MELANOCORTIN RECEPTOR BINDING MIMETIBODIES, COMPOSITIONS, METHODS AND USES

Field of the Invention

The present invention relates to melanocortin receptor binding mimetibodies, polynucleotides encoding these, cells comprising the polynucleotides or expressing the mimetibodies, and methods of making and using the foregoing.

Background of the Invention

Obesity is a chronic disease manifested by an excess of fat mass in proportion to body size. Today, every third American is considered over-weight (Body Mass Index (BMI) >25 kg/m^2), thus prompting the United States Centers for Disease Control and Prevention (CDC) to declare that obesity is reaching epidemic proportions (Cummings and Schwartz, *Annu. Rev. Med.* 54:453-471((2003)). The importance of treating obesity is emphasized by the fact that this disease is either the underlying cause, or a risk factor, for developing diseases such as Type 2 Diabetes, congestive heart failure, osteoarthritis and sleep apnea among others.

Additionally, obesity is linked to "Metabolic Syndrome" which is a medical condition characterized by obesity, atherogenic dyslipidemia, elevated blood pressure and insulin resistance. Metabolic Syndrome affects an increasing number of people in the United States. Importantly, it has been shown that even a modest decrease in body weight (5-10% of initial body weight) may significantly improve Metabolic Syndrome conditions and decrease the risk factors for developing obesity-associated disease (Wing *et al.*, *Arch. Intern. Med.* 147:1749-1753 (1987); Tuomilehto *et al.*, *New Engl. J. Med.* 344:1343-1350 (2001); Knowler *et al.*, *New Engl. J Med.* 346:393-403 (2002); Franz *et al.*, *Diabetes Care* 25:148-198 (2002)). Additionally, treatment of obesity may be important from a mental health perspective due to the social stigma often attached to obese individuals in some cultures.

Melanocortin receptors play a major role in the regulation of overall energy balance and obesity in both humans and rodents. Alpha-melanocyte stimulating hormone (alpha-MSH) is a 13 amino acid peptide hormone that is an important component of the melanocortin system. Alpha-MSH is produced by the proteolytic processing of proopiomelanocortin (POMC) released by the pituitary gland. Alpha-MSH binds with high affinity to the melanocortin 4 receptor (MC4R), but also binds melanocortin receptor 3 (MC3R) and melanocortin receptor 5 (MC5R) with lower affinity. MC4R is a G-coupled protein receptor found in the brain which, when stimulated by alpha-MSH binding, causes decreased food intake and increased fat oxidation. Ultimately, stimulation of melanocortin receptors such as MC4R results in weight loss.

In humans and rodents, loss of function mutations in the different components of the melanocortin system are closely correlated with obesity and related conditions. In mice, mutations within POMC, or MC4R and MC3R produce obesity, insulin resistance and hyperphagia (Goodfellow and Saunders, *Curr. Topics Med. Chem. 3*: 855-883 (2003); Huszar *et al.*, *Cell 88*:131-141 (1997); Yaswen *et al.*, *Nat. Med. 5*: 1066-1070 (1999)). In man mutations within POMC or MC4R lead to the development of obesity associated with increased food intake (Krude *et al.*, *Nat. Genet. 19*:155-157 (1998); Yeo *et al.*, *Nature Genetics 20*:111-112 (1998); Branson *et al.*, *New Engl. J. Med. 348*: 1096-1103 (2003); Vaisse *et al.*, *J. Clin. Invest. 106*):253-262 (2000); Ho and MacKenzie, *J. Biol. Chem. 275*: 35816-35822 (1999)).

Weight loss can result from the pharmacological stimulation of melanocortin system activity. In rodents pharmacological stimulation of melanocortin receptors such as MC4R leads to decreased food intake, increased energy expenditure and weight loss (Pierroz *et al.*, *Diabetes 51*: 1337-1345 (2002)). In man the intranasal administration of alpha-MSH to stimulate MC4R in non-obese men results in decreased body weight due to the loss of fat- but not lean body mass (Fehm *et al.*, *J. Clin. Endo. Metabol. 86*: 1144-1148 (2001)).

Obesity is currently treated, with only limited success, by several different strategies. These strategies primarily involve

"life-style" changes (e.g. diet and exercise), small molecule based pharmaceutical therapies or surgical removal of a portion of the stomach (gastric by-pass surgery). Additionally, weight loss stimulating melanocortin receptor binding peptides such as alpha-MSH are of limited use as pharmaceuticals due to the extremely short serum half-life of such peptides. Thus, a need exists for additional obesity treatments and in particular for melanocortin receptor binding molecules that overcome the short serum half-life of melanocortin receptor binding peptides such as alpha-MSH.

Brief Description of the Drawings

Fig. 1 shows elements of a melanocortin receptor binding mimetibody polypeptide.

Fig 2 shows a cartoon of a melanocortin receptor binding mimetibody.

Fig. 3 shows the amino acid (SEQ ID NO: 62) and cDNA (SEQ ID NO: 61) sequences of a melanocortin receptor binding alpha-MSH mimetibody. The amino terminal portions of individual mimetibody elements are underlined.

Fig. 4 shows alpha-MSH mimetibody binding to MC4R in a competitive binding assay.

Fig. 5 shows alpha-MSH mimetibody activation of MC4R in cells expressing a high level of MC4R.

