the competing antibody binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by a PCSK9 antibody described herein. Methods for mapping an antibody epitope are known in the art are described in, for example, Morris (1996, "Epitope Mapping Protocols," in Methods in Molecular Biology vol. 66 (Humana Press, Totowa, NJ). In one example, immobilized PCSK9 is incubated in a solution that contains a first labeled antibody that can bind to PCSK9 (e.g., a PCSK9 antibody described herein) and a second unlabeled antibody that is being tested for its ability to compete with the first antibody for binding to PCSK9. The second antibody can optionally be present in a hybridoma supernatant. As a control, immobilized PCSK9 is incubated in a solution comprising the first labeled antibody but not the second unlabeled antibody. After incubation under conditions that permit binding of the first antibody to PCSK9, excess unbound antibody is removed, and the amount of label associated with immobilized PCSK9 is measured.

If the amount of label associated with immobilized PCSK9 is substantially reduced in the test sample relative to the control sample, then that indicates that the second antibody is competing with the first antibody for binding to PCSK9 (e.g., Harlow and Lane, 1988, Antibodies: A Laboratory Manual ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

An activity assay can be used to identify a PCSK9 antibody having a biological activity. Biological activity of a PCSK9 antibody can include, e.g., blocking, antagonizing, suppressing, interfering, modulating and/or reducing one or more biological activities of PCSK9. Such an assay can be an *in vitro* assay, for example an ELISA assay measuring the ability of the antibody to block PCSK9-LDLR binding, or a cell-based LDL uptake assay. In some cases, the assay is an *in vivo* assay.

In certain embodiments, a PCSK9 antibody can specifically bind human PCSK9 and can prevent interaction of PCSK9 with the LDLR. In some embodiments, a PCSK9 antibody as provided herein can inhibit binding of human PCSK9 to LDLR by at least about 20%-40%, 40-60%, 60-80%, 80-85%, or more (as determined, for example, by measuring binding in an *in vitro* competitive binding assay), for example by at least 20%, at least 40%, at least 60%, at least 80%, at least 85%, at least 90%, or at least 95%. In certain embodiments, the invention provides isolated PCSK9 antibodies that specifically bind to PCSK9 and which antagonize the PCSK9-mediated effect on LDLR levels when measured *in vitro* using an LDLR down regulation assay in HepG2 cells.

Immunoconjugates

In some embodiments, a PCSK9 antibody is conjugated to at least one heterologous agent, creating an immunoconjugate. Such agents can be cytotoxic agents, including a chemotherapeutic agent or drug, a growth inhibitory agent, a toxin (e.g., a protein toxin, toxic enzyme, or radioactive isotope). In one embodiment, agent is a drug such that the PCSK9 antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (U.S. Pat. Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235); an auristatin such as monomethylauristatin drug moieties DE and DF (MMAE and MMAF; U.S. Pat. Nos. 5,635,483 and 5,780,588, and 7,498,298); a dolastatin; a calicheamicin or derivative thereof (U.S. Pat. Nos. 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, and 5,877,296; Hinman et al., Cancer Res 53:3336-3342, 1993); and Lode et al., Cancer Res. 58:2925-2928,

1998)); an anthracycline such as daunomycin or doxorubicin (Kratz et al., Curr Med Chem 13:477-523, 2006); Jeffrey et al., Bioorganic Med Chem Lett 16:358-362, 2006); Torgov et al., Bioconj. Chem. 16:717-721 (2005); Nagy et al., Proc. Natl. Acad. Sci. USA 97:829-834 (2000); Dubowchik et al., Bioorg. & Med. Chem. Letters 12:1529-1532 (2002); King et al., J. Med. Chem. 45:4336-4343 (2002); and U.S. Pat. No. 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but 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.

In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, Re^{188}, Sm^{153}, Bi^{212}, P^{32}, Pb^{212} and radioactive isotopes of Lu. When the radioconjugate is used for detection, it can include a radioactive atom for scintigraphic studies, or a spin label for nuclear magnetic resonance (NMR) imaging, e.g., $iodine^{123}$, $iodine^{131}$, $indium^{111}$, $fluorine^{19}$, $carbon^{13}$, $nitrogen^{15}$, $oxygen^{17}$, gadolinium, manganese or iron.

Conjugates of an antibody and cytotoxic agent can be made using methods known in the art, typically using bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of

radionucleotide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Res. 52:127-131 (1992); U.S. Pat. No. 5,208,020) may be used.

The immunuoconjugates herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SLAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, Ill., U.S.A).

Detection of PCSK9 and Diagnostics

A PCSK9 antibody as described herein is useful for detecting the presence of PCSK9, for example, in a biological sample. "Detecting" as used herein encompasses quantitative or qualitative detection. A biological sample can be blood, serum, plasma, CSF, or other samples of biological origin including a cell or a tissue.

Provided herein is a PCSK9 antibody for use in a method of diagnosis or detection, for example, in a method of detecting the presence of PCSK9 in a biological sample. In certain embodiments, the method comprises detecting the presence of PCSK9 protein in a biological sample. In certain embodiments, PCSK9 is human PCSK9. In certain embodiments, the method comprises contacting the biological sample with a PCSK9 antibody as described herein under conditions permissive for binding of the PCSK9 antibody to PCSK9, and detecting whether a complex is formed between the PCSK9 antibody and PCSK9. Such method may be an *in vitro* or *in vivo* method. In one embodiment, a PCSK9 antibody is used to select subjects eligible for therapy with a PCSK9 antibody, e.g. where PCSK9 or LDL-cholesterol is a biomarker for selection of patients.

Disorders that can be diagnosed using a PCSK9 inhibitor as described herein or for which assay of PCSK9 is otherwise useful include without limitation, cholesterol-related disorders such as hypercholesterolemia, heart disease, metabolic syndrome, diabetes, coronary heart disease, stroke, cardiovascular diseases, Alzheimer's disease and generally dyslipidemias, which can be manifested, for example, by an elevated total serum cholesterol, elevated LDL, elevated

triglycerides, elevated very low density lipoprotein (VLDL), and/or low HDL. An embodiment includes a method for treating or preventing hypercholesterolemia, and/or at least one symptom of dyslipidemia, atherosclerosis, cardiovascular disease (CVD) or coronary heart disease, in a subject by administering to the subject a therapeutically effective amount of a PCSK9 inhibitor. In certain embodiments, a therapeutically effective amount of an PCSK9 inhibitor for use in treating or preventing hypercholesterolemia, and/or at least one symptom of dyslipidemia, atherosclerosis, CVD or coronary heart disease, in a subject. The invention further provides the use of an effective amount of a PCSK9 inhibitor that antagonizes extracellular or circulating PCSK9 in the manufacture of a medicament for treating or preventing hypercholesterolemia, and/or at least one symptom of dyslipidemia, atherosclerosis, CVD or coronary heart disease, in an individual.

Labeled PCSK9 inhibitors (e.g., PCSK9 antibodies, fragments, and derivatives thereof) are also provided. Labels include, but are not limited to, labels or moieties that can be directly detected (e.g., fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Examples of labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase, luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, f3-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Articles of Manufacture

In certain embodiments, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described herein using a PCSK9 antibody is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes,

IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). At least one active agent in the composition is a PCSK9 antibody as described herein. The label or package insert indicates that the composition is used for treating the condition of choice. The article of manufacture can comprise (a) a first container with a composition that includes a PCSK9 antibody; and (b) a second container with a composition containing a cytotoxic or other therapeutic agent that is not a PCSK9 antibody. In certain embodiments, the second container comprises a second therapeutic agent, wherein the second therapeutic agent is a cholesterol-lowering drug of the statin class. In certain embodiments, the statin is and/or comprises atorvastatin (e.g., LIPITOR® or Torvast), fluvastatin (e.g., LESCOL™), lovastatin (e.g., MEVACOR®, ALTOCOR®, or ALTOPREV™), mevastatin (pitavastatin (e.g., LIVALO® or PITAVA®), pravastatin (e.g., PRAVACHOL®, SELEKTINE®, LIPOSTAT®), rosuvastatin (e.g., CRESTOR®), simvastatin (e.g., ZOCOR®, LIPEX®), or any combination thereof, e.g., VYTORIN®, ADVICOR® or SIMCOR®.

