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(54) Title: M-CSF MUTEINS AND USES THEREOF

(57) Abstract: M-CSF muteins are provided, along with pharmaceutical compositions containing a M-CSF mutein, kits containing a pharmaceutical composition, methods of preventing and treating bone metastases in a subject afflicted with metastatic cancer, and methods of screening for M-CSF muteins.

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M-CSF MUTEINS AND USES THEREOF

This application claims the benefit of U.S. Provisional Application No. 60/537,985, filed January 21, 2004.

TECHNICAL FIELD

This invention relates to methods for preventing and treating cancer metastasis and bone loss associated with cancer metastasis by administering a M-CSF mutein to a subject.

BACKGROUND OF THE INVENTION

Cancer metastasis is the primary cause of post-operation or post-therapy recurrence in cancer patients. Despite intensive efforts to develop treatments, cancer metastasis remains substantially refractory to therapy. Bone is one of the most common sites of metastasis of various types of human cancers (e.g., breast, lung, prostate and thyroid cancers). The occurrence of osteolytic bone metastases causes serious morbidity due to intractable pain, high susceptibility to fracture, nerve compression and hypercalcemia. Despite the importance of these clinical problems, there are few available treatments for bone loss associated with cancer metastasis.

Osteoclasts mediate bone readsorption. Osteoclasts are multinucleated cells differentiating from haemopoietic cells. It is generally accepted that osteoclasts are formed by the fusion of mononuclear precursors derived from haemopoietic stem cells in the bone marrow, rather than incomplete cell divisions (Chambers, Bone and Mineral Research, 6: 1- 25, 1989; Göthling et al., Clin Orthop Relat R. 120: 201-228, 1976; Kahn et al., Nature 258: 325-327, 1975, Suda et al., Endocr Rev 13: 66-80, 1992; Walker, Science 180: 875, 1973; Walker, Science 190: 785-787, 1975; Walker, Science 190: 784-785, 1975). They share a common stem cell with monocyte-macrophage lineage cells (Ash et al., Nature 283: 669-670, 1980, Kerby et al., J. Bone Miner Res 7: 353-62, 1992). The differentiation of osteoclast precursors into mature multinucleated osteoclasts requires different factors including hormonal and local stimuli (Athanasou et al., Bone Miner 3: 317-333, 1988; Feldman et al., Endocrinology 107: 1137-1143, 1980; Walker, Science 190: 784-785, 1975; Zheng et al., Histochem J 23: 180-188, 1991) and living bone and bone cells have been shown to play a critical role in osteoclast development (Hagenaars et al., Bone Miner 6: 179-189, 1989). Osteoblastic or bone marrow stromal cells are also required for osteoclast differentiation. One of the factors produced by these cells that supports osteoclast formation is macrophage-

colony stimulating factor, M-CSF (Wiktor-Jedrzejczak et al., Proc Natl Acad Sci USA 87: 4828-4832, 1990; Yoshida et al., Nature 345: 442-444, 1990). Receptor activator for NF-κ B ligand (RANKL, also known as TRANCE, ODF and OPGL) is another signal (Suda et al., Endocr Rev 13: 66-80, 1992) through which osteoblastic/stromal cells stimulate osteoclast formation and resorption via a receptor, RANK (TRANCER), located on osteoclasts and osteoclast precursors (Lacey et al., Cell 93: 165-176, 1998; Tsuda et al., Biochem Biophys Res Co 234: 137-142, 1997; Wong et al., J Exp Med 186: 2075-2080, 1997; Wong et al., J Biol. Chem 272: 25190-25194, 1997; Yasuda et al., Endocrinology 139: 1329-1337, 1998; Yasuda et al., Proc Natl Acad Sci US 95: 3597-3602, 1998). Osteoblasts also secrete a protein that strongly inhibits osteoclast formation called osteoprotegerin (OPG, also known as OCIF), which acts as a decoy receptor for the RANKL thus inhibiting the positive signal between osteoclasts and osteoblasts via RANK and RANKL.

Osteoclasts are responsible for dissolving both the mineral and organic bone matrix (Blair et al., J Cell Biol 102: 1164-1172, 1986). Osteoclasts represent terminally differentiated cells expressing a unique polarized morphology with specialized membrane areas and several membrane and cytoplasmic markers, such as tartrate resistant acid phosphatase (TRAP) (Anderson et al. 1979), carbonic anhydrase II (Väänänen et al., Histochemistry 78: 481-485, 1983), calcitonin receptor (Warshafsky et al., Bone 6: 179-185, 1985) and vitronectin receptor (Davies et al., J Cell Biol 109: 1817-1826, 1989). Multinucleated osteoclasts usually contain less than 10 nuclei, but they may contain up to 100 nuclei being between 10 and 100 μm in diameter (Göthling et al., Clin Orthop Relat R 120: 201-228, 1976). This makes them relatively easy to identify by light microscopy. They are highly vacuolated when in the active state, and also contain many mitochondria, indicative of a high metabolic rate (Mundy, in Primer on the metabolic bone diseases and disorders of mineral metabolism, pages 18-22, 1990). Since osteoclasts play a major role in osteolytic bone metastases, there is a need in the art for new agents and methods for preventing osteoclast stimulation.