Fig. 6 shows alpha-MSH mimetibody activation of MC4R in cells expressing a low level of MC4R.

Fig. 7 shows alpha-MSH mimetibody-mediated decrease in animal food intake.

Fig. 8 shows alpha-MSH mimetibody-mediated decrease in animal body weight.

Summary of the Invention

One aspect of the invention is a polypeptide according to formula (I):

$$(Mp\text{-}Lk\text{-}V2\text{-}Hg\text{-}C_H2\text{-}C_H3)_{(t)}$$

(I)

where Mp is a melanocortin receptor binding molecule, Lk is a polypeptide or chemical linkage, V2 is a portion of a C-terminus of an immunoglobulin variable region, Hg is at least a portion of an immunoglobulin variable hinge region, C_H2 is an immunoglobulin heavy chain C_H2 constant region and C_H3 is an immunoglobulin heavy chain C_H3 constant region and t is independently an integer from 1 to 10.

Another aspect of the invention is a polypeptide comprising SEQ ID NO: 60 or 62.

Another aspect of the invention is a polynucleotide comprising SEQ ID NO: 59 or SEQ ID NO: 61 or a polynucleotide complementary to SEQ ID NO: 59 or SEQ ID NO: 61.

Another aspect of the invention is a polynucleotide comprising a polynucleotide encoding the polypeptide of SEQ ID NO: 60 or SEQ ID NO: 62.

Another aspect of the invention is a method of modifying the biological activity of a melanocortin receptor in a cell, tissue or organ, comprising contacting a mimetibody composition of the invention with the cell, tissue or organ.

Another aspect of the invention is a method of modulating at least one melanocortin receptor mediated condition comprising administering a mimetibody composition of the invention to a patient in need thereof.

Detailed Description of the Invention

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

The present invention provides polypeptides having the properties of binding a melanocortin receptor and mimicking different isotypes of antibody immunoglobulin molecules such as IgA, IgD, IgE, IgG, or IgM, and any subclass thereof, such as IgA_1, IgA_2, IgG_1, IgG_2, IgG_3 or IgG_4, or combinations thereof, herein after generally referred to as "mimetibodies." In some embodiments, the mimetibody polypeptides of the invention contain an alpha melanocyte

stimulating hormone peptide (alpha-MSH) sequence and are designated melanocortin receptor binding alpha-MSH mimetibody. Such alpha-MSH mimetibody polypeptides can bind melanocortin receptor 4 (MC4R) and, with equal and lower affinity, for MC3R and MC5R respectively. One result of such melanocortin receptor binding can be the stimulation or inhibition of melanocortin receptor activity. Stimulation can cause weight loss while inhibition may cause weight gain.

In one embodiment the polypeptides of the invention have the generic formula (I):

$$(Mp\text{-}Lk\text{-}V2\text{-}Hg\text{-}C_H2\text{-}C_H3)_{(t)} \quad (I)$$

where Mp is a melanocortin receptor binding molecule, Lk is a polypeptide or chemical linkage, V2 is a portion of a C-terminus of an immunoglobulin variable region, Hg is at least a portion of an immunoglobulin variable hinge region, C_H2 is an immunoglobulin heavy chain C_H2 constant region and C_H3 is an immunoglobulin heavy chain C_H3 constant region and t is independently an integer of 1 to 10.

As used herein, "melanocortin receptor binding molecule" means a molecule, which can bind at least one melanocortin receptor such as *Homo sapiens* MC4R (SEQ ID NO: 77). Examples of other *Homo sapiens* melanocortin receptors include MCR1 (SEQ ID NO: 71), MCR2 (SEQ ID NO: 73), MCR3 (SEQ ID NO: 75), and MCR5 (SEQ ID NO: 79). A given peptide chain is a "melanocortin receptor" if it has at least 85% amino acid sequence identity to a known melanocortin receptor sequence or the mature form of a known melanocortin receptor and can function as a G-protein coupled receptor. Percent identity between two peptide chains can be determined by pairwise alignment using the default settings of the AlignX module of Vector NTI v.9.0 .0 (Invitrogen Corp., Carslbad, CA). An exemplary melanocortin receptor binding molecule is the 13 amino acid alpha-MSH peptide having the amino acid sequence shown in (SEQ ID NO: 2). Other melanocortin receptor binding molecules include biologically active fragments of SEQ ID NO: 2 and other amino acid sequences that can bind a melanocortin receptor. The term "biologically active fragment" as used herein, refers to a portion of an alpha-MSH peptide that can bind to a melanocortin receptor such as MC4R. The peptide sequence HFRW (SEQ. ID. NO. 81) is an exemplary

"biologically active fragment" of the alpha-MSH peptide sequence SYSMEHFRWGKPV (SEQ ID NO: 2). The HFRW fragment has been incorporated into the structure of the synthetic melanocortin receptor activator molecule melanotan II (MTII) (Fan *et al., Nature 385:* 165-168 (1997)).