The article of manufacture in this embodiment can also include a package insert indicating that the compositions can be used to treat a particular condition. Alternatively, or additionally, the article of manufacture can include a second or third container containing a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It can further include additional useful materials, including other buffers, diluents, filters, needles, and syringes.

An article of manufacture as described herein can include an immunoconjugate of the invention in place of or in addition to a PCSK9 antibody.

Inhibitor molecules derived from antibodies

A PCSK9 antibody of the invention can be a fragment of an antibody that specifically binds to PCSK9, e.g., to the prodomain of PCSK9, provided that the fragment comprises an antigen binding site. Antibody fragments of the invention are generally obtained starting with a reference (parent) antibody molecule such as those comprising one or more CDRs as disclosed herein. Antibody fragments can be generated using methods known in the art such as

recombinant DNA, enzymatic cleavage (for example, using pepsin or papain), chemical cleavage of an antibody (for example, chemical reduction of disulfide bridges). Antibody fragments that comprise an antibody antigen-binding site include, but are not limited to, molecules such as Fab, Fab', Fab'-SH, scFv, Fv, dAb, Fd and disulfide stabilized variable region (dsFv). Various other antibody molecules including one or more antibody antigen-binding sites can be engineered, including for example F(ab')2, F(ab)3, diabodies, triabodies, tetrabodies, and minibodies. Examples of antibody molecules and methods for their construction and use are described in Holliger and Hudson, 2005, Nat Biotechnol. 23:1126-1136. Non-limiting examples of binding fragments are a Fab fragment composed of VL, VH, constant light chain domain (CL) and constant heavy chain domain 1 (CH1) domains; an Fd fragment composed of VH and CH1 domains; an Fv fragment composed of the VL and VH domains of a single antibody; a dAb fragment composed of a VH or a VL domain; isolated CDR regions; an F(ab')2 fragment, a bivalent fragment comprising two linked Fab fragments; a single chain Fv molecule (scFv), in which a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site; a bispecific single chain Fv dimer (for example as disclosed in WO 1993/011161) and a diabody, which is a multivalent or multispecific fragment constructed using gene fusion (for example as disclosed in WO94/13804 and [50]). Fv, scFv, or diabody molecules can be stabilized by the incorporation of disulfide bridges linking the VH and VL domains. Minibodies comprising an scFv joined to a CH3 domain can also be used as a PCSK9 inhibitor. Other fragments and derivatives of an antibody that can be used as a PCSK9 inhibitor include an Fab', which differs from a Fab fragment by the addition of a few amino acid residues at the carboxyl terminus of the heavy chain CH1 domain, including one or more cysteines from the antibody hinge region, and Fab'-SH, which is a Fab' fragment in which the cysteine residue(s) of the constant domains bear a free thiol group.

In some cases, a PCSK9 inhibitor that is an antibody fragment has been chemically modified to improve or introduce a desirable property, for example PEGylation to increase half-life or incorporation.

A dAb (domain antibody) is a small monomeric antigen-binding fragment of an antibody (the variable region of an antibody heavy or light chain). VH dAbs occur naturally in camelids (e.g., camels and llamas) and can be produced by immunizing a camelid with a target antigen, isolating antigen-specific B cells and directly cloning dAb genes from individual B cells. A

PCSK9 inhibitor of the present invention can be a dAb comprising a VH or VL domain substantially as set out herein, or a VH or VL domain comprising a set of CDRs substantially as set out herein.

Disease Treatment

PCSK9 inhibitors are useful for treatment of diseases in which a reduction in PCSK9 activity is desirable (PCSK9 related diseases). The term 'disease' as used herein is intended to include conditions that cause or could cause discomfort, dysfunction, distress, and/or death to the person affected, and includes, for example, injuries, disabilities, disorders, syndromes, infections, isolated symptoms, and atypical (not within the norm) variations in function. Examples of such PCSK9 related diseases include hypercholesterolemia, coronary heart disease, metabolic syndrome, acute coronary syndrome and related conditions, for example, diabetes, stroke, cardiovascular diseases, Alzheimer's disease and dyslipidemias exhibiting elevated total serum cholesterol, elevated LDL, elevated triglycerides, elevated VLDL, and/or low HDL. Non-limiting additional examples of primary and secondary dyslipidemias that can be treated using a PCSK9 inhibitor include familial combined hyperlipidemia, familial hypertriglyceridemia, familial hypercholesterolemias, including heterozygous hypercholesterolemia, homozygous hypercholesterolemia, familial defective apoplipoprotein B-100; polygenic hypercholesterolemia; remnant removal disease, hepatic lipase deficiency; dyslipidemia. Dyslipidemia may be the result of, for example diet, hypothyroidism, drugs such as estrogen and progestin, beta-blockers, thiazide diuretics; nephrotic syndrome, chronic renal failure, Cushing's syndrome, primary biliary cirrhosis, glycogen storage diseases, hepatoma, cholestasis, acromegaly, insulinoma, isolated growth hormone deficiency, and alcohol-induced hypertriglyceridemia.

PCSK9 inhibitors as described herein can also be useful in preventing or treating atherosclerotic diseases, such as coronary heart disease, coronary artery disease, peripheral arterial disease, stroke (ischaemic or hemorrhagic), angina pectoris, or cerebrovascular disease and acute coronary syndrome, myocardial infarction. Prophylactic uses of PCSK9 inhibitors include reducing the risk of heart attack, fatal and non-fatal stroke, in certain heart surgeries, hospitalization for heart failure, chest pain in patients with heart disease, and/or cardiovascular events due to prior conditions such as heart attack, heart surgery, and/or chest pain. In certain

embodiments, the PCSK9 antibodies and methods described herein can be used to reduce the risk of recurrent cardiovascular events.

Treatment and Dosage

Subjects in need of treatment with a PCSK9 inhibitor are treated with a therapeutically effective amount of the inhibitor, e.g., an amount that is effective at dosages and administration regimes sufficient to achieve a desired result as a therapeutic or prophylactic. For example, a therapeutically effective amount of a PCSK9 inhibitor decreases serum cholesterol levels in a subject, e.g., reduces serum LDL-cholesterol levels in a subject compared to levels in the subject before initiating treatment with the PCSK9 inhibitor. In some cases, a therapeutically effective amount is sufficient to decrease serum cholesterol levels in a subject to below a predetermined level.

PCSK9 inhibitors described herein are useful for reducing at least one activity of PCSK9 such as binding of PCSK9 to LDLR and/or reducing or ameliorating at least one sign or symptom of a PCSK9-associated disease. Inhibition of one or more of PCSK9-associated functional properties can be determined using methods known to the art (e.g., Barak and Webb, 1981 J Cell Biol 90:595-604; Stephan and Yurachek, 1993 J Lipid Res 34:325330; and McNamara et al., 2006 Clin Chim Acta 369:158-167) including those described herein. Inhibition of PCSK9 activity is typically assayed relative to activity observed in the absence of the inhibitor. In general, a PCSK9-inhibitor as described herein inhibits a PCSK9 activity by at least 10% compared to a control, for example, decreasing activity by at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 95% compared to a control. As used herein, the term "control" when applied to assays of PCSK9 activity includes an assay in which PCSK9 activity is compared to a sample that does not contain a component of the assay, typically, the PCSK9 inhibitor, or refers to an assay in which activity of a PCSK9 inhibitor is compared to a predetermined reference activity level.

A PCSK9 inhibitor as described herein can be used in a method that includes contacting a cell, population of cells, or a tissue that expresses or contains an LDL receptor with a PCSK9 inhibitor disclosed herein under conditions that allow the PCSK9 inhibitor to bind to PCSK9. In general, the binding of PCSK9 by the PCSK9 inhibitor results in increased uptake of cellular LDL. The cell, population of cells or tissue can be *in vitro* or *in vivo* and are generally

mammalian, e.g., rodents (including rats, mice, rabbits, and guinea pigs), canines (including dogs), bovines (including cattle), ovines, porcine, cats, equines, and camelids.

PCSK9 inhibitors described herein can be used in methods for inhibiting PCSK9 in a subject in need of such methods by administering to the subject a therapeutically effective amount of a PCSK9 inhibitor described herein. In general, the method is for treating a disease associated with PCSK9 activity. The PCSK9 inhibitor is useful for the preparation of a medicament for treating a PCSK9 associated disease, for example, an undesirably high LDL level, e.g., hypercholesterolemia, coronary heart disease, metabolic syndrome, acute coronary syndrome, or related conditions. Treatment of a subject can be prophylactic or therapeutic.