Thus, there remains a need in the art to identify new agents and methods for preventing or treating cancer metastasis, including osteolytic bone metastases.

SUMMARY OF THE INVENTION

Methods of preventing bone metastases are provided by the instant invention. In one aspect, a method of preventing bone metastases is provided comprising administering to a subject afflicted with metastatic cancer a therapeutically effective amount of a M-CSF mutein or

mutein product thereby preventing bone loss associated with the metastatic cancer. In another aspect of the invention, a method of treating a subject afflicted with a metastatic cancer to bone comprising administering to the subject a therapeutically effective amount of a M-CSF mutein or mutein product thereby reducing the severity of bone loss associated with the metastatic cancer is provided.

In related aspects of the invention, the aforementioned methods are provided wherein the subject is a mammal or a human. In another related aspect, a method is provided wherein the mutein or mutein product inhibits the interaction between M-CSF and its receptor (M-CSFR). In yet another aspect, a method wherein the M-CSF mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells is provided. In still another related aspect, a method is provided wherein the metastatic cancer is breast, lung, renal, multiple myeloma, thyroid, prostate, adenocarcinoma, blood cell malignancies, including leukemia and lymphoma; head and neck cancers; gastrointestinal cancers, including stomach cancer, colon cancer, colorectal cancer, pancreatic cancer, liver cancer; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers and cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; and skin cancer, including malignant melanoma or squamous cell cancer.

Methods of screening M-CSF muteins or mutein products are provided by the present invention. In one aspect, a method of screening for a M-CSF mutein or mutein product is provided comprising the steps of contacting metastatic tumor cell medium, osteoclasts and a candidate M-CSF mutein or mutein product; detecting osteoclast formation, proliferation and/or differentiation; and identifying the candidate as an M-CSF mutein or mutein product if a decrease in osteoclast formation, proliferation and/or differentiation is detected. In a related aspect, a method is provided wherein the metastatic tumor cell medium includes tumor cells. In still another related aspect, a method wherein the contacting step (a) occurs *in vivo*, the detecting step (b) comprises detecting size and/or number of bone metastases, and the candidate is identified as a M-CSF mutein or mutein product if a decrease in size and/or number of bone metastases is detected is provided. In yet another related embodiment, a method is provided further comprising the step of determining if the candidate M-CSF mutein or mutein product inhibits interaction between M-CSF and its receptor M-CSFR.

In another aspect of the invention, a method of identifying a M-CSF mutein or mutein product that can prevent or treat metastatic cancer to bone is provided, comprising the steps of detecting binding of a candidate M-CSF mutein or mutein product to M-CSFR; and

assaying the ability of the candidate M-CSF mutein or mutein product to prevent or treat metastatic cancer to bone *in vitro* or *in vivo*. In another aspect, a method of identifying a M-CSF mutein or mutein product that can prevent or treat metastatic cancer to bone, comprising the steps of identifying a candidate M-CSF mutein or mutein product that inhibits the interaction between M-CSF and M-CSFR; and assaying the ability of the candidate M-CSF mutein or mutein product to prevent or treat metastatic cancer to bone *in vitro* or *in vivo* is provided.

In another aspect, a method of preventing bone metastases and tumor growth is provided comprising administering to a subject afflicted with metastatic cancer therapeutically effective amounts of M-CSF mutein or mutein product and a therapeutic agent, thereby preventing bone loss associated with the metastatic cancer and preventing tumor growth. In another aspect, a method of treating a subject afflicted with a metastatic cancer comprising administering to the subject therapeutically effective amounts of M-CSF mutein or mutein product and a therapeutic agent, thereby reducing the severity of bone loss associated with the metastatic cancer and inhibiting tumor growth is provided. In a related aspect, a method is provided wherein the subject is a mammal or human. In another related aspect, a method is provided wherein the M-CSF mutein or mutein product inhibits the interaction between M-CSF and its receptor M-CSFR. In yet another related aspect, a method is provided wherein the M-CSF mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells.

In still another related aspect, a method is provided wherein the therapeutic agent is a bisphosphonate. In a related aspect, the bisphonate is zeledronate, pamidronate, clodronate, etidronate, tilundronate, alendronate, or ibandronate. In still another related aspect, the aforementioned methods are provided wherin the therapeutic agent is a chemotherapeutic agent. In yet another related aspect, the aforementioned methods are provided wherein the subject is precluded from receiving bisphophonate treatment. In a related aspects of the invention, the aforementioned methods are provided wherein the M-CSF mutein or mutein product is effective to reduce the dosage of therapeutic agent required to achieve a therapeutic effect. In still another related aspect, a method is provided further comprising the step of administering a non-M-CSF colony stimulating factor, for example G-CSF.