Incorporation of melanocortin receptor binding molecules in the mimetibody polypeptides of the invention provides for binding to melanocortin receptors with a wide range of affinities. The mimetibody polypeptides of the invention may bind a melanocortin receptor with a K_d less than or equal to about 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} or 10^{-12} M. The range of obtained IC50 values for aMSH peptide, MTII peptide and aMSHMMB were 260-400 nM, 5-30 nM and 200-300 nM respectively. The affinity of a mimetibody polypeptide for a melanocortin receptor can be determined experimentally using any suitable method. Such methods may utilize Biacore or KinExA instrumentation, ELISA or competitive binding assays. Mimetibody polypeptides binding specific melanocortin receptors with a desired affinity can be selected from libraries of variants or fragments by techniques known to those skilled in the art.

An alpha-MSH peptide having the amino acid sequence shown in SEQ ID NO: 2 may be modified to obtain other melanocortin receptor binding molecules. Such modifications may comprise the incorporation of C-$[X]_n$-C motifs into the peptide to conformationally constrain the peptide through the formation of disulfide bonds. In a C-$[X]_n$-C motif, C is a cysteine residue, X is a amino acid residues and n is an integer necessary to acheive the required conformational constraint. In this instance n can be as little as 1 residue and as high as 50. Exemplary C-$[X]_n$-C modified peptide sequences are shown in SEQ ID NOs: 4, 6, 8 and 10.

The modification may also comprise the incorporation of a Wa-$[X]_n$-Wa motif into the peptide to conformationally constrain the peptide through the formation of a tryptophan zipper. In a Wa-$[X]_n$-Wa motif W is tryptophan residue, X is an amino acid, a is an integer ususlly 2, but can be from 1 to 10, and n is an integer necessary to acheive the required conformational constraint. In this instance n can be as little a 1 residue and as high as 50. Exemplary Wa-$[X]_n$-Wa peptides are shown in SEQ ID NOs: 12, 14, 16

and 18. Further, the sequence HFRW (SEQ ID NO: 81) present in the alpha-MSH peptide may also be modified by substituting any residue in this sequence with any one of F, H, W and M; for example, HFRW (SEQ ID NO: 81) can be substituted to FHWM (SEQ ID NO: 83).

In the polypeptides of the invention, the linker portion (Lk) provides structural flexibility by allowing the mimetibody to have alternative orientations and binding properties. Exemplary linkers include non-peptide chemical linkages or one to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids or other amino acids (e.g. D-amino acids, non-naturally occurring amino acids, or rare naturally occuring amino acids). The linker portion can include a majority of amino acids that are sterically unhindered, such as glycine, alanine and serine and can include GS, poly GS (e.g. GSGS (SEQ ID NO: 20)), GGSG (SEQ ID NO: 22), GSGGGS (SEQ ID NO: 24), GSGGGSG (SEQ ID NO: 26), GSSG (SEQ ID NO: 28), or GSGGGS (SEQ ID NO: 30) or GGGS (SEQ ID NO: 85) or any combination or polymer thereof. Other exemplary linkers within the scope of the invention may be longer than 20 residues and may include residues other than glycine, alanine and serine.

In the polypeptides of the invention, V2 is a portion of a carboxy terminal domain of an immunoglobulin variable region such as a heavy chain variable region. Exemplary V2 amino acid sequences are GTLVTVSS (SEQ ID NO: 32) and TLVAVSS (SEQ ID NO: 34).

In the polypeptides of the invention, Hg is a portion of the hinge domain of an immunoglobulin variable region such as a heavy chain variable region. Exemplary Hg amino acid sequences include EPKSCDKTHTCPPCP (SEQ ID NO: 36), EPKSADKTHTCPPCP (SEQ ID NO: 38), ESKYGPPCPSCP (SEQ ID NO: 40), ESKYGPPCPPCP (SEQ ID NO: 42), CPPCP (SEQ ID NO: 44) and CPSC (SEQ ID NO: 46).

In the polypeptides of the invention, C_H2 is an immunoglobulin heavy chain C_H2 constant region. Exemplary C_H2 amino acid sequences include:

APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK (SEQ ID NO: 48),

APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK (SEQ ID NO: 50),

APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK (SEQ ID NO: 52) and APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK (SEQ ID NO: 54).

In the polypeptides of the invention, C_H3 is an immunoglobulin heavy chain C_H3 constant region. Exemplary C_H3 amino acid sequences include:

GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 56) and GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS RLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK (SEQ ID NO: 58). It will be recognized by those skilled in the art that the C_H3 region of the polypeptides of the invention may have its C-terminal amino acid cleaved off when expressed in certain recombinant systems.

In the mimetibody polypeptides of invention Hg, C_H2 or C_H3 may be of the IgG_1 or IgG_4 subclass. A sequence is of the IgG_1 or IgG_4 subclass if it is formed or developed from a γ1 or γ4 heavy chain respectively. A given peptide chain is a γ1 or γ4 heavy chain if it is at least 80% identical to a known γ1 or γ4 heavy chain sequence of a given species. Percent identity between two peptide chains can be determined by pairwise alignment using the default settings of the AlignX module of Vector NTI v.9.0.0 (Invitrogen Corp., Carlsbad, CA).

In the mimetibody polypeptides of the invention Hg, C_H2 or C_H3 may individually be of the IgG_1 or IgG_4 subclass. The mimetibodies of the invention may also comprise combinations of Hg, C_H2 or C_H3 elements from each subclass For example, Hg may be of the IgG_4 subclass while C_H2 and C_H3 are of the IgG_1 subclass. Alternatively, Hg, C_H2 and C_H3 may all of the IgG_4 or IgG_1 subclass. The polypeptide EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVE VHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTL PPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 65) is exemplary of a polypeptide in which Hg (residues 1-15 of SEQ ID NO: 65), C_H2 (residues 16-125 of SEQ ID NO: 65), and C_H3 (residues 126-232 of SEQ ID NO: 65) are all of the IgG_1 subclass.

