ENDO180-TARGETED PARTICLES FOR SELECTIVE DELIVERY OF THERAPEUTIC AND DIAGNOSTIC AGENTS

~~RELATED APPLICATIONS~~

~~[0001] This application claims the benefit of U.S. Provisional Application Serial No. 61/582373 filed January 1, 2012 entitled "ENDO180-Targeted Particles for Selective Delivery of Therapeutic and Diagnostic Agents" and incorporated herein by reference in its entirety and for all purposes.~~

SEQUENCE LISTING

[00021] This application incorporates-by-reference nucleotide and/or amino acid sequences which are present in the file named "230-PCTI_SEQLISTI G.ST25.txt", which is 33 kilobytes in size, and which was created December 31 2012 in the IBM-PCT machine format, having an operating system compatibility with MS-Windows.

FIELD OF THE INVENTION

[00032] Disclosed herein are compositions comprising carrier moieties (such as lipid based particles), and anti-ENDO180 targeting moieties (such as anti-ENDO180 antibodies) and to methods of using the same for delivery of therapeutic and/or diagnostic agents to cells and tissue expressing ENDO180, including tumor cells, macrophages, endothelial cells and fibrotic cells.

[0003] The compositions and methods are useful for treating cell proliferative diseases, or disorders including fibrosis, cancer, or inflammation, and for controlling (modulating) tumor progression.

BACKGROUND OF THE INVENTION

[0004] The ENDO180 Receptor, also known as CD280, uPARAP (urokinase plasminogen activator receptor associated protein) and mannose receptor C type 2 (MRC2), is a recycling endocytic receptor that directs bound ligands to degradation in the endosomes. It is part of a triple complex with urokinase type plasmin activator (uPA) and urokinase-type plasmin activator receptor (uPAR), and is involved in the production of plasmin from plasminogen. Plasmin, in turn, is known to play a role in both extracellular matrix (ECM) turnover and proteolytic conversion of latent TGF-beta into its active form.

[0005] ENDO180 shares homology with the macrophage mannose receptor family: mannose receptor, phospholipase A2 and DEC-205/MR6 (Isacke et al., 1990 Mol. Cell. Biol. 10:2606

various preferred embodiments the therapeutic agent is a double stranded oligonucleotide and preferably siRNA. In some embodiments a chemically modified siRNA molecule is preferred.

[00100] The selection and synthesis of siRNA corresponding to known genes has been widely reported; (see for example Ui-Tei et al., 2006. J Biomed Biotechnol.; 2006:65052; Chalk et al., 2004. BBRC. 319(1): 264-74; Sioud & Leirdal, 2004. Met. Mol Biol.; 252:457-69; Levenkova et al., 2004, Bioinform. 20(3):430-2; Ui-Tei et al., 2004. NAR 32(3):936-48).

[00101] For examples of the use of, and production of, modified siRNA see for example Braasch et al., 2003. Biochem., 42(26):7967-75; Chiu et al., 2003, RNA, 9(9):1034-48; PCT publications WO 2004/015107 (atugen AG) and WO 02/44321 (Tuschl et al). US Patent Nos. 5,898,031 and 6,107,094 teach chemically modified oligomers. US Patent No. 7,452,987 relates to oligomeric compounds having alternating unmodified and 2' sugar modified ribonucleotides. US patent publication No. 2005/0042647 describes dsRNA compounds having chemically modified internucleoside linkages.

[00102] Amarzguioui et al., (2003, NAR, 31(2):589-595) showed that siRNA activity depended on the positioning of the 2'-O-methyl modifications. Holen et al (2003, NAR, 31(9):2401-2407) report that an siRNA having small numbers of 2'-O-methyl modified nucleosides showed good activity compared to wild type but that the activity decreased as the numbers of 2'-O-methyl modified nucleosides was increased. Chiu and Rana (2003, RNA, 9:1034-1048) teach that incorporation of 2'-O-methyl modified nucleosides in the sense or antisense strand (fully modified strands) severely reduced siRNA activity relative to unmodified siRNA. The placement of a 2'-O-methyl group at the 5'-terminus on the antisense strand was reported to severely limit activity whereas placement at the 3'-terminus of the antisense and at both termini of the sense strand was tolerated (Czauderna et al., 2003, NAR, 31(11), 2705-2716).

[00103] PCT Patent Application Nos. PCT/IL2008/000248 and PCT/IL2008/001197, ~~are hereby incorporated by reference in their entirety~~ disclose motifs useful in the preparation of chemically modified siRNA compounds. PCT Patent Publication No. WO 2008/020435 discloses inhibitors, including some siRNA compounds to the target genes set forth herein.