The terms "therapeutically effective" or "prophylactically effective" refers to the amount necessary at the intended dosage to achieve the desired therapeutic or prophylactic effect for the period of time desired. The desired effect can be, for example, amelioration of at least one symptom associated with condition being treated. Those skilled in the art will appreciate that the amount of PCSK9 inhibitor administered will vary according to various factors, including but not limited to, the disease state, age, sex and weight of the individual. The response can be assessed using methods known in the art including assay of circulating LDL levels and/or amelioration of other symptoms of disease or predisposition to disease.

A PCSK9 inhibitor is generally administered as a pharmaceutical composition. Such compositions include a PCSK9 inhibitor and, generally, a pharmaceutically acceptable carrier that can include an excipient, diluent, stabilizer, buffer, or other component that facilitates administration or storage of the inhibitor.

Pharmaceutical compositions can be formulated using methods known in the art, e.g., McGoff and Scher, 2000 Solution Formulation of Proteins/Peptides, In: McNally, E. J., ed. Protein Formulation and Delivery, New York, NY, Marcel Dekker; pp. 139-158; Akers and Defilippis, 2000, Peptides and Proteins as Parenteral Solutions, In: Pharmaceutical Formulation Development of Peptides and Proteins, Philadelphia, PA, Taylor and Francis, pp. 145-177; Akers et al., 2002, Pharm Biotechnol 14:47-127.

The composition can be in liquid or solid form. Solid formulations can be produced using methods known in the art including, but not limited to, lyophilization, spray drying, or drying by supercritical fluid technology. An oral formulation can be in, for example, a tablet, capsule, or powder. A solid formulation can be a lyophilized composition and is made into a solution prior

to administration for either single or multiple dosing according to methods known to the skilled artisan. Compositions are generally formulated within a biologically relevant pH range and can be buffered to maintain an acceptable pH during storage. Both liquid and solid formulations can require storage at lower temperatures (e.g., 2-8ºC). Compositions may contain a bacteriostat to prevent or minimize proteolysis during storage, including but not limited to effective concentrations. Examples include benzyl alcohol, phenol, m-cresol, chlorobutanol, methylparaben, and/or propylparaben. A bacteriostat may be contraindicated for some patients. Therefore, a lyophilized formulation may be reconstituted in a solution either containing or not containing such a component. Additional components may be added to either a buffered liquid or solid inhibitor formulation, including but not limited to a cryoprotectant (including but not limited to polyhydroxy hydrocarbons such as sorbitol, mannitol, glycerol, and dulcitol and/or disaccharides such as sucrose, lactose, maltose, or trehalose) and, a salt (including but not limited to NaCl, KCl, or LiCl). Osmolarity of certain formulations is selected to support long term stability at temperatures of, for example, 2-8ºC or higher, and parenteral injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be included in a formulation. A formulation can contain a divalent cation (including but not limited to $MgCl_2$, $CaCl_2$, and $MnCl_2$); and/or a non-ionic surfactant (including but not limited to polysorbate-80 (Tween® 80), polysorbate-60 (Tween® 60), polysorbate-40 (Tween® 40), and polysorbate-20 (Tween 20), polyoxyethylene alkyl ethers, including but not limited to Brij™ 58, Brij™ 3, Triton® X-100, Triton® X-114, NP40, Span 85 and the Pluronic® series of non-ionic surfactants (e.g., Pluronic® 121)).

Pharmaceutical compositions in liquid format can include a liquid carrier, e.g., water, petroleum, animal oil, vegetable oil, mineral oil, or synthetic oil. The liquid format may also include physiological saline solution, dextrose or other saccharide solution or glycols, such as ethylene glycol, propylene glycol or polyethylene glycol.

Pharmaceutical compositions can be formulated for administration after dilution in isotonic vehicles such as sodium chloride, Ringer's solution, or lactated Ringer's solution.

A PCSK9 pharmaceutical composition can include 50 to 200 mg/mL of a PCSK9-specific inhibitor described herein. In addition, such a composition can include at least one of the following; a polyhydroxy hydrocarbon (including but not limited to sorbitol, mannitol, glycerol and dulcitol) and/or a disaccharide (including but not limited to sucrose, lactose,

maltose and trehalose) at 1% to 6% weight per volume ("w/v") of the formulation; 5 mM to about 200 mM of histidine, imidazole, phosphate or acetic acid, 5 mM to about 200 mM of arginine, proline, phenylalanine, alanine, glycine, lysine, glutamic acid, aspartic acid or methionine; 0.01M to about 0.1M of hydrochloric acid ("HCl") in an amount sufficient to achieve a pH in the range of about 5.5 to about 7.5; and a carrier including but not limited to sterile water, petroleum, animal oil, vegetable oil, mineral oil, synthetic oil, physiological saline solution, dextrose or other saccharide solution or glycols, such as ethylene glycol, propylene glycol or polyethylene glycol; such that the pharmaceutical composition has a pH in the range of about 5.5 to about 7.5. In some cases, the pharmaceutical composition contains 0.01% to about 1% w/v of the formulation of a non-ionic surfactant. HCl can be added to a composition as free acid, histidine-HCl or arginine-HCl.

Use of the term "about" with respect to amounts disclosed in the specification means within at least 10%, e.g., within 5%, of the specified numbers provided. A range provided as, for example" in "about 50 to about 200" expressly includes as distinct embodiments each number within said range. As such in the above example, embodiments including but not limited to those having 50, 100, 125, 150 and 200 form specific embodiments herein. Pharmaceutical compositions as disclosed herein have general applicability despite the mode of administration. In specific embodiments, the disclosed pharmaceutical compositions are useful for subcutaneous administration as a liquid or upon reconstitution of a lyophilized form.

In some cases, the pharmaceutical composition comprises about 50 to about 200 mg/mL of a PCSK9 inhibitor as described herein. Optionally, the composition can include about 1% to about 6% (e.g., from about 2% to about 6%) w/v mannitol, trehalose or sucrose; about 10 mM to about 100 mM of histidine; about 25 mM to about 100 mM of arginine or proline; about 0.02 M to about 0.05M of hydrochloric acid ("HCl") in an amount sufficient to achieve a pH in the range of about 5.8 to about 7; and a liquid including but not limited to sterile water, petroleum, animal oil, vegetable oil, mineral oil, synthetic oil, physiological saline solution, dextrose or other saccharide solution or glycols, such as ethylene glycol, propylene glycol or polyethylene glycol; wherein said pharmaceutical composition has a pH in the range of about 5.8 to about 7. T he pharmaceutical composition optionally can include about 0.01% to about 1% w/v of the formulation of a non-ionic surfactant (including but not limited to Polysorbate-80, Polysorbate-60, Polysorbate-40, and Polysorbate-20, polyoxyethylene alkyl ethers, including but not limited

to Brij 58, Brij35, Triton X-100, Triton X-114, NP40, Span 85 and the Pluronic series of non-ionic surfactants (e.g., Pluronic 121)).

Also provided herein are pharmaceutical compositions that are lyophilized and can be reconstituted. In some cases, the PCSK9 inhibitor concentration in a lyophilized and reconstituted solution is up to 2-fold higher than in the pre-lyophilized composition. In specific embodiments, the PCSK9 inhibitor concentration in the lyophilized and/or reconstituted pharmaceutical composition is in the range of about 50 mg/mL to about 300 mg/mL. Diluents useful for reconstituting the lyophilized pharmaceutical compositions include but are not limited to sterile water, bacteriostatic water for injection ("BWFI"), phosphate-buffered saline, a sterile saline solution, physiological saline solution, Ringer's solution or dextrose solution. Other constitutents such as those described *supra* can be included in such solutions used for reconstitution.