The IgG_1 and IgG_4 subclasses differ in the number of cysteines in the hinge region. Most IgG type antibodies, such as IgG_1, are homodimeric molecules made up of two identical heavy (H) chains and two identical light (L) chains, typically abbreviated H_2L_2. Thus, these molecules are generally bivalent with respect to antigen binding due to the formation of inter-heavy chain disulfide bonds and both antigen binding (Fab) arms of the IgG molecule have identical binding specificity. IgG_4 isotype heavy chains, in contrast, contain a CPSC (SEQ ID NO: 46) motif in their hinge regions capable of forming either inter- or intra-heavy chain disulfide bonds, *i.e.*, the two Cys residues in the CPSC motif may disulfide bond with the corresponding Cys residues in the other H chain (inter) or the two Cys residues within a given CPSC motif may disulfide bond with each other (intra). Since the HL pairs in those IgG_4 molecules with intra-heavy chain bonds in the hinge region are not covalently associated with each other, they may dissociate into HL monomers that then reassociate with HL monomers derived from other IgG_4 molecules forming bispecific, heterodimeric IgG_4 molecules. *In vivo* isomerase enzymes may facilitate this process. In a bispecific IgG antibody the two Fab "arms" of the antibody molecule differ in the epitopes that they bind. Substituting Ser residues in the hinge region of IgG_4 with Pro results in "IgG_1-like behavior," *i.e.*, the molecules form stable disulfide bonds between heavy chains and therefore, are not susceptible to HL exchange with other IgG_4 molecules.

The mimetibody polypeptides of the invention may be made more IgG_4-like, or IgG_1-like by the modification of sites which are involved in disulfide bond formation and are present in the Hg-C_H2-C_H3 portion of the mimetibody polypeptides. Such sites may be modified by removal, deletion, insertion or substitution with other amino acids. Typically, the cysteine residues present in disulfide bond associated motifs are removed or substituted. Removal of these sites may avoid covalent disulfide bonding with other cysteine-containing proteins present in the mimetibody producing host cell or intra-heavy chain disulfide bonding in IgG_4-based constructs while still allowing for noncovalent dimerization of mimetibody Hg-C_H2-C_H3 domains. Modification of such sites can permit the formation of bispecific mimetibody polypeptides with two different M portions or

prevent the formation of such bispecific species.

The IgG_1 and IgG_4 subclasses also differ in their ability to mediate complement dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). CDC is the lysing of a target cell in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule complexed with a cognate antigen. IgG_1 is a strong inducer of the complement cascade and subsequent CDC activity, while IgG_4 has little complement-inducing activity. ADCC is a cell-mediated process in which nonspecific cytotoxic cells that express Fc receptors (FcRs) involved in ADCC (*e.g.*, natural killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The IgG_1 subclass binds with high affinity to Fc receptors involved in ADCC and contributes to ADCC, while IgG_4 binds only weakly to such receptors and has little ADCC inducing activity. The relative inability of IgG_4 to activate effector functions such as ADCC is desirable since delivery of the mimetibody polypeptide to cells without cell killing is possible.

The CDC and ADCC activity of the mimetibody polypeptides of the invention may be modified by altering sites involved in CDC and ADCC present in the Hg-C_H2-C_H3 portion of the mimetibody polypeptide. Such sites may be modified by removal, deletion, insertion or substitution with other amino acids. In the mimetibodies of the invention sites involved in CDC, such as the C1q binding site, are typically removed or otherwise modified to minimize CDC activity. Additionally, Fc receptor binding sites involved in ADCC can also be similarly modified in the mimetibodies of the invention. In general, such modification will remove Fc receptor binding sites involved in ADCC activity from the mimetibodies of the invention. The substitution of Leu residues with Ala residues in the C_H2 portion of the polypeptides of the invention is one example of a modification which can minimize ADCC activity in the polypeptides of the invention. The C_H2 amino acid sequence APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK (SEQ ID NO: 52) is

exemplary of such a Leu to Ala substitution at residues 4 and 5 (in sequence above). Further, the V1 domain can be removed such that the N-terminus of the peptide is free following cleavage of the signal peptide, and is accessible to and could be modified by enzymes such as acetylases.

Antibodies of both the IgG_4 and IgG_1 isotypes contain FcRn salvage receptor binding sites. The FcRn salvage receptor helps maintain IgG antibody levels in the body by recycling or transporting IgG type antibodies across enodothelial cell layers such as those lining the inside of body cavities and blood vessels. The FcRn salavage receptor does this by binding IgGs that have entered endothelial cells by nonspecific pinocytosis and preventing these IgG antibody molecules from being degraded in the lysosome of the cell. The result of such FcRn receptor activity is that the serum half-life of a molecule with an FcRn binding site is extended relative to an otherwise identical molecule lacking such a site.