[00104] The compound comprises at least one modified nucleotide selected from the group consisting of a sugar modification, a base modification and an internucleotide linkage modification and may contain DNA, and modified nucleotides such as LNA (locked nucleic acid), ENA (ethylene-bridged nucleic acid), PNA (peptide nucleic acid), arabinoside,

[00129] (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule which can be produced by digestion of whole antibody with the enzyme papain to yield a light chain and a portion of the heavy chain;

[00130] (2) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab'2) is a dimer of two Fab fragments held together by two disulfide bonds;

[00131] (3) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and

[00132] (4) Single chain antibody (SCA), defined as a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain linked by a suitable polypeptide linker as a genetically fused single chain molecule, including a scFv.

[00133] CDR grafting may be performed to alter certain properties of the antibody molecule including affinity or specificity. A non-limiting example of CDR grafting is disclosed in US Patent No. 5,225,539.

[00134] Single-domain antibodies are isolated from the unique heavy-chain antibodies of immunized Camelidae, including camels and llamas. The small antibodies are very robust and bind the antigen with high affinity in a monomeric state. US Patent 6838254 describes the production of antibodies or fragments thereof derived from heavy chain immunoglobulins of Camelidae.

[00135] A monoclonal antibody (mAb) is a substantially homogeneous population of antibodies to a specific antigen. Monoclonal antibodies (mAbs) are obtained by methods known to those skilled in the art. See, for example Kohler et al (1975); US patent 4,376,110; Ausubel et al (1987-1999); Harlow et al (1988); and Colligan et al (1993), ~~the contents of which are incorporated entirely herein by reference~~. The mAbs disclosed herein may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and any subclass thereof. A hybridoma producing a mAb may be cultivated in vitro or in vivo. High titers of mAbs are obtained in vivo for example wherein cells from the individual hybridomas are injected intraperitoneally into pristine-primed Balb/c mice to produce ascites fluid containing high concentrations of the desired mAbs. mAbs of isotype IgM or IgG may be purified from such ascites fluid, or from culture supernatants, using column chromatography methods well known to those of skill in the art.

immunoglobulin molecules, e.g. the human framework regions replace the non-human regions. In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions remain unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind the antigen, ENDO180.

[00142] A "humanized" antibody would retain a similar antigenic specificity as the original antibody, i.e. the ability to bind ENDO180, specifically human ENDO180 receptor and would similarly be internalized by the receptor.

[00143] One skilled in the art would know how to produce the humanized antibodies of the subject invention. Various publications, ~~several of which are hereby incorporated by reference into this application,~~ describe how to make humanized antibodies.

[00144] For example, the methods described in U.S. Patent Nos. 4,816,567 and 6,331,415 comprise the production of chimeric antibodies having a variable region of one antibody and a constant region of another antibody.

[00145] U.S. Patent No. 5,225,539; 6,548,640 and 6,982,321 describes the use of recombinant DNA technology to produce a humanized antibody wherein the CDRs of one immunoglobulin are replaced with the CDRs from an immunoglobulin with a different specificity such that the humanized antibody would recognize the target antigen but would not illicit an immune response. Specifically, site directed mutagenesis is used to introduce the CDRs onto the framework region.

[00146] Other approaches for humanizing an antibody are described in WO 90/07861 and corresponding patents including U.S. Patent Nos. 5,585,089; 5,693,761; 6,180,370 and 7,022,500. These patents describe a method to increase the affinity of an antibody for the desired antigen by combining the CDRs of a mouse monoclonal antibody with human immunoglobulin framework and constant regions. Human framework regions can be chosen to maximize homology with the mouse sequence. Computer modeling can be used to identify amino acids in the framework region which are likely to interact with the CDRs or the specific antigen and then mouse amino acids can be used at these positions to create the humanized antibody.

[00147] The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies.

[00148] The monoclonal antibody E3-8D8 represents a suitable anti-ENDO180 antibody for use in the compositions and methods disclosed herein. The hybridoma cell E3-8D8 was deposited with the Belgian Co-ordinated Collections of Micro-Organisms (BCCM), under the terms of the Budapest Treaty and given Accession Number LMBP 7203CB.

Epitope Mapping

[00149] Epitope mapping studies identify the residues that are important for antibody binding. Various methods are known in the art for epitope mapping and are readily performed by one skilled in the art. Certain methods are described in Epitope Mapping: A Practical Approach (O. M. R. Westwood, F. C. Hay; Oxford University Press, 2000), ~~incorporated herein by reference~~.