In one aspect of the invention, a pharmaceutical composition comprising a M-CSF mutein or mutein product and a cancer therapeutic agent is provided. In another aspect of the invention, a package, vial or container is provided comprising a medicament comprising an M-CSF mutein or mutein product and instructions that the medicament should be used in

combination with surgery or radiation therapy.

In another aspect of the invention, a method of preventing or treating metastatic cancer to bone is provided comprising the steps of administering a M-CSF mutein or mutein product to a subject and treating the subject with surgery or radiation therapy. In another aspect, a method of treating a subject suffering from a cancer, wherein the cells comprising the cancer do not secrete M-CSF is provided, comprising the step of administering a M-CSF mutein or mutein product.

Numerous uses are also contemplated by the instant invention. B way of example, use of a M-CSF mutein or mutein product in the manufacture of a medicament for preventing bone metastases in a subject afflicted with metastatic cancer is provided by the present invention. In another embodiment, a use of a M-CSF mutein or mutein product in the manufacture of a medicament for preventing, in a subject afflicted with metastatic cancer, bone loss associated with the cancer is provided. In still another embodiment, a use of a M-CSF mutein or mutein product in the manufacture of a medicament for treating a subject afflicted with a metastatic cancer to bone is provided by the instant invention. In yet another embodmiment, a use of a M-CSF mutein or mutein product is provided for the manufacture of a medicament for reducing, in a subject afflicted with a metastatic cancer to bone, the severity of bone loss associated with the cancer.

Numerous subjects are contemplated to benefit from the uses of the instant invention. In one embodiment, the subject is a mammal. In another embodiment, the mammal is human.

In another embodiment of the invention, an aforementioned use is provided wherein the mutein or mutein product inhibits the interaction between M-CSF and its receptor (M-CSFR). In a related embodiment, the mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells. In another related embodiment, an aforementioned use is provided wherein the metastatic cancer is breast, lung, renal, multiple myeloma, thyroid, prostate, adenocarcinoma, blood cell malignancies, including leukemia or lymphoma; head or neck cancers; gastrointestinal cancers, including stomach cancer, colon cancer, colorectal cancer, pancreatic cancer, liver cancer; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers or cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; or skin cancer, including malignant melanoma or squamous cell cancer.

In still another embodiment of the invention, a use of a M-CSF mutein or mutein

product and a second therapeutic agent in the manufacture of a medicament for preventing, in a subject afflicted with metastatic cancer, bone metastases and tumor growth is provided. In another embodiment, a use of a M-CSF mutein or mutein product and a second therapeutic agent in the manufacture of a medicament for preventing, in a subject afflicted with metastatic cancer, bone loss associated with the cancer is provided. In yet another embodiment, a use of a M-CSF mutein or mutein product and a second therapeutic agent is provided for the manufacture of a medicament for treating a metastatic cancer to bone. In still another embodiment, a use of a M-CSF mutein or mutein product and a second therapeutic agent in the manufacture of a medicament for reducing the severity of bone loss associated with the cancer and inhibiting tumor growth in a subject afflicted with metstatic cancer is provided.

In one embodiment of the invention, a product comprising an M-CSF mutein or mutein product and a second therapeutic agent as a combined preparation is provided for use in treating cancer. In another embodiment, a use of a M-CSF mutein or mutein product in preparation of a medicament for preventing or treating metastatic cancer to bone, wherein the medicament is simultaneously separately or sequentially administered with a second cancer therapeutic agent is provided.

In another embodiment of the invention, a use of a M-CSF mutein or mutein product in preparation of a medicament for preventing or treating metastatic cancer to bone,wherein the medicament is coordinated with treatment using a second therapeutic agent is provided. In still another embodiment, a use of a M-CSF mutein or mutein product is provided for preparation of a medicament for treating a subject afflicted with a metastatic cancer to bone,wherein the subject has been pre-treated with the second therapeutic agent. In yet another embodiment, a use of a synergistic combination of a MCSF mutein or mutein product in the manufacture of a medicament for treating a patient having an osteolytic disease wherein said medicament is coordinated with treatment using an anti-MCSF antibody, anti-RANKL antibody, soluble RANKL receptor or bisphosphonate is provided.

In still another embodiment, a use of a second therapeutic agent in preparation of a medicament for preventing or treating metastatic cancer to bone, wherein the medicament is simultaneously separately or sequentially administered with an M-CSF mutein or mutein product is provided.