It is desirable that the Hg-C_H2-C_H3 portion of the mimetibodies of the invention contain a FcRn binding site at the junction of the C_H2 and C_H3 regions. It is expected that such FcRn sites will increase the serum half-life of the mimetibodies of the invention as well as improve other pharmacokinetic properties relative to a melanocortin receptor binding molecule, such as alpha-MSH alone. In the mimetibodies of the invention FcRn sites may be modified or added by removal, deletion, insertion or substitution of amino acids. Typically, such modifications are used to improve the binding of a given site to the FcRn. One example of a human FcRn binding sites is the sequence MISRTPTVLHQHNHY (SEQ. ID. NO.: 69) found in both IgG_1 and IgG_4 antibodies. Other FcRn binding sites are well known by those skilled in the art.

Antibodies with different isotypes, such as IgG_4 and IgG_1, may contain glycosylation sites. Glycosylation of these sites can alter the properties and activites of antibody molecules. Antibody molecules may be N-glycosylated or O-glycosylated. N-glycosylation of antibody amino acid residue side chains containing nitrogen atoms (*e.g.*, Asn) can modulate antibody Fc effector functions such as ADCC by conferring a cytolytic activity to N-glycosylated antibody molecules. This ADCC associated cytolytic activity causes the lysis

of cells effected by such N-glycosylated antibodies. Alternatively, an antibody molecule may be O-glycosylated by modification of amino acid residue side chains containing oxygen atoms (*e.g.*, Ser or Thr). O-glycosylation can decrease the serum half-life of an antibody molecule through increased lectin mediated clearance of O-glycosylated antibody molecules from the serum. Additionally, O-glycosylation can cause undesirable increases in antibody heterogeneity due to differing extents of O-glycosylation between various antibody molecules. Lastly, both O-glycosylation and N-glycosylation can alter the structure dependent properties of antibody molecules such as binding affinity and immunogenicity.

Like the antibody molecules they mimic, the mimetibody polypeptides of the invention may also be post-translationally modified by N-glycosylation and O-glycosylation. In most instances, it is desirable to limit the N-glycosylation of the mimetibodies of the invention to minimize cytolytic activity. N-glycosylation can be limited by the removal or substitution of amino acid residues, such as Asn, which are typically N-glycosylated. It is also desirable to limit mimetibody O-glycosylation to minimize lectin-mediated clearance, mimetibody heterogeneity and the alteration of structure dependent mimetibody properties such as binding affinity and immunogenicity. One way to minimize O-linked glycosylation in the mimetibodies of the invention is to substitute Ala residues for Thr residues in the V2 portion of the polypeptides of the invention. The V2 amino acid sequence TLVAVSS (SEQ ID NO: 34) is exemplary of such a Thr to Ala substitution; this particular V2 substitution can also be obtained by a Thr to Ala substitution at postion 47 of SEQ ID NO: 62. Those skilled in the art also will recognize other ways to control N-linked and O-linked glycosylation including modulation of glycosylase enzyme activity.

The monomeric structure Mp-Lk-V2-Hg-C_H2-C_H3 of the mimetibody polypeptides of the invention can be linked to "t" other monomers where t is an integer from 1 to 10. Such linking can occur through non-covalent interactions or covalent linkages such as a Cys-Cys disulfide bond. In this way multimeric structures such as dimers and higher order multimers of the polypeptides of the invention can be formed. It is expected that dimerization of the polypeptides of

the invention will increase the affinity of these polypeptides to melanocortin receptors such as MC4R. The term "multimers" as used herein means molecules that have quaternary structure and are formed by the association of two or more subunits.

The polypeptides of the invention can optionally comprise at the amino terminus, a amino terminal portion of an immunoglobulin variable region, designated V1 as shown in Formula II:

$$(V1\text{-}Mp\text{-}Lk\text{-}V2\text{-}Hg\text{-}C_H2\text{-}C_H3)_{(t)} \quad (II)$$

Exemplary V1 amino acid sequences include QIQ and QVQ.

The polypeptides of the invention may also comprise secretory signals necessary to facilitate protein secretion or other signals necessary for protein trafficking in the cell. An exemplary secretory signal sequence is MAWVWTLLFLMAAAQSIQA (SEQ ID NO: 69). Those skilled in the art will recognize other secretory signals.

In one embodiment the polypeptides of the invention comprise SEQ ID NO: 60 or 62. SEQ ID NO: 62 represents a $(V1\text{-}Mp\text{-}Lk\text{-}V2\text{-}Hg\text{-}C_H2\text{-}C_H3)_{(t)}$ melanocortin receptor binding alpha-MSH polypetide of generic formula (II) which has the secretory signal MAWVWTLLFLMAAAQSIQA (SEQ ID NO: 69) fused to its amino terminus. SEQ ID NO: 60 represents a $(Mp\text{-}Lk\text{-}V2\text{-}Hg\text{-}C_H2\text{-}C_H3)_{(t)}$ melanocortin receptor binding alpha-MSH polypetide of generic formula (I). No secretory signal is present in SEQ ID NO: 60.

Another aspect of the present invention is a polynucleotide comprising, complementary to or having significant identity with, a polynucleotide encoding at least one melanocortin receptor binding mimetibody. Other aspects of the present invention include vectors comprising at least one polynucleotide molecule encoding a melanocortin receptor binding mimetibody. In a different aspect the invention provides a cell comprising a vector of the invention or a cell expressing a mimetibody polypeptide of the invention. The polynucleotides, vectors and cells may be used to produce the mimetibody polypeptides of the invention.