In general, a composition comprising a PCSK9 inhibitor is stable. "Stable" as used herein refers to the property of a PCSK9 inhibitor to retain its physical or chemical stability, conformational integrity, at 2-37°C for at least 30 days, for example, for at least 3 months, at least 6 months, at least 12 months, at least 2 years, or at least 3 years when maintained in a specified temperature range, for example, 2°C-37°C, 2°C-10°C, or 2°C-15°C. Degradation of the PCSK9 inhibitor within a composition is generally less than 10%, for example, less than 5%, 3%, or 2% compared with a freshly prepared composition. Stability of a formulation can be tested using methods known in the art including, but not limited to, size exclusion chromatography (SEC-HPLC), dynamic light scattering (DLS), capillary SDS-PAGE, and capillary iso-electric focusing (cIEF) or cation exchange chromatography ("CEX").

Determination of dose of a PCSK9 inhibitor can be determined by those skilled in the art. (e.g., Lederman et al., Int J Cancer 47:659-664, 1991; Bagshawe et al., Antibody, Immunoconjugates and Radiopharmaceuticals 4:915-922, 1991), and will vary based on a number of factors including but not limited to the particular PCSK9-specific inhibitor, the subject being treated, the condition of the subject, the tissue or system being treated, the route of administration, and the desired outcome. A physician or veterinarian of skill can determine and prescribe the therapeutically effective amount of the inhibitor. Dosage ranges can be from about 0.01 to 100 mg/kg, e.g., 0.05 to 25 mg/kg, of the host body weight. For example, dosages can be 0.3 mg/kg body weight, 1 mg/kg body weight, 3 mg/kg body weight, 5 mg/kg body weight or

10 mg/kg body weight or within the range of 1-10 mg/kg. In one non-limiting example, a dosage of 5 mg to 2.0 g is used for systemic delivery. A PCSK9 inhibitor as described herein can be used alone at appropriate dosages. Alternatively, the inhibitor is administered according to a dosing regime with other agents, for example, with a statin. For example, a PCSK9 inhibitor can be used in combination or in conjunction with other drugs (therapeutic and/or prophylactic), including but not limited to cholesterol-lowering drugs, for example, cholesterol absorption inhibitors (e.g., Zetia®) and cholesterol synthesis inhibitors (e.g., Zocor® and Vytorin®). The present invention contemplates such combinations and they form an important embodiment hereof. Accordingly, the present invention relates to methods of treatment as described above where the PCSK9-specific inhibitor is administered/delivered simultaneously with, following or prior to another drug or drugs (therapeutic and/or prophylactic), including but not limited to cholesterol-lowering drugs, cholesterol absorption inhibitors and cholesterol absorption inhibitors.

Individuals (subjects) that can be treated with a composition described herein include primates, human and non-human, and include any non-human mammal or vertebrate of commercial or domestic veterinary importance.

A PCSK9 inhibitor (e.g., antibody, fragment, or derivative) can be administered to an individual by a route of administration known in the art, including but not limited to oral administration, administration by injection (specific embodiments of which include intravenous, subcutaneous, intraperitoneal or intramuscular injection), administration by inhalation, intranasal, or topical administration, either alone or in combination with other agents designed to assist in the treatment of the individual. The PCSK9 inhibitor can also be administered using an injection device, injector pen, needleless device, or patch delivery systems. The route of administration should be determined based on a number of considerations appreciated by those of skill in the art including, but not limited to, the desired physiochemical characteristics of the treatment. Treatment may be provided on a daily, weekly, biweekly, or monthly basis, or any other regimen that delivers the appropriate amount of PCSK9 inhibitor to the individual at the prescribed times such that the desired treatment is effected and maintained. The formulations may be administered in a single dose or in more than one dose at separate times.

A PCSK9 inhibitor (e.g., antibody, fragment, or derivative thereof) described herein can be used in the manufacture of a medicament for treatment of a PCSK9-associated disease. The

medicament is useful in a subject exhibiting a disease or symptoms associated with undesirable PCSK9 activity. In some embodiments, the condition is hypercholesterolemia, coronary heart disease, metabolic syndrome, acute coronary syndrome or a related condition.

Accordingly, the invention also relates to use of a PCSK9 inhibitor (e.g., a PCSK9 antibody, fragment, or derivative thereof) in the manufacture of a medicament for the treatment of a PCSK9-associated disease in a subject, wherein the PCSK9 inhibitor can specifically bind to the prodomain of a human PCSK9. In some embodiments, a subject in need of treatment with a PCSK9 inhibitor is identified. In some embodiments, the PCSK9 inhibitor is an antibody, fragment, or variant thereof that exhibits pH-dependent binding to a PCSK9. In some embodiments, the subject's plasma is further tested for free PCSK9 before and after treatment, wherein the subject has decreased free PCSK9 in their serum after treatment with the PCSK9 inhibitor compared to the level of free PCSK9 after treatment. In some embodiments, the subject's plasma is further tested for free PCSK9 after treatment and comparing it to a reference. In some embodiments, the subject is at risk for or has been diagnosed with a cholesterol-related disorder. In some embodiments, the subject is at risk for or has been diagnosed with hypercholesterolemia, heart disease, metabolic syndrome, diabetes, coronary heart disease, stroke, cardiovascular diseases, Alzheimer's disease, atherosclerosis, or a dyslipidemia, e.g., elevated total serum cholesterol, elevated LDL, elevated triglycerides, elevated very low density lipoprotein (VLDL), and/or low HDL. In some embodiments, the PCSK9 inhibitor is used in combination with a statin.

The invention further relates a PCSK9 inhibitor (e.g., a PCSK9 antibody, fragment, or derivative thereof) for use in treating a PCSK9-associated disease in a subject, wherein the PCSK9 inhibitor can specifically bind to the prodomain of a human PCSK9. In some embodiments, a subject in need of treatment with a PCSK9 inhibitor is identified. In some embodiments, the PCSK9 inhibitor is an antibody, fragment, or variant thereof that exhibits pH-dependent binding to a PCSK9. In some embodiments, the subject's plasma is further tested for free PCSK9 before and after treatment, wherein the subject has decreased free PCSK9 in their serum after treatment with the PCSK9 inhibitor compared to the level of free PCSK9 after treatment. In some embodiments, the subject's plasma is further tested for free PCSK9 after treatment and comparing it to a reference. In some embodiments, the subject is at risk for or has been diagnosed with a cholesterol-related disorder. In some embodiments, the subject is at risk

for or has been diagnosed with hypercholesterolemia, heart disease, metabolic syndrome, diabetes, coronary heart disease, stroke, cardiovascular diseases, Alzheimer's disease, atherosclerosis, or a dyslipidemia, e.g., elevated total serum cholesterol, elevated LDL, elevated triglycerides, elevated very low density lipoprotein (VLDL), and/or low HDL. In some embodiments, the PCSK9 inhibitor is used in combination with a statin.

Genetic Therapy

Also provided herein are methods for administering a of disclosed PCSK9 antibody for purposes of gene therapy. In this method, one or more cells of a subject are transformed with nucleic acid encoding a PCSK9 antibody as described herein. Subjects treated in this manner can endogenously produce the PCSK9 antibody. An example of the method is provided by Alvarez, et al. (Clin Cancer Res 6:3081-3087, 2000) in which single-chain anti-ErbB2 nucleic acid was introduced into subjects using a gene therapy approach.

A subject can be treated for a PCSK9 disease using a nucleic acid-based therapy, e.g., a using a nucleic acid that codes for a PCSK9 inhibitor, e.g., antibody. In general, in such methods, a nucleic acid encoding a PCSK9 inhibitor is introduced into the cells of a subject using methods known in the art, for example, by incorporating sequences encoding the PCSK9 inhibitor into a viral vector. Examples of such viral vectors include those derived from lentivirus, herpes virus, adenovirus, adeno-associated virus, vaccinia virus, baculovirus, alphavirus, and influenza virus. Such vectors are commercially available, e.g., from Avigen, Inc. (Alameda, CA; AAV vectors), Cell Genesys (Foster City, CA; retroviral, adenoviral, AAV vectors, and lentiviral vectors), Clontech (retroviral and baculoviral vectors), Genovo, Inc. (Sharon Hill, PA.; adenoviral and AAV vectors), Genvec (adenoviral vectors), IntroGene (Leiden, Netherlands; adenoviral vectors), Molecular Medicine (retroviral, adenoviral, AAV, and herpes viral vectors), Norgen (adenoviral vectors), Oxford BioMedica (Oxford, United Kingdom; lentiviral vectors), and Transgene (Strasbourg, France; adenoviral, vaccinia, retroviral, and lentiviral vectors).