A package, vial or container is contemplated by the present invention comprising a medicament comprising an M-CSF mutein or mutein product and instructions that the medicament should be used in combination with surgery or radiation therapy. Numerous

subjects are contemplated to benefit from the aforementioned uses of the instant invention. In one embodiment, the subject is a mammal. In another embodiment, the mammal is human.

In another embodiment of the invention, an aformetioned use is provided wherein said mutein or mutein product inhibits the interaction between M-CSF and its receptor M-CSFR. In a related embodiment, the mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells. In still another related embodiment, the second therapeutic agent is a bisphosphonate. In another related embodiment, the bisphonate is zeledronate, pamidronate, clodronate, etidronate, tilundronate, alendronate, or ibandronate. In yet another related embodiment, the second therapeutic agent is a chemotherapeutic agent. In another related embodiment, the subject is precluded from receiving bisphophonate treatment. In another embodiment of the invention, an aforementioned use is provided wherein the second therapeutic agent is a non-M-CSF colony stimulating factor. In a related embodiment, an aforementioned use is provided wherein the non-M-CSF colony stimulating factor is G-CSF.

The present invention also contemplates a use of a M-CSf mutein or mutein product in the manufacture of a medicament for reducing the dose of a therapeutic agent administered to a subject to treat or prevent bone metastases and tumor growth. In another embodiment of the invention, a use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, in the manufacture of a medicament for preventing bone metastases is provided. In still another embodiment, a use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, is provided for the manufacture of a medicament for neutralizing M-CSF produced by a subject's cells.

The invention also contemplates a use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, in the manufacture of a medicament for treating a subject afflicted with a metastatic cancer to bone. In another embodiment, a use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, in the manufacture of a medicament for treating cancer is provided.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a topology diagram showing the disulfide bonds in truncated dimeric M-CSF.

Figure 2 is a stereodiagram of the C-alpha backbone with every tenth residue labeled and with the non-crystallographic symmetry axis indicated by a dotted line.

Figure 3 is a comparison of osteoclast inducing activity between purified M-CSF and conditioned medium (CM) from MDA 231 cells and MCF7 cells.

Figure 4 is the amino acid sequence of M-CSFα (SEQ ID NO: 2).

Figure 5 is is the amino acid sequence of M-CSFβ (SEQ ID NO: 4).

Figure 6 is is the amino acid sequence of M-CSFγ (SEQ ID NO: 6).

DETAILED DESCRIPTION

The ability to metastasize is a defining characteristic of a cancer. Metastasis refers to the spread of cancer cells to other parts of the body or to the condition produced by this spread. Metastasis is a complex multi-step process that includes changes in the genetic material of a cell, uncontrolled proliferation of the altered cell to form a primary tumor, development of a new blood supply for the primary tumor, invasion of the circulatory system by cells from the primary tumor, dispersal of small clumps of primary tumor cells to other parts of the body, and the growth of secondary tumors in those sites.

Bone is one of the most common sites of metastasis in human breast, lung, prostate and thyroid cancers, as well as other cancers, and in autopsies as many as 60% of cancer patients are found to have bone metastasis. Osteolytic bone metastasis shows a unique step of osteoclastic bone resorption that is not seen in metastasis to other organs. Bone loss associated with cancer metastasis is mediated by osteoclasts (multinucleated giant cells with the capacity to resorb mineralized tissues), which seem to be activated by tumor products.

Colony stimulating factor (CSF 1), also known as macrophage colony stimulating factor (M-CSF), has been found crucial for osteoclast formation. In addition, M-CSF has been shown to modulate the osteoclastic functions of mature osteoclasts, their migration and their survival in cooperation with other soluble factors and cell to cell interactions provided by osteoblasts and fibroblasts (Fixe and Praloran, Cytokine 10: 3-7, 1998; Martin et al., Critical Rev. in Eukaryotic Gene Expression 8: 107-23 (1998)).

The full-length human M-CSF mRNA encodes a precursor protein of 554 amino acids (SEQ ID NO: 4). Through alternative mRNA splicing and differential post-translational proteolytic processing, M-CSF can either be secreted into the circulation as a glycoprotein or

chondroitin sulfate containing proteoglycan or be expressed as a membrane spanning glycoprotein on the surface of M-CSF producing cells. The three-dimensional structure of the bacterially expressed amino terminal 150 amino acids of human M-CSF, the minimal sequence required for full in vitro biological activity, indicates that this protein is a disulfide linked dimer with each monomer consisting of four alpha helical bundles and an anti-parallel beta sheet (Pandit et al., Science 258: 1358-62 (1992)). Three distinct M-CSF species are produced through alternative mRNA splicing. The three polypeptide precursors are M-CFSα of 256 amino acids (DNA and amino acid sequences set forth in SEQ ID NOS: 1 and 2), M-CSFβ of 554 amino acids (DNA and amino acid sequences set forth in SEQ ID NOS: 3 and 4), and M-CSFγ of 438 amino acids (DNA and amino acid sequences set forth in SEQ ID NOS: 5 and 6). M-CSFβ is a secreted protein that does not occur in a membrane-bound form. M-CSFα is expressed as an integral membrane protein that is slowly released by proteolytic cleavage. M-CSFα is cleaved at amino acids 191-197 of SEQ ID NO: 2. The membrane-bound form of M-CSF can interact with receptors on nearby cells and therefore mediates specific cell-to-cell contacts.