In one embodiment, the polynucleotides of the invention comprise SEQ ID NO: 59 or SEQ ID NO: 61 or a polynucleotide complementary to SEQ ID NO: 59 or SEQ ID NO: 61. SEQ ID NO: 59 is a

cDNA encoding a (Mp-Lk-V2-Hg-C_H2-C_H3)$_{(t)}$ melanocortin receptor binding alpha-MSH polypetide of generic formula (I) which lacks a signal sequence. SEQ ID NO: 61 is a cDNA encoding a (V1-Mp-Lk-V2-Hg-C_H2-C_H3)$_{(t)}$ melanocortin receptor binding alpha-MSH polypetide of generic formula (II) which has a secretory signal fused to its amino terminus.

In one embodiment, the polynucleotides of the invention comprise a polynucleotide encoding the polypeptide of SEQ ID NO: 60 or SEQ ID NO: 62. Exemplary nucleic acid sequences that encode the polypeptide sequences shown in SEQ ID NO 60 or SEQ ID NO: 62 are shown in SEQ ID NO 59 or SEQ ID NO: 61, respectively. Also provided are polynucleotides that are substantially identical to the above described polynucleotides.

The term "substantially identical" in the context of polynucleotides means that a given polynucleotide sequence is identical to a polynucleotide sequence of the invention, or portion thereof, in at least 60% or at least about 70% or at least about 80% or at least about 90% or at least about 95-98% of the nucleotides. Percent identity between two polynucleotide sequences can be determined by pairwise alignment using the default settings of the AlignX module of Vector NTI v.9.0.0 (Invitrogen Corp., Carlsbad, CA).

Typically, the polynucleotides of the invention are used in expression vectors for the preparation of the mimetibody polypeptides of the invention. Vectors within the scope of the invention provide necessary elements for eukaryotic expression and include viral promoter driven vectors, such as CMV promoter driven vectors, e.g., pcDNA3.1, pCEP4, and their derivatives, Baculovirus expression vectors, *Drosophila* expression vectors, and expression vectors that are driven by mammalian gene promoters, such as human Ig gene promoters. Other examples include prokaryotic expression vectors, such as T7 promoter driven vectors, e.g. pET41, lactose promoter driven vectors and arabinose gene promoter driven vectors.

The present invention also relates to a cell that expresses a mimetibody of the invention or comprises a vector of the invention. Such a cell can be prokaryotic or eukaryotic. Exemplary eukaryotic cells are mammalian cells, such as but not limited to, COS-1, COS-7,

HEK293, BHK21, CHO, BSC-1, HepG2, 653, SP2/0, NS0, 293, HeLa, myeloma, lymphoma cells or any derivative thereof. Most preferably, the eukaryotic cell is a HEK293, NS0, SP2/0, or CHO cell. *E. coli* is an exempary prokaryotic cell. A cell according to the invention may be generated by transfection, cell fusion, immortalization, or other procedures that are well known in the art. Polynucleotides transfected into a cell may be extrachromasomal or stably integrated into the chromosome of the cell.

The mimetibodies of the invention can be made more compatible with a given host cell by modification of the Hg-C_H2-C_H3 portion of the polypeptide. For example, when a mimetibody of the invention is expressed recombinantly in a bacterial cell such as *E. coli*, the Pro-Ala sequence in the Hg element may be removed to prevent digestion by the *E. coli* enzyme proline iminopeptidase. Similarly, a portion of the Hg element can be deleted or substituted with other amino acids in the mimetibodies of the invention to prevent heterogeneity in the products expressed in a selected host cell.

The present invention further provides a method to produce a mimetibody polypeptide comprising the steps of culturing a cell of the invention and purifying an expressed mimetibody polypeptide of the invention. Cell components, such as those necessary for *in vitro* transcription and translation, may also be used to express the polypeptides of the invention. The present invention encompasses mimetibodies produced by both methods. Expressed mimetibody polypeptides can be recovered and purified from cells or cell component based systems by methods well known in the art including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylatpatite chromatography and lectin chromatography. High performance liquid chroatography (HPLC) can also be employed for purification. Typically purfication will require a combination of several different methods.

Another aspect of the present invention is a pharmaceutical composition comprising an effective amount of at least one mimetibody polypeptide and a pharmaceutically acceptable carrier or

diluent. The term "effective amount" generally refers to the quantity of mimetibody necessary for effective therapy, *i.e.*, the partial or complete alleviation of the symptom or disorder for which treatment was sought. The composition can optionally comprise at least one further compound, protein or composition useful for treating obesity and the other conditions described below. The parmaceutically acceptable carrier or diluent in the compositions can be a solution, suspension, emulsion, colloid or powder. Those skilled in the art will recognize other parmaceutically acceptable carriers and diluents.

Another aspect of the present invention is a method of modifying the biological activity of a melanocortin receptor in a cell, tissue or organ comprising contacting the pharmaceutical compositions of the invention with the cell, tissue or organ. The method may be used to modify melanocortin receptor activity in the brain, brain tissue, or brain cells. Alternatively, the method of the invention may be used to modify melanocortin receptor activity in other peripheral cells or tissues such as muscle, or other organs such as the stomach. Those skilled in the art will recognize other cells, tissues or organs, which may be used.