Methods for constructing and using viral vectors are known in the art (e.g., Miller, et al, BioTechniques 7:980-990, 1992). Typically, the vector is replication defective or attenuated in some other way. Examples of such vectors include, for example a herpes virus vector (Kanno et al, Cancer Gen Ther 6:147-154, 1999; Kaplitt et al, J Neurosci Meth 71:125-132, 1997 and Kaplitt et al, J Neuro Onc 19:137-147, 1994); adenovirus vectors (e.g., Strafford-Perricaudet et

al, J Clin Invest 90:626-630, 1992; PCT Publication Nos. WO94/26914, WO94/28938, WO94/28152, WO94/12649, WO95/02697 and WO96/22378). Retroviral vectors can also be used to introduce a sequence encoding a PCSK9 inhibitor, for example, see U.S. Patent Nos. 5,399,346, 4,650,764, 4,980,289, and 5,124,263; Mann et al, Cell 33:153, 1983; Markowitz et al, J. Virol., 62:1120, 1988; EP 453242 and EP178220.

Lentiviral vectors can be used as agents for the direct delivery and sustained expression of nucleic acids encoding a PCSK9-specific inhibitor of the invention in several tissue types, including brain, retina, muscle, liver and blood. The vectors can efficiently transduce dividing and nondividing cells in these tissues, and maintain long-term expression of the PCSK9-specific inhibitor. For a review, see Zufferey et al, J. Virol. 72:9873-80, 1998 and Kafri et al, Curr. Opin. Mol. Ther. 3:316-326, 2001. Lentiviral packaging cell lines are available and known generally in the art. They facilitate the production of high-titer lentivirus vectors for gene therapy. An example is a tetracycline-inducible VSV-G pseudotyped lentivirus packaging cell line which can generate virus particles at titers greater than 10.sup.6 IU/ml for at least 3 to 4 days; see Kafri et al, J. Virol. 73:576-584, 1999. The vector produced by the inducible cell line can be concentrated as needed for efficiently transducing nondividing cells in vitro and in vivo.

Sindbis virus is a member of the alphavirus genus and has been studied extensively since its discovery in various parts of the world beginning in 1953. Gene transduction based on alphavirus, particularly Sindbis virus, has been well-studied in vitro (see Straus et al., Microbiol Rev., 58:491-562, 1994; Bredenbeek et al., J Virol, 67:6439-6446, 1993; Ijima et al., Int J Cancer 80:110-118, 1999 and Sawai et al, Biochim Biophys Res Comm 248:315-323, 1998). Many properties of alphavirus vectors make them a desirable alternative to other virus-derived vector systems being developed, including rapid engineering of expression constructs, production of high-titered stocks of infectious particles, infection of nondividing cells, and high levels of expression (Strauss et al, 1994 *supra*). Use of Sindbis virus for gene therapy has been described. (Wahlfors et al., Gene Ther 7:472-480, 2000 and Lundstrom, J Recep Sig Transduct Res 19:673-686, 1999).

In another embodiment, a vector can be introduced to cells by lipofection or with other transfection facilitating agents (peptides, polymers, etc.). Synthetic cationic lipids can be used to prepare liposomes for in vivo and in vitro transfection of a gene encoding a marker (Feigner et al., Proc Natl Acad Sci USA 84:7413-7417, 1987 and Wang et al., Proc Natl Acad Sci USA

84:7851-7855, 1987). Useful lipid compounds and compositions for transfer of nucleic acids are described in PCT Publication Nos. WO 95/18863 and WO 96/17823, and in U.S. Pat. No. 5,459,127.

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into desired host cells by methods known in the art, e.g., electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (see, e.g., Wilson, et al., J Biol Chem 267:963-967, 1992; Williams et al., Proc Natl Acad Sci USA 88:2726-2730, 1991). Other reagents commonly used for transfection of plasmids include, but are by no means limited to, FuGENE®, Lipofectin®, and Lipofectamine®. Receptor-mediated DNA delivery approaches can also be used (Wu et al., J Biol Chem 263:14621-14624, 1988). U.S. Pat. Nos. 5,580,859 and 5,589,466 disclose delivery of exogenous DNA sequences, free of transfection facilitating agents, in a mammal. Electrotransfer, a relatively low voltage, high efficiency *in vivo* DNA transfer technique can be used (Vilquin et al., Gene Ther 8:1097, 2001; Payen et al., Exp Hematol 29:295-300, 2001; Mir, Bioelectrochemistry 53:1-10, 2001; PCT Publication Nos. WO 99/01157, WO 99/01158 and WO 99/01175).

Pharmaceutical compositions suitable for such gene therapy approaches and comprising nucleic acids encoding a PCSK9 antibody of the present invention are included within the scope of the present invention.

Research and Related Uses

In another aspect, the present invention provides a method for identifying, isolating, quantifying or antagonizing PCSK9 in a sample of interest using a PCSK9 antibody provided herein. For example, a PCSK9 antibody or variant thereof can be utilized as a research tool in immunochemical assays, such as Western blots, ELISAs, radioimmunoassay, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art (see, e.g., Immunological Techniques Laboratory Manual, ed. Goers 1993, Academic Press) or various purification protocols. The inhibitors may have a label incorporated therein or affixed thereto to facilitate ready identification or measurement of the activities associated therewith. Various types of detectable labels (e.g., enzymes, dyes, or other suitable molecules

that are either readily detectable or cause some activity/result that is readily detectable) that can be useful in such protocols are known in the art.

Formulation

In some embodiments, treatment includes administering a pharmaceutical PCSK9 inhibitor composition to a patient, the composition comprising the PCSK9 inhibitor composition and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition is an oral dosage form. In some embodiments, the pharmaceutical compositions comprise, as the active ingredient, a PCSK9 inhibitor, e.g., PCSK9 antibody, in combination with one or more pharmaceutically acceptable carriers (excipients). In making the compositions of the invention, the agent is typically mixed with an excipient, diluted by an excipient or enclosed within such a carrier in the form of, for example, a capsule, sachet, paper, or other container. When the excipient serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the formulations can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

In preparing a formulation, the PCSK9 inhibitor composition can be milled to provide the appropriate particle size prior to combining with the other ingredients. If the PCSK9 inhibitor composition is substantially insoluble, it can be milled to a particle size of less than 200 mesh. If the agent is substantially water soluble, the particle size can be adjusted by milling to provide a substantially uniform distribution in the formulation, e.g. about 40 mesh.

Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring agents. The compositions can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

A PCSK9 inhibitor composition can be formulated in a unit dosage form, each dosage containing from about 5 to about 1000 mg (1 g), more usually about 100 to about 500 mg, of the agent. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

A PCSK9 inhibitor composition can be effective over a wide dosage range and is generally administered in a pharmaceutically effective amount. It will be understood by those in the art that the amount of the agent actually administered is typically determined by a physician, according to the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the like.

For preparing a solid PCSK9 inhibitor formulation such as a tablet, the PCSK9 inhibitor composition is mixed with a pharmaceutical excipient to form a solid composition containing a homogeneous mixture of a PCSK9 inhibitor composition. Such formulations are typically provided in unit dosage forms, for example, about 0.1 to about 1000 mg of the PCSK9 inhibitor composition.

A tablet or pill comprising a PCSK9 inhibitor composition can be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

A liquid formulation comprising a PCSK9 inhibitor composition can be prepared for oral delivery or for injection, for example, in an aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, or flavored emulsions with edible oils such as cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

Formulations for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described supra. In some embodiments, the formulations are administered by the oral or nasal respiratory route for local or systemic effect. Solution, suspension, or powder formulations can be administered orally or nasally from devices that deliver the formulation in an appropriate manner.

The amount and frequency of a PCSK9 inhibitor composition administered to a patient will vary depending upon what is being administered, the purpose of the administration, such as prophylaxis or therapy, the state of the patient, the manner of administration, and the like. In therapeutic applications, compositions can be administered to a patient already suffering from a disease in an amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. Effective doses will depend on the disease condition being treated as well as by the judgment of the attending clinician depending upon factors such as the severity of the disease, the age, weight and general condition of the patient, and the like.