Various forms of M-CSF function by binding to its receptor M-CSFR on target cells. M-CSFR (DNA and amino acid sequences set forth in SEQ ID NOS: 7 and 8) is a membrane spanning molecule with five extracellular immunoglobulin-like domains, a transmembrane domain and an intracellular interrupted Src related tyrosine kinase domain. M-CSFR is encoded by the *c-fms* proto-oncogene. Binding of M-CSF to the extracellular domain of M-CSFR leads to dimerization of the receptor, which activates the cytoplasmic kinase domain, leading to autophosphorylation and phosphorylation of other cellular proteins (Hamilton J. A., J Leukoc Biol.,62(2):145-55 (1997); Hamilton J, A., Immuno Today., 18(7): 313-7(1997).

Phosphorylated cellular proteins induce a cascade of biochemical events leading to cellular responses: mitosis, secretion of cytokines, membrane ruffling, and regulation of transcription of its own receptor (Fixe and Praloran, Cytokine 10: 32-37 (1998)).

M-CSF is expressed in stromal cells, osteoblasts, and other cells. It is also expressed in breast, uterine, and ovarian tumor cells. The extent of expression in these tumors correlates with high grade and poor prognosis (Kacinski Ann. Med. 27: 79-85 (1995); Smith et al., Clin. Cancer Res. 1: 313-25 (1995)). In breast carcinomas, M-CSF expression is prevalent in invasive tumor cells as opposed to the intraductal (pre-invasive) cancer (Schöll et al., J. Natl. Cancer Inst. 86: 120-6 (1994)). In addition, M-CSF is shown to promote progression of

mammary tumors to malignancy (Lin et al., J. Exp. Med. 93: 727-39 (2001)). For breast and ovarian cancer, the production of M-CSF seems to be responsible for the recruitment of macrophages to the tumor.

It has been discoverd that M-CSF neutralizing antibody inhibits osteoclast induction by factors produced by metastatic type cancer cells (See the disclosure of, United States Serial No. 10/713, 895; incorporated by reference in its entirety). It is expected that M-CSF muteins, via neutralizing active M-CSF activity through means such as blocking ligand-receptor interaction, will block osteoclast induction by metastatic cancer cels. Thus, the present invention provides compositions and methods for treating or preventing cancer metastasis and bone loss associated with cancer metastasis.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma,; lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include breast cancer, prostate cancer, colon cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

"Treatment" is an intervention performed with the intention of preventing the development or altering the pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. In tumor (e.g., cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, or render the tumor cells more susceptible to treatment by other therapeutic agents, e.g., radiation and/or chemotherapy. The "pathology" of cancer includes all phenomena that compromise the well being of the patient. This includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, etc.

As used herein, the phrase "metastatic cancer" is defined as cancers that have

potential to spread to other areas of the body, particularly bone. A variety of cancers can metastasize to the bone, but the most common metastasizing cancers are breast, lung, renal, multiple myeloma, thyroid and prostate. By way of example, other cancers that have the potential to metastasize to bone include but are not limited to adenocarcinoma, blood cell malignancies, including leukemia and lymphoma; head and neck cancers; gastrointestinal cancers, including stomach cancer, colon cancer, colorectal cancer, pancreatic cancer, liver cancer; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers and cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; and skin cancer, including malignant melanoma and squamous cell cancer. The present invention especially contemplates prevention and treatment of tumor-induced osteolytic lesions in bone.

M-CSF Muteins

The invention provides M-CSF muteins that may be used as MCSF antagonists according to the methods of the invention.

"Mutein" as used herein with respect to polypeptides means a variant of the intact native molecule or a variant of a fragment of the native molecule, in which one or more amino acids have been substituted, inserted or deleted and that exhibit ability to inhibit M-CSF osteoclast-stimulating activity. Such substitutions, insertions or deletions can be at the N-terminus, C-terminus or internal to the molecule. Thus the term "muteins" includes within its scope fragments of the native molecule. Insertional muteins include fusions at the N- or C-terminus, e.g. fusion to the Fc portion of an immunoglobulin to increase half-life.

"Mutein product" as used herein means a mutein or modification thereof that retains the desired activity.

"Fragment" as used herein means a portion of the intact native molecule; for example, a fragment polypeptide is a fragment of the native polypeptide in which one or more amino acids from either the N-terminal or C-terminal have been deleted.