Another aspect of the invention is a method of modulating at least one melanocortin receptor-mediated condition comprising administering a pharmaceutical composition of the invention to a patient in need thereof. The pharmaceutical compositions of the invention can be administered by any suitable route. Such routes may be intrathecal, intranasal, peripheral (*e.g.*, subcutaneous, intramuscular, intradermal, intravenous) or by any other means known in the art. As described previously, abnormal melanocortin receptor activity has been implicated in a number of pathological conditions, such as obesity and Type 2 diabetes. The mimetibody polypeptides of the invention may be also be used to modulate other melanocortin receptor mediated conditions such as male and female erectile dysfunction, inflammation, congestive heart failure, central nervous system disorders, nerve damage, infectious disease, pulmonary disease, skin disease, fever and pain.

The present invention is further described with reference to the following examples. These examples are merely to illustrate aspects of the present invention and are not intended as limitations of this invention.

Example 1

Alpha-MSH Mimetibody and Expression Vector Construction

An alpha-MSH mimetibody protein comprising a secretory signal sequence, an alpha-MSH peptide sequence, a linker sequence, V_H sequence, a hinge sequence, a human IgG_1 C_H2 sequence and a human IgG_1 C_H3 sequence was designed (Fig. 3 and SEQ ID NO. 62) Analytical data, *e.g.*, mass spectroscopy, has confirmed that a mature polypeptide is generated (61,344.6 for G1/G1 form). Nucleic acid sequences encoding this alpha-MSH mimetibody protein (Fig. 3; SEQ ID NO: 61) were generated using standard molecular biology techniques. Nucleic acid sequences encoding the alpha-MSH mimetibody sequence were subcloned into the p2389 expression vector to generate an alpha-MSH mimetibody expression vector (SEQ ID NO: 63).

Example 2

Alpha-MSH Mimetibody Expression

The alpha-MSH mimetibody was transiently expressed in HEK293E cells. Cells were cultured using standard conditions and transiently transfected with the alpha-MSH mimetibody expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as directed by the manufacturer. 24 h after transfection cells were transferred to a serum free media formulation and cultured for 5 days. The culture media was then removed and centrifuged to remove debris. Clarified media was incubated with Protein A-Sepharose™ (HiTrap rProtein A FF, Amersham Biosciencies, Piscataway, NJ) and proteins were eluted from the Protein A-Sepharose™ conjugate as directed by the manufacturer. The eluted protein solution was then further purified via Superose™ 12 size exclusion chromatography (Superose 12 10/300 GL, Amersham Biosciencies, Piscataway, NJ) using standard methods. Column eluant was then subjected to SDS-PAGE and visualized by silver and Coomassie blue staining. Western blots

were then prepared and the blots were probed with either an Fc specific primary antibody or an alpha-MSH specific primary antibody. Together, the Western Blot and SDS-PAGE staining results indicated that a purified alpha-MSH mimetibody, composed of two polypeptide chains, had been obtained from the transiently transfected HEK293 cells.

Example 3

Alpha-MSH Mimetibody Binds MC4R

The alpha-MSH mimetibody binds to MC4R and can compete with radiolabeled [Nle(4), D-Phe(7)]-alpha-MSH (NDP-alpha-MSH) agonist molecules for MC4R binding (Fig. 4). MC4R is a receptor for alpha-MSH. alpha-MSH binding to recombinantly expressed MC4R in HEK293 cell membranes (Perkin Elmer Life and Analytical Sciences, Boston, MA) was examined by competive binding assays in which increasing amounts of unlabeled MC4R agonists (positive controls) and the Fc domain of a human antibody (negative control) were added to assay cocktails containing [^{125}I]-NDP-alpha-MSH as indicated in Fig. 4. The unlabeled MC4R agonists were melanotan II (MTII; an alpha MSH analog), alpha-MSH, and NDP-alpha-MSH. Alpha-MSH mimetibody binding to MC4R was stable after two weeks of storage at 4°C, -20°C, and -80°C in PBS (phosphate buffered saline) as assessed by competive binding assays.

Competivive binding assays were performed using Scintillation Proximity Assays® (Amersham Biosciences Corp, Piscataway, NJ) as directed by the assay manufacturer. Assay cocktails contained [^{125}I]-NDP-alpha-MSH at EC80, *i.e.*, ~0.5 nM, 0.1 μg of MC4R membranes, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 25 mM Hepes, 0.2% BSA, 1 mM 1,10-phenthroline, an assay manufacturer recommended quantity of protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN) and SPA beads. Light emission from Scintillation Proximity Assay® beads was measured with a Packard Top Count NXT Instrument (Perkin Elmer Life and Analytical Sciences, Boston, MA) for 5 minutes.

Example 4

Alpha-MSH Mimetibody Activates MC4R

The alpha-MSH mimetibody can activate MC4R signalling to increase cAMP production in CHOK1 cells expressing MC4R (Fig. 5 and Fig. 6). MC4R is a seven transmembrane (7TM) G-protein coupled receptor. Activation of MC4R by ligand or agonist results in an increase in cyclic AMP levels (cAMP).