The therapeutic dosage of a PCSK9 inhibitor composition agents can vary according to, for example, the particular use for which the treatment is made, the manner of administration of the compound, the health and condition of the patient, and the judgment of the prescribing physician. The proportion or concentration of an agent in a pharmaceutical composition can vary depending upon a number of factors including dosage, chemical characteristics (e.g., hydrophobicity), and the route of administration. For example, the agents can be provided in an aqueous physiological buffer solution containing about 0.1 to about 10% w/v of the compound for parenteral administration. Some typical dose ranges are from about 1 μg/kg to about 1 g/kg of body weight per day. In some embodiments, the dose range is from about 0.01 mg/kg to about 100 mg/kg of body weight per day. The dosage is likely to depend on such variables as the type and extent of progression of the disease or disorder, the overall health status of the particular patient, the relative biological efficacy of the compound selected, formulation of the excipient, and its route of administration. Effective doses can be extrapolated from dose-response curves derived from in vitro or animal model test systems.

Summary of Sequences

SEQ ID NO	Description	Sequence
1	Human PCSK9 (aa)	1 mgtvssrrsw wplpllllll lllgpagara qededgdyee lvlalrseed glaeapehgt 61 tatfhrcakd pwrlpgtyvv vlkeethlsq sertarrlqa qaarrgyltk ilhvfhgllp 121 gflvkmsgdl lelalklphv dyieedssvf aqsipwnler itppryrade yqppdggslv 181 evylldtsiq sdhreiegrv mvtdfenvpe edgtrfhrqa skcdshgthl agvvsgrdag 241 vakgasmrsl rvlncqgkgt vsgtliglef irksqlvqpv gplvvllpla ggysrvlnaa 301 cqrlaragvv lvtaagnfrd daclyspasa pevitvgatn aqdqpvtlgt lgtnfgrcvd 361 lfapgediig assdcstcfv sqsgtsqaaa hvagiaamml saepeltlae lrqrlihfsa 421 kdvineawfp edqrvltpnl vaalppsthg agwqlfcrtv wsahsgptrm atavarcapd 481 eellscssfs rsgkrrgerm eaqggklvcr ahnafggegv yaiarccllp qancsvhtap 541 paeasmgtrv hchqqghvlt gcsshweved lgthkppvlr prgqpnqcvg hreasihasc 601 chapgleckv kehgipapqe qvtvaceegw tltgcsalpg tshvlgayav dntcvvrsrd 661 vsttgstseg avtavaiccr srhlaqasqe lq
2	2C6: HC CDR1	GYTFTDYYMN
3	2C6: HC CDR2	DINPNNGGPS
4	2C6: HC CDR3	GGPIYYGNSAWFAY
5	2C6: LC CDR1	SVSSSISSSNLH
6	2C6: LC CDR2	GTSNLAS
7	2C6: LC CDR3	QQWSGYPLT
8	6H7: HC CDR1	GYTFTSYWIT
9	6H7: HC CDR2	DIYPGSGSTD

10	6H7: HC CDR3	WAYGEDY
11	6H7: LC CDR1	KASQNVGTNVA
12	6H7: LC CDR2	SASYRYS
13	6H7: LC CDR3	QQYNSYPYT
14	7F8: HC CDR1	GYTFTTYPID
15	7F8: HC CDR2	NFHPYNDDTN
16	7F8: HC CDR3	RGELAWFAY
17	7F8: LC CDR1	RTSENVYSYLT
18	7F8: LC CDR2	NAKTLAE
19	7F8: LC CDR3	QHHYGTPYT
20	Residues 61-152 of hPCSK9	tatfhrcakd pwrlpgtyvv vlkeethlsq sertarrlqa qaarrgyltk ilhvfhgllp gflvkmsgdl lelalklphv dyieedssvf aq
21	Residues 31-151 of hPCSK9	qededgdyee lvlalrseed glaeapehgt tatfhrcakd pwrlpgtyvv vlkeethlsq sertarrlqa qaarrgyltk ilhvfhgllp gflvkmsgdl lelalklphv dyieedssvf a
22	Residues 153-447 of hPCSK9	sipwnler itppryrade yqppdggslv evylldtsiq sdhreiegrv mvtdfenvpe edgtrfhrqa skcdshgthl agvvsgrdag vakgasmrsl rvlncqgkgt vsgtliglef irksqlvqpv gplvvllpla ggysrvlnaa cqrlaragvv lvtaagnfrd daclyspasa pevitvgatn aqdqpvtlgt lgtnfgrcvd lfapgediig assdcstcfv sqsgtsqaaa hvagiaamml saepeltlae lrqrlihfsa kdvineawfp edqrvltpnl vaalpps
23	Residues 453-662 of hPCSK9	wqlfcrtv wsahsgptrm atavarcapd eellscssfs rsgkrrgerm eaqggklvcr ahnafggegv yaiarccllp qancsvhtap paeasmgtrv hchqqghvlt gcsshweved lgthkppvlr prgqpnqcvg hreasihasc chapgleckv kehgipapqe qvtvaceegw tltgcsalpg tshvlgayav dntcvvrsrd vs

24	Residues 31-152 of hPCSK9	qededgdyee lvlalrseed glaeapehgt tatfhrcakd pwrlpgtyvv vlkeethlsq sertarrlqa qaarrgyltk ilhvfhgllp gflvkmsgdl lelalklphv dyieedssvf aq

HC=heavy chain; LC=light chain

EQUIVALENTS

All technical features can be individually combined in all possible combinations of such features.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein.

EXAMPLES

The following non-limiting examples further illustrate embodiments of the inventions described herein.

Example 1: Identification of PCSK9 antibodies

To develop antibodies that specifically bind to PCSK9, mice were immunized with a human PCSK9. Hybridoma supernatants were initially screened for binding to human, murine, and rhesus monkey PCSK9.

In binding experiments, an anti-mouse IgG was immobilized on a plate surface. PCSK9 antibody was then bound to the anti-mouse capture antibody, the plate was washed, and labeled human PCSK9 added, incubated, then washed and bound PCSK9 was detected. Clones binding to all three species were preferred, but at a minimum selected clones bound to human and rhesus PCSK9.

All supernatants were also initially screened for pH sensitive binding by ELISA. These experiments were carried out as above for binding to human PCSK9 but were done at both pH 7.4 and pH 5.5. Clones that were promising based on this initial screen were examined in more detail to obtain affinities at both high and low pH. Table 3 illustrates the results of this

experiment, which demonstrated that selected antibodies had differential binding at pH 7.4 and pH 5.5.

Table 3

Antibody	2C10	6E1	6H7
K_d at pH7.4	3.2 nM	8.4 nM	1.3 nM
K_d at pH 5.5	281 nM	578 nM	165 nM
Fold change	88X	69X	127X

These data demonstrate that PCSK9 antibodies that differentially bind to PCSK9 can be generated.

Hybridoma cultures for selected antibodies demonstrating characteristics of interest from initial screening, i.e. binding to at least human and rhesus (and in some cases mouse) PCSK9, and/or differential binding to PCSK9 at pH 7.4 and pH 5.5 were cultured for six days. Conditioned medium was purified over protein A/G columns according to the manufacturer's instructions and the antibodies further characterized.

Example 2: LDL uptake assay screen

Twenty six purified candidate antibodies prepared as described in Example 1 were used in an LDL uptake assay screen. Briefly, human PCSK9 at 3 ug/mL was incubated with a purified candidate antibody at 50 ug/mL for 30 minutes then added to HepG2 cells in 96 well platesalong with fluorescently labeled LDL (FL-LDL). After six hours, plates were washed with PBS and fluorescence assayed to determine relative LDL uptake by cells. Controls in these experiments included cells incubated with FL-LDL and PCSK9, cultures incubated only with FL-LDL, cultures incubated with FL-LDL and an antibody without PCSK9, and cultures with no additions. In these experiments, relative fluorescence for five of the candidate antibodies was greater than a control containing PCSK9 and FL-LDL but no antibody. Of these five, two antibodies resulted in complete restoration of fluorescence to the same level as the control containing FL-LDL without PCSK9. One of these antibodies, 6H7, had been shown to exhibit pH sensitive binding.

These data demonstrate that selected antibodies were effective for increasing LDL uptake by cells. Of these antibodies, only 6H7 was from the pH selected group.

Example 3: Subcloning and Characterization

Five clones expressing candidate antibodies were selected for subcloning, four of which appeared to at least partially inhibit PCSK9 based on initial screening, and one additional clone that was pH sensitive.