Preferred muteins according to the invention exhibit at least about 65%, 70%. 75%, 80%, 85%, 90%, 95%, 97% or more sequence identity (homology) to the native polypeptide, as determined by the Smith-Waterman homology search algorithm (Meth. Mol. Biol. 70:173-187 (1997)) as implemented in the MSPRCH program (Oxford Molecular) using an affine gap search with the following search parameters: gap open penalty of 12, and gap extension penalty of 1. Other well-known and routinely used homology/identity scanning

algorithm programs include Pearson and Lipman, PNAS USA, 85:2444-2448 (1988); Lipman and Pearson, Science, 222:1435 (1985); Devereaux et al., Nuc. Acids Res., 12:387-395 (1984); or the BLASTP, BLASTN or BLASTX algorithms of Altschul, et al., Mol. Biol., 215:403-410 (1990). Computerized programs using these algorithms are also available and include, but are not limited to: GAP, BESTFIT, BLAST, FASTA and TFASTA, which are commercially available from the Genetics Computing Group (GCG) package, Version 8, Madison Wis., USA; and CLUSTAL in the PC/Gene program by Intellegenetics, Mountain View Calif. Preferably, the percentage of sequence identity is determined by using the default parameters determined by the program.

"Modification" as used herein means any modification of the native polypeptide, fragment or mutein, such as glycosylation, phosphorylation, polymer conjugation (such as with polyethylene glycol), or other addition of foreign moieties, so long as the desired activity (agonist or antagonist) is retained.

U.S. Patent No. 6,025,146, and Koths, Mol. Reprod. Dev. 1997 Jan;46(1):31-38 both of which are incorporated herein by reference in their entirety, describe the crystallization of M-CSF alone and M-CSF complexed to MCSF-R, and characterize the three-dimensional structure of M-CSF as well as residues involved in receptor-binding. U.S. Patent No. 6,025,146 also describes methods for selecting candidate amino acid substitutions in M-CSF, based on structural information. FIG. 1 is a topology diagram showing the disulfide bonds in truncated dimeric M-CSF; FIG. 2 is a stereodiagram of the C-alpha backbone with every tenth residue labelled and with the non-crystallographic symmetry axis indicated by a dotted line. The overall topology of this form of M-CSF as shown in FIG. 1 is that of an antiparallel four alpha-helical bundle, in which the helices run up-up-down-down, unlike the more commonly observed up-down-up-down connectivity of most four helical bundles. A long crossover connection links helix A to helix B and a similar connection is found between helices C and D. In the disulfide-linked dimeric form, the bundles are linked end-to-end, forming an extremely flat, elongated structure (approximate dimensions 85 x 35 x 25 ′). There are three intramolecular disulfide bonds in each monomer (Cys7-Cys90, Cys48-Cys139, Cys102-Cys146) all of which are at the distal end of the molecule. One interchain disulfide bond (Cys31--Cys31) is located at the dimer interface with the noncrystallographic two-fold symmetry axis passing through it as shown in FIG. 2. Mutation experiments indicate that all of the cysteine residues in this form of M-CSF may be necessary for full biological activity. The structure described herein suggests that their role is primarily structural rather than being related to receptor recognition. U.S. Patent No. 6,025,146 provides the three-dimensional structure of the truncated recombinant M-CSF α dimer

as identified by the alpha-carbon positions of the amino acid residues in the sequence.

Specific residues in helices A, C, and D appear to be involved in the specificity of the receptor-binding interaction. Since M-CSFβ has intrachain disulfide bonds involving cysteines 157 and/or 159, the C-terminal region of M-CSF likely extends from the "rear" of the structure, providing a variable-length "tether" for membrane-bound forms of M-CSF. Thus, the "front" or receptor-binding region of M-CSF is on the opposite side of the molecules, consisting of solvent-accessible residues in or near helices A, C, and D, including residues from about 6 to 26, 71 to 90, and 110 to 130, respectively, of native M-CSF. Altering solvent accessible residues in these regions by site directed mutagenesis to increase or decrease side-chain interactions with the receptor may generate M-CSF agonists or antagonists. Residues having a solvent accessible surface area of greater than about 0.25 and preferably greater than about 0.4 are preferred based on normalization of the surface area of the amino acid accessible when in the trypeptide gly-x-gly (Kabsch, W. et al., Biopolymers 22:2577 (1983)). Preferably residues are chosen which do not interact with other parts of the protein such as the dimer interface in order to maintain the relative orientation of monomers and to avoid disturbing the process of protein folding.