[00150] One example of an epitope mapping techniques is Synthetic Labeled Peptides Epitope Mapping whereby a set of overlapping synthetic peptides is synthesized, each corresponding to a small segment of the linear sequence of the protein antigen, i.e. extracellular domain of ENDO180, and arrayed on a solid phase. The panel of peptides is then probed with the test antibody, and bound antibody is detected using an enzyme-labeled secondary antibody.

[00151] Other techniques include fragmentation or cleavage and gel separation of the protein antigen, transfer to a membrane, probing by test antibody and bound antibody is detected using an enzyme-labeled secondary antibody.

Antibody drug development

[00152] In general monoclonal antibodies need to be designed to preserve binding properties (selectivity, internalization etc) and to reduce an immune response in the recipient. Specifically, the monoclonal antibody secreted from hybridoma 3E-8D8 may be optimized for human therapeutics by one of several methods known to those with skill in the art. In one method the variable heavy chain (V_H) and variable light chain (V_L) of the monoclonal antibody are sequenced. Once the amino acid sequence is known, the complementarity determining regions (CDR), heavy chain and light chain CDR3 are identified and degenerate oligonucleotides are used to clone synthetic CDR3 into a vector to produce a recombinant vector or construct. The construct may be for example a Fab fragment, a F(ab')2 fragment, a Fv fragment, a single chain fragment or a full IgG molecule. The construct(s) is expressed and a polypeptide is isolated. In some embodiments the monoclonal antibody may be further

are disclosed in PCT Patent Publication No. WO 2009/044392, WO 2011/066475, WO 2011/085056 ~~and are hereby incorporated by reference in its entirety~~.

[00176] The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art. The compositions disclosed herein are administered by any of the conventional routes of administration. It should be noted that the composition can be administered alone or with pharmaceutically acceptable carriers, solvents, diluents, excipients, adjuvants and vehicles. The compounds can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques. Implants of the compounds are also useful. Liquid forms may be prepared for injection, the term including subcutaneous, transdermal, intravenous, intramuscular, intrathecal, and other parental routes of administration. The liquid compositions include aqueous solutions, with and without organic cosolvents, aqueous or oil suspensions, emulsions with edible oils, as well as similar pharmaceutical vehicles.

[00177] In addition, under certain circumstances the compositions for use in the novel treatments of the present invention may be formed as aerosols, for intranasal and like administration. The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, solvents, diluents, excipients, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients disclosed herein and they include liposomes, lipidated glycosaminoglycans and microspheres. Examples of delivery systems useful in the present invention include US Patent Nos. 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

[00178] In general, the active dose of compound for humans is in the range of from 1ng/kg to about 20-100 mg/kg body weight per day, preferably about 0.01 mg to about 2-10 mg/kg body weight per day, in a regimen of one dose per day or twice or three or more times per day for a period of 1-2 weeks or longer, preferably for 24-to 48 hrs or by continuous infusion during a period of 1-2 weeks or longer.

available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

EXAMPLES

General methods in molecular biology

[00214] Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons, New York (1988), and in Watson et al., Recombinant DNA, Scientific American Books, New York and in Birren et al (eds) Genome Analysis: A Laboratory Manual Series, Vols. 1-4 Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 ~~and incorporated herein by reference~~. Polymerase chain reaction (PCR) was carried out generally as in PCR Protocols: A Guide To Methods And Applications, Academic Press, San Diego, CA (1990). In situ (In cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences (Testoni et al., 1996, Blood 87:3822.)

[00215] General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al (eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

[00216] Immunoassays: ELISA immunoassays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate, other immunoassays such as radioimmunoassays (RIA) can be used as are known to those skilled in the art. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States Patent Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor, New York, 1989.

Antibody Production

[00217] By the term "antibody" as used herein is meant both polyclonal and monoclonal complete antibodies as well as fragments thereof, such as Fab, F(ab')2, scFv and Fv, which are

Example 1: Anti-ENDO180 antibodies

[00229] ENDO180 is also known as the C-type mannose receptor 2 precursor.The polynucleotide sequence of human ENDO180 mRNA is set forth in accession number NM_006039.3: 5983 bases, of that the open reading frame (ORF) is 4439 bases (from 117-4441); the polypeptide sequence of 1479 amino acids (aa) is set forth in accession number NP_006030 with gene identifier number: GI:110624774. The mouse mRNA sequence is 5818 bases, accession number MMU56734 with ORF of 1479 aa.