MC4R receptor activation assays were performed using two different clonal CHOK1 cell lines stably transfected with a MC4R expression vector and expressing MC4R. Clone 1 (Fig. 5) expressed MC4R at high levels relative to Clone 2 (Fig. 6). Clone 1 and Clone 2 cells were grown as a monolayer using standard culture conditions to a density of approximately 100,000 cells/well and then incubated with increasing amounts (0-100 μM) of alpha-MSH, MTII, or alpha-MSH mimetibody for 15 minutes as indicated in Fig. 5 and Fig. 6. Cells were then lysed and cAMP assays were performed using the cAMP-Screen Direct™ Chemiluminescent Immunoassay System (Applied Biosystems, Foster City, CA) as directed by the manufacturer. EC_{50} values from cAMP assays using Clone 1 (Fig. 5) and Clone 2 (Fig. 6) are listed in Table 1 below

Table 1

	Clone 1	Clone 2
alpha-MSH peptide (Positive control)	EC_{50} = 3.29 nM	EC_{50} = 9.46 nM
MT II (Positive control)	EC_{50} = 0.52 nM	EC_{50} = 0.52 nM
alpha-MSH mimetibody	EC_{50} = 14.36 nM	EC_{50} = 52.4 nM

Example 5

Alpha-MSH Mimetibody Administration Decreases Animal Food Intake and Body Weight

Alpha-MSH mimetibody administration to *Rattus norvegicus* brain ventricules decreases animal food intake (Fig. 7) and body weight (Fig. 8). Alpha-MSH mimetibody was supplied to brain ventricules by intracerebroventricular injections (ICV) via a cannula surgically inserted into the left lateral brain ventricle.

Cannulae were surgically inserted into male Sprague-Dawley or Wistar rats weighing 250 g to 350 g. Cannula placement coordinates were as follows: -0.8 mm from bregma, -4.5 mm ventral and -1.5 posterior-anterior. Animals recovered for 7 to 10 days after surgery. Animals were acclimatized to the experimental procedures by both daily handling and mock injection, in order to minimize stress. In addition animals were submitted to the reversal of dark-light cycle.

Proper cannula placement was confirmed by an angiotensin II test. The test confirmed proper cannula placement if the ICV administration of 10 ng of angiotensin II via the cannula caused the rats to drink 5-10 ml of water in 30 minutes. Only animals that passed this angiotensin II test were used in food intake experiments.

Animals were fasted for 18-24 hours and alpha-MSH mimetibody, alpha-MSH (positive control), or PBS (negative control) were then administered to the brain ventricles via the cannula at an injection rate of 9 μl/min. Each treatment group had a minimum of 7 animals. Treatments and dosages were as indicated in Fig. 7 and Fig. 8.

Food and water was given to the animals after injection. The amount of food and water consumed was measured at 0 h, 4 h, 24 h, 48 h and 72 h (Fig. 7) after injection. Body weight at 72 hours post injection was measured as ahown in Fig. 6.

The present invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

1. A polypeptide according to formula (I):

$$(Mp\text{-}Lk\text{-}V2\text{-}Hg\text{-}C_H2\text{-}C_H3)_{(t)}$$

(I)

where Mp is a melanocortin receptor binding molecule, Lk is a polypeptide or chemical linkage, V2 is a portion of a C-terminus of an immunoglobulin variable region, Hg is at least a portion of an immunoglobulin variable hinge region, C_H2 is an immunoglobulin heavy chain C_H2 constant region and C_H3 is an immunoglobulin heavy chain C_H3 constant region and t is independently an integer from 1 to 10.

2. The polypeptide of claim 1 wherein M is a biologically active fragment of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, or 18.

3. The polypeptide of claim 1 wherein M has the amino acid sequence shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, or 18.

4. The polypeptide of claim 1 wherein the polypeptide binds to at least one melanocortin receptor.

5. The polypeptide of claim 4 wherein the melanocortin receptor is a melanocortin 4 receptor.

6. A polypeptide comprising SEQ ID NO: 60 or 62.

7. A polynucleotide encoding a polypeptide according to any one of claims 1 to 6.

8. A polynucleotide comprising SEQ ID NO: 59 or SEQ ID NO: 61 or a polynucleotide complementary to SEQ ID NO: 59 or SEQ ID NO: 61.

9. A polynucleotide comprising a polynucleotide encoding the polypeptide of SEQ ID NO: 60 or SEQ ID NO: 62.

10. A vector comprising the polynucleotide of claim 8 or 9.

11. The vector of claim 10 comprising SEQ ID NO: 63.

12. A cell expressing a polypeptide according to any one of claims 1 to 6.

13. A cell comprising the vector of claim 10.

14. The cell of claim 13 wherein the cell is a HEK293 derived cell.

15. A method to produce a polypeptide comprising the steps of culturing the cell of claim 12 and purifying the expressed polypeptide.

16. A pharmaceutical composition comprising an effective amount of at least one polypeptide according to any one of claims 1 to 6 and a pharmaceutically acceptable carrier or diluent.

17. A method of modifying the biological activity of a melanocortin receptor in a cell, tissue or organ comprising contacting the pharmaceutical composition of claim 16 with the cell, tissue or organ.

18. A method of modulating at least one melantocortin receptor mediated condition comprising administering the pharmaceutical composition of claim 16 to a patient in need thereof.

19. The method of claim 18 wherein the melanocortin receptor mediated condition is obesity.