An optional additional consideration is selecting residues not conserved between human and mouse M-CSF, which does not recognize the human M-CSF receptor. Candidate amino acids are preferably selected for substitution with non-conservative amino acids, so as to disrupt hydrogen bonding and/or hydrophobic interactions with MCSF-R residues. For example, changing one or more histidines to non-hydrogen-donor amino acids of similar size may create an M-CSF with altered receptor binding ability. Preferred amino acids for substitution include but are not limited to: H15; Q79; R86; E115; E41; K93; D99; L55; S18; Q20; I75; V78; L85; D69; N70; H9; N63; and T34. M-CSF residues important in receptor signaling are believed to be composed of discontinuous regions of M-CSF. To minimize the likelihood of antibody formation to potentially administered M-CSF-based proteinaceous drugs, it is desirable to retain the solvent-accessible parental M-CSF residues (to resemble the native molecule) whenever possible.

Mutagenesis of amino acids H15 and H9 in the N-terminal/A helix region resulted in muteins with significantly lower biological activity and significantly lower MCSF-R binding ability. These results indicated that the reduced biological activity was due to decreased receptor binding affinity; thus, these histidine amino acids represent contacts that are important for M-CSF receptor binding affinity and should be left unchanged if full receptor-binding ability

is desired. Nearby solvent accessible residues such as Y6 and S13 and others may also represent M-CSF receptor contact residues. A double mutant of M-CSF (Q20A, V78K) was constructed to test the importance of solvent accessible residues in the central portion of helices A and C. This double mutein had slightly lower (8-10 fold) biological activity and correspondingly lower receptor-binding activity. Mutagenesis of residues Q17, R21, E115 and E119 changed side chain properties of solvent-accessible amino acids in the areas of interest but did not affect biological specific activity, suggesting that these residues need not be altered in muteins designed to have antagonist activity.

In one embodiment, the invention contemplates M-CSF muteins in which residues of helices A and/or C and/or D involved in receptor-binding (for example, amino acids 6 to 26, 71 to 90 and/or 110 to 130) have been mutated non-conservatively. Such muteins preferably retain at least 65%, 70%, 75%, 80%, 85% or 90% similarity (i.e. amino acids that are identical or have similar properties) to the native sequence within helices A, C or D, but have higher similarity to the native sequence in the remainder of the polypeptide, e.g. , at least 95%, 98% or 99% similarity. In addition, residues that support the three-dimensional confirmation of the receptor-binding site may be mutated non-conservatively.

In another embodiment, the M-CSF mutein is a monomeric form of M-CSF. The dimeric form of M-CSF is the biologically active form, and monomeric forms of M-CSF are generally not active. Disulfide bonding of the monomers appears to occur through the Cys31-Cys31 interchain linkage. Thus, it is contemplated that monomeric forms of M-CSF may be suitable for use as antagonists. Such forms include muteins comprising cysteine deletions and/or cysteine replacements (e.g., cysteine to alanine substitutions) of Cys31 and/or other cysteines, or muteins in which the cysteine(s), particularly Cys31, have been chemically modified so that they are not available for disulfide bonding.

In yet another embodiment, the M-CSF mutein comprises one or more of helices A, C or D, or portions thereof involved in receptor-binding, alone or fused to other polypeptides that allow display of the fragments in proper three-dimensional conformation.

Muteins containing any desired conservative and/or non-conservative muteins are readily prepared using techniques well known in the art, including recombinant production or chemical synthesis.

Conservative substitutions, particularly substitutions outside of regions directly involved in ligand-receptor binding, are not expected to significantly change the binding properties of the M-CSF muteins (or M-CSFR muteins). Amino acids can be classified

according to physical properties and contribution to secondary and tertiary protein structure. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are set out in Table 2 (from WO 97/09433, page 10, published March 13, 1997 (PCT/GB96/02197, filed 9/6/96), immediately below.

Table 2

Conservative Substitutions I

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Aliphatic	
Non-polar	G A P I L V
Polar-uncharged	C S T M N Q
Polar-charged	D E K R
Aromatic	H F W Y
Other	N Q D E

Alternatively, conservative amino acids can be grouped as described in Lehninger, (Biochemistry, Second Edition; Worth Publishers, Inc. NY:NY (1975), pp.71-77) as set out in Table 3, immediately below.

Table 3

Conservative Substitutions II

SIDE CHAIN CHARACTERISTIC	AMINO ACID
Non-polar (hydrophobic)	
A. Aliphatic:	A L I V P
B. Aromatic:	F W
C. Sulfur-containing:	M
D. Borderline:	G

Uncharged-polar	
A. Hydroxyl:	S T Y
B. Amides:	N Q
C. Sulfhydryl:	C
D. Borderline:	G
Positively Charged (Basic):	K R H
Negatively Charged (Acidic):	DE

As still an another alternative, exemplary conservative substitutions are set out in Table 4, immediately below.