To further identify candidate antibodies, the LDL uptake assay (described *supra)* was repeated with the purified antibodies isolated from the clones using a range of antibody concentrations. Three of the clones increased uptake of LDL in a dose-dependent fashion. These clones are identified as 2C6, 6H7, and 7F8. Their rank order of percent inhibition was 2C6>7F8>6H7. The rank order for IC_{50} was 7F8<2C6<6H7. The amino acid sequence for the CDRs of each of these is provided in Table 1 *(supra).*

These data further demonstrate a method of identifying inhibitors of PCSK9 and provide novel antibodies that can bind to PCSK9.

Epitope mapping

To further characterize candidate antibodies, epitope mapping was performed. PCSK9 can be divided into three domains, the prodomain, catalytic domain, and C-terminal domain. To epitope map the binding sites of candidate antibodies, PCSK9 containing point mutations and a histidine tag (His tag) were generated and expressed in HEK293 cells, purified via their His tag. Candidate antibodies 2C6, 6H7, and 7F8 monoclonal antibodies were then tested for their ability to bind the mutated PCSK9 compared to binding to a wild type molecule.

Epitope mapping of 7F8 revealed binding to the prodomain of human PCSK9 at S127 and D129. Epitope mapping of 2C6 revealed binding at D192, R194, D238, D374, S376 in the catalytic domain of human PCSK9. Epitope mapping of 6H7 revealed binding within residues 453 to 662, inclusive, a C terminal domain. These data demonstrate that antibodies can be generated to each of the three domains of PCSK9 and demonstrates specific epitope sites. In particular, these data demonstrate that antibodies can be generated to the prodomain of PCSK9 and provides specific antibodies that bind to the prodomain of PCSK9.

Example 4: Chimeric PCSK9 antibodies

To produce antibodies that can be used in humans, it is necessary to humanize the PCSK9 antibodies. Accordingly, the variable domains of the murine 2C6, 6H7, and 7F8 antibodies were sequenced. The variable domains were then cloned into human IgG1 constant regions in pTT5 vectors to yield human chimeric antibodies. The cloned sequences were expressed in HEK293 6E cells followed by protein A purification and characterization by ELISA. Table 4 illustrates binding data (K_D) for these chimeric (ch) candidate antibodies.

Table 4

Clone	Human (nM)	Mouse (nM)	Rhesus (nM)
ch2C6	1.2	~1.0	6.7
ch6H7	1.3	Below detection	4.8
ch7F8	0.2	Below detection	1.5

All three clones were found to bind to human and rhesus PCSK9, but ch6H7 and ch7F8 demonstrated little or no binding to murine PCSK9.

All three clones were found to inhibit PCSK9 function in the cell-based assay of LDL uptake in HepG2 cells. In general, these chimeric antibodies potently inhibited PCSK9 and restored 90-100% of LDL uptake in the cell-based assay. Table 5 illustrates these results.

Table 5

Clone	Inhibition (%)	IC_{50} (μg/ml)	IC_{50} (nM)
ch2C6	100	2.5	16.7
ch6H7	93	2.6	17.3
ch7F8	100	1.1	7.3

Example 5: *In vivo* inhibition of PCSK9

To test the ability of PCSK9 antibodies to influence LDL levels, experiments were designed using cynomolgus monkeys and carried out by the Sinclair Research Center. In these experiments, six healthy male cynomolgus macaque monkeys (divided into three groups of two

monkeys each) received a single bolus intravenous injection of 3 mg/kg chimeric antibody, either ch2C6 (catalytic domain targeted), ch6H7 (C terminal domain targeted) or ch7F8 (prodomain targeted). Blood samples were collected immediately pre-injection (day 0), and then at the following time points post-injection: 15 minutes, 24 hours, days 4, 7, 10, 14, 17, 21 and 30. Monkeys were fasted overnight before each blood collection. For each time point, freshly collected serum was aliquoted and analyzed for LDL cholesterol on a Beckman Coulter AU480 Chemistry System and the remainder of the serum was frozen at -70°C for subsequent analysis.

In all three groups, LDL-c levels decreased at one day following injection (Fig. 1). At later time points, efficacy of the three antibodies was varied. Monkeys dosed with ch7F8 or ch2C6 continued to exhibit reduced LDL-c throughout the time course, returning to approximately baseline (pre-injection) levels by day 30. The ch6H7 group showed a large rebound in LDL-c at day 4, remained higher than baseline through day 14 and then began decreasing toward pre-injection levels at about day 17.

These data demonstrate the novel finding of LDL-c reduction *in vivo* by a PCSK9 antibody targeted to the PCSK9 prodomain.

PK Assay

Serum samples from all three groups of monkeys were analyzed for antibody PK using the Human Therapeutic IgG1 EIA kit (Cayman Chemical, Ann Arbor, MI). In these analyses, microplate wells were pre-coated with an anti-human IgG capture antibody, and captured IgG is detected with a separate anti-human IgG-HRP detection antibody. Standard curves for each group were generated using the respective chimeric antibody diluted in 5% monkey serum (Bioreclamation Inc., Westbury, NY) at concentrations between 6.25 ng/ml and 400 ng/mL. Test samples were also diluted to appropriate concentrations (between 20- and 200-fold) in 5% monkey serum and were run in duplicate.

All three PCSK9 chimeric antibodies exhibited an average terminal half-life of approximately eight days (Fig. 2).

PCSK9 Assay

Total PCSK9 levels

For all three groups of monkeys, the total amount (i.e., both freely circulating and antibody-bound) of cynomolgus PCSK9 was measured in serum samples using a Human PCSK9 Quantikine ELISA kit (R&D Systems, Minneapolis, MN). The kit utilizes a microplate coated with a monoclonal anti-human PCSK9 antibody, and a polyclonal anti-human PCSK9-HRP detection antibody. It was verified both that the assay cross-reacted with monkey PCSK9, and that the chimeric antibodies did not interfere with detection of PCSK9. Rhesus PCSK9 was used between 0.625 and 40 ng/mL to generate a standard curve. Test samples were diluted 100-fold in the assay and run in duplicate.

For all three groups of monkeys, the total PCSK9 levels transiently rose following antibody injection, peaking at a 200%-500% increase from pre-injection levels, likely due to antibody-mediated extended PCSK9 half-life. PCSK9 levels began returning to baseline levels at approximately 10 days after injection (Fig. 3).

Free (unbound) PCSK9 levels

For the ch7F8 group only, freely circulating PCSK9 levels were quantified using a sandwich ELISA. Plates were coated with ch7F8 as a capture antibody then blocked with PBS + 2% BSA. Standards (rhesus PCSK9 at 0.5 ng/ml to 400 ng/mL) or samples (serum samples at a 5-fold dilution) were prepared in PBS + 2% BSA + 0.1% Tween-20 and incubated in the plate for 1 hour. Murine anti-PCSK9 antibody that does not compete with ch7F8 was then added to the wells and incubated for 1 hour. Finally, an anti-mouse IgG-HRP antibody was used for detection. Samples were run in duplicate.

Analysis of both monkeys showed that within 15 minutes following antibody injection, all PCSK9 was antibody-bound, leaving no free circulating PCSK9 in serum (Fig. 4). In both monkeys, a return to baseline levels was observed over the course of the experiment. The trend of these data is similar to that observed for LDL-c levels in this group – a sharp initial decrease followed by a slow, steady return to baseline.

Immunogenicity

For all three groups, the immunogenicity of dosed chimeric antibody was measured by ELISA. Two assays were developed, one to measure IgG response and one to measure IgM. For the IgM ELISA, plates were coated with the dosed anti-PCSK9 chimera and blocked. Serum

from various time points was titrated at 10-fold dilutions and incubated. Plates were washed, then any bound monkey antibodies were detected using an anti-monkey IgM-HRP antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The IgG assay was carried out similarly, except that plates were coated with the purified murine version of the dosed antibody, and the detection antibody was an anti-monkey IgG-HRP (AbD Serotec, Raleigh, NC).

The IgG assay showed that two monkeys developed antibodies to the chimera, one in the 2C6 group and one in the 6H7 group. The data for fold increase in IgG titer compared to day 0 are shown in Table 6.

No change in IgM titer was observed in any monkeys over the course of the experiment.