[00230] ENDO180 comprises several protein domains, as follows: 1-31 aa SP (signal peptide); 41-161 aa cysteine rich N-terminal domain; 180-228 aa FNII (fibronectin type II) domain; 8 CDR (carbohydrate recognition domain) domains 1CRD-8CRD (235-360 aa 1CRD, 382-505 aa 2CRD, 521-645 aa 3CRD, 669-809 aa 4CRD, 825-951 aa 5CRD, 972-1108 aa 6CRD, 1161-1244 aa 7CRD, 1261-1394 aa 8CRD); 1413-1435 aa 1 TM (transmembrane domain), 1437-1479 aa-cytoplasmic domain. In some embodiments the ENDO180 polypeptide is substantially identical to an amino acid sequence set forth in SEQ ID NO:2, (NCBI identifier: gi|110624774|ref|NP_006030.2|) encoded by a polynucleotide substantially identical to a nucleic acid sequence set forth in SEQ ID NO:1 (NCBI identifier: gi|110624773|ref|NM_006039.3|).

[00231] Provided below are polynucleotide and amino acid sequences disclosed herein: polynucleotide sequence of extracellular domain of human ENDO180 (amino acids 1-522) with FLAG sequence, (pcDNA3-5'hENDO180-FLAG construct, SEQ ID NO:3); polypeptide sequence of SEQ ID NO:3 (SEQ ID NO:4); polynucleotide sequence of scFv clone G7V (SEQ ID NO:5); polypeptide sequence of scFv clone G7V (SEQ ID NO:6; also known as minibody or "MB"); heavy chain CDR3 of G7V (SEQ ID NO:7); light chain CDR3 of G7V (SEQ ID NO:8).

[00232] The antibody produced from hybridoma cell line E3-8D8 (also known herein as 8D8 or e3b3 or 8d8e3b3; deposited in BCCM under Accession Number LMBP 7203CB) and the recombinant anti-ENDO180 antibodies disclosed herein are described in PCT patent publication WO2010/111198, ~~hereby incorporated by reference in its entirety~~. In some embodiments the preferred ENDO180 targeting agent is selected from

a. an isolated monoclonal antibody or an antigen-binding fragment thereof, produced by the hybridoma cell line E3-8D8 deposited with the BCCM Accession Number under LMBP 7203CB;

383(2):185-90; Peer and Margalit, 2004, Neoplasia 6(4):343-53; Peer et al., 2008, Science 319(5863):627-630). The lipid film was hydrated with doxorubicin resuspended in saline at pH of 7.4 to create MLV. Lipid mass was measured as previously described (Peer et al, 2008). Resulting MLV were extruded into small unilamellar nano-scale vesicles (SUV) with a Thermobarrel Lipex extruder (Lipex Biomembranes Inc., Vancouver, British Columbia, Canada) at 60°C under nitrogen pressure of 300 to 550 psi. The extrusion was carried out in a stepwise manner using progressively decreasing pore-sized membranes (from 1, 0.8, 0.6, 0.4, 0.2, to 0.1 μm) (Nucleopore, Whatman), with 10 cycles per pore-size.

Surface modification and purification of anti-ENDO180-coated lipid-nanoparticles and isotype control particles

[00244] Anti-ENDO180 (clone 8D8) or Isotype control (non-binding mouse IgG2a) antibodies were concentrated using Amicon Tube (MW cut off of 100KDa) to a final concentration of 10mg/mL as determine by IgG absorbance at 280nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific).

[00245] Covalent association of antibody to lipid was performed with EDC-NHS crosslinkers. at room temperature overnight in PBS in 1mL reaction vials that include 50μL of mAb (10mg/mL) and lipid particle at 10mg/mL at 950μL.

[00246] Purification of excessive 8D8 mAb was made using a Sepharose CL-4B column equilibrated with HEPES buffer-saline at pH 7.4.

[00247] Doxorubicin (DOX) was quantified by fluorescence with a calibration curve freshly made for each experiment.

Composition 2. Lipid-based nanoparticle preparation –hyaluronan spacer coated with anti-ENDO180 antibody and carrying labeled siRNA

[00248] Multilamellar vesicles (MLV) comprising Dioleoyl Phosphatidylethanolamine (DOPE), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) and cholesterol (Chol) all from Avanti Polar Lipids, Inc., (Alabaster, AL, USA) at molar ratios of about 4:2:1 (DOPE:DOTMA:Chol), were prepared by a lipid-film method (Peer and Margalit 2004). The lipid film was hydrated with Cy3-labeled siRNA suspended in DEPC-water to create MLV.

[00249] Effcacy of siRNA encapsulation: Cargo loading was performed accoring to methods disclosed in Landesman-Milo, et al., (2012, Cancer Lett. pii: S0304-3835 (12)00512-5), ~~incorporated herein by reference in its entirety~~. Briefly, siRNA encapsulation efficiency was determined by the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen) and was performed by