Table 6

Monkey		Day 0	Day 7	Day 14	Day 25	Day 30
ch2C6	1	1	5	50	100	300
	2	1	1	1	1	1
ch6H7	1	1	1	1	1	1
	2	1	10	20	20	20
ch7F8	1	1	1	1	1	1
	2	1	1	1	1	1

Other embodiments are within the scope of the following claims.

What is claimed is:

1. An antibody, antibody fragment, or derivative thereof that can specifically bind to the prodomain of a human PCSK9.

2. The antibody of claim 1, wherein the antibody can inhibit an activity of a PCSK9.

3. The antibody, antibody fragment, or derivative thereof of claim 1, wherein the antibody exhibits pH-dependent binding to PCSK9.

4. The antibody, fragment, or derivative thereof of claim 1 comprising at least one of SEQ ID NO:14, 15, or 16 and at least one of SEQ ID NO:17, 18, or 19.

5. The antibody or fragment thereof of claim 1, comprising sequences that are at least 90% identical to SEQ ID NO:14, 15, or 16 and at least 80% identical to at least one of SEQ ID NO:17, 18, or 19.

6. The antibody, fragment, or derivative thereof of claim 1, wherein the antibody, fragment, or derivative thereof can specifically bind to S127, D129, or S127 and D129 of SEQ ID NO:1.

7. The antibody, fragment, or derivative thereof of claim 1, wherein the antibody, fragment, or derivative thereof binds to PCSK9 with a K_d equal to or less than 8.4 nM, 3.2 nM, or 1.3 nM at pH 7.4 in a cell-based assay.

8. The antibody, fragment, or derivative thereof of claim 7, wherein the antibody, fragment, or derivative thereof binds to PCSK9 with a K_d equal to or greater than 165 nM, 281 nM, or 578 nM at pH 5.5 in a cell-based assay.

9. A method of inhibiting PCSK9, the method comprising contacting a sample comprising a PCSK9 with a PCSK9 antibody, fragment, or derivative thereof that can specifically bind to the prodomain of human PCSK9.

10. A method of detecting a PCSK9 polypeptide in a sample, the method comprising

a. providing a sample,

b. contacting the sample with a PCSK9 antibody, fragment, or derivative thereof of claim 1 under conditions suitable for specific binding of a PCSK9 polypeptide, and

c. detecting a complex comprising the PCSK9 antibody.

11. A method of treating a subject in need thereof, the method comprising

a. identifying a subject in need of treatment with a PCSK9 inhibitor; and

b. administering a therapeutically effective amount of a PCSK9 antibody, fragment, or derivative thereof to the subject, wherein the PCSK9 antibody can specifically bind to the prodomain of human PCSK9.

12. The method of claim 11, wherein the PCSK9 antibody, fragment, or derivative thereof exhibits pH-dependent binding to a PCSK9.

13. The method of claim 11, further comprising testing the subject's plasma for free PCSK9 before and after treatment, wherein the subject has decreased free PCSK9 in their serum after treatment with the PCSK9 inhibitor compared to the level of free PCSK9 after treatment.

14. The method of claim 11, further comprising testing the subject's plasma for free PCSK9 after treatment and comparing it to a reference.

15. The method of claim 11, wherein the subject is at risk for or has been diagnosed with a cholesterol-related disorder.

16. The method of claim 11, wherein the subject is at risk for or has been diagnosed with hypercholesterolemia, heart disease, metabolic syndrome, diabetes, coronary heart disease,

stroke, cardiovascular diseases, Alzheimer's disease, atherosclerosis, or a dyslipidemia, e.g., elevated total serum cholesterol, elevated LDL, elevated triglycerides, elevated very low density lipoprotein (VLDL), and/or low HDL.

17. The method of claim 11, further comprising treating the subject with a statin.

18. A vector comprising a nucleic acid sequence encoding an antibody, fragment, or derivative thereof of claim 1.

19. A host cell comprising a nucleic acid sequence encoding an antibody, fragment, or derivative thereof of claim 1.

20. A pharmaceutical composition comprising the antibody, fragment, or derivative thereof of claim 1 and a pharmaceutically acceptable excipient.

21. A kit comprising an antibody, fragment, or derivative thereof of claim 1 and instructions for use of the antibody, fragment, or derivative thereof.

Fig. 1

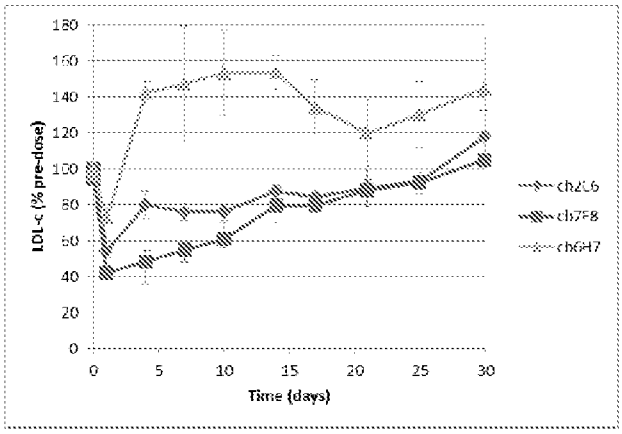


Fig. 2

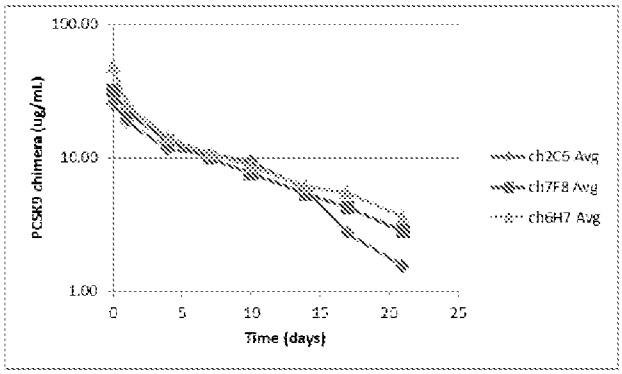


Fig. 3

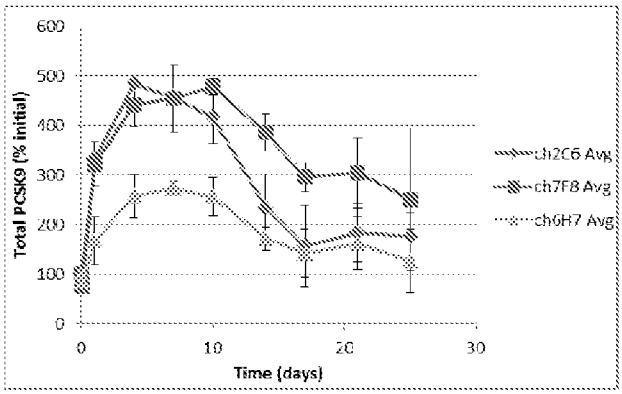
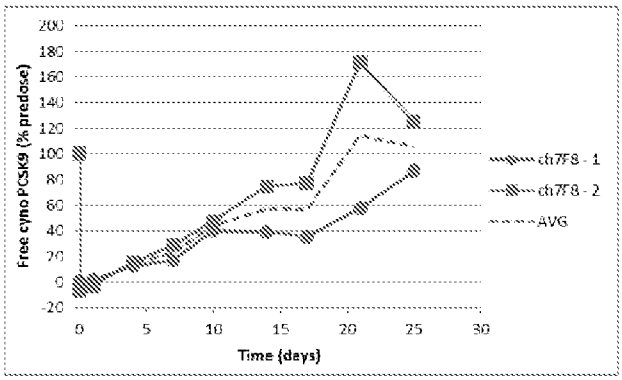


Fig. 4


Free cyno PCSK9 (% predose)
200
180
160
140
120
100
80
60
40
20
0
-20
0
5
10
15
20
25
30
Time (days)
ch7F8 - 1
ch7F8 - 2
AVG

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/010536

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/40 A61K39/395
ADD.

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/166768 A1 (SLEEMAN MARK W [US] ET AL) 1 July 2010 (2010-07-01)	1-5,7-21
Y	the whole document in particular paragraphs [0006]-[0018], [0029], [0031], [0043], [0044] examples ----- -/--	6

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 May 2014	17/06/2014

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2014/010536

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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