Table 4

Conservative Substitutions III

Original Residue	Exemplary Substitution
Ala (A)	Val, Leu, Ile
Arg (R)	Lys, Gln, Asn
Asn (N)	Gln, His, Lys, Arg
Asp (D)	Glu
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
His (H)	Asn, Gln, Lys, Arg
Ile (I)	Leu, Val, Met, Ala, Phe,
Leu (L)	Ile, Val, Met, Ala, Phe
Lys (K)	Arg, Gln, Asn
Met (M)	Leu, Phe, Ile
Phe (F)	Leu, Val, Ile, Ala
Pro (P)	Gly

Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser
Val (V)	Ile, Leu, Met, Phe, Ala

The availability of a DNA sequence encoding M-CSF permits the use of various expression systems to produce the desired polypeptides. Construction of expression vectors and recombinant production from the appropriate DNA sequences are performed by methods well known in the art. These techniques and various other techniques are generally performed according to Sambrook et al., Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989), and Kriegler, M., Gene Transfer and Expression, A Laboratory Manual, Stockton Press, New York (1990), both of which are incorporated herein by reference.

Certain modifications to the primary sequence of M-CSF can be made by deletion, addition, or alteration of the amino acids encoded by the DNA sequence without destroying the desired structure (e.g., the receptor binding ability of M-CSF) in accordance with well-known recombinant DNA techniques. Further, a skilled artisan will appreciate that individual amino acids may be substituted or modified by oxidation, reduction or other modification, and the polypeptide may be cleaved to obtain fragments that retain the active binding site and structural information. Such substitutions and alterations result in polypeptides having an amino acid sequence which falls within the definition of polypeptide "having substantially the same amino acid sequence as the mature M-CSFα (SEQ ID NO: 2), M-CSFβ (SEQ ID NO: 4), and M-CSFγ (SEQ ID NO: 4)polypeptides."

Polypeptides may be synthesized using standard solution phase or solid phase peptide synthesis techniques known in the art. In solution phase synthesis, a wide variety of coupling methods and protecting groups may be used (see Gross and Meienhofer, eds., "The Peptides: Analysis, Synthesis, Biology," Vol. 1-4 (Academic Press, 1979); Bodansky and Bodansky, "The Practice of Peptide Synthesis," 2d ed. (Springer Verlag, 1994)). In addition, intermediate purification and linear scale up are possible. Those of ordinary skill in the art will appreciate that solution synthesis requires consideration of main chain and side chain protecting groups and activation method as well as segment selection to minimize racemization.

Solid-phase peptide synthesis may generally be performed according to the method of Merrifield et al., J. Am. Chem. Soc. 85:2149, 1963, which involves assembling a linear peptide chain on a resin support using protected amino acids. Solid phase peptide synthesis typically utilizes either the Boc or Fmoc strategy. The Boc strategy uses a 1% cross-linked polystyrene resin. The standard protecting group for α -amino functions is the tert-butyloxycarbonyl (Boc) group. This group can be removed with dilute solutions of strong acids such as 25% trifluoroacetic acid (TFA). The next Boc-amino acid is typically coupled to the amino acyl resin using dicyclohexylcarbodiimide (DCC). Following completion of the assembly, the peptide-resin is treated with anhydrous HF to cleave the benzyl ester link and liberate the free peptide. Side-chain functional groups are usually blocked during synthesis by benzyl-derived blocking groups, which are also cleaved by HF. The free peptide is then extracted from the resin with a suitable solvent, purified and characterized. Newly synthesized peptides can be purified, for example, by gel filtration, HPLC, partition chromatography and/or ion-exchange chromatography, and may be characterized by, for example, mass spectrometry or amino acid sequence analysis. In the Boc strategy, C-terminal amidated peptides can be obtained using benzhydrylamine or methylbenzhydrylamine resins, which yield peptide amides directly upon cleavage with HF. An alternative approach using 9-fluorenylmethyloxycarbonyl (Fmoc) uses different reagents which allow the side-chain protecting groups and the peptide-resin link to be completely stable to the secondary amines used for cleaving the N-α-Fmoc group. The side-chain protection and the peptide-resin link are cleaved by mild acidolysis. The repeated contact with base makes the Merrifield resin unsuitable for Fmoc chemistry, and p-alkoxybenzyl esters linked to the resin are generally used. Deprotection and cleavage are generally accomplished using TFA. Acetylation of the N-terminal can be accomplished by reacting the final peptide with acetic anhydride before cleavage from the resin. C-amidation is accomplished using an appropriate resin such as methylbenzhydrylamine resin using the Boc technology.

In general, modifications of the genes encoding the M-CSF polypeptide are readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see, Gillman and Smith, Gene 8:81-97 (1979) and Roberts, S. et al., Nature 328:731-734 (1987) and U.S. Pat. No. 5,032,676, all of which are incorporated herein by reference). Most modifications are evaluated by screening in a suitable assay for the desired characteristic. For instance, a change in the M-CSF receptor-binding character of the polypeptide can be detected by competitive assays with an appropriate reference polypeptides or by the bioassays described in U.S. Pat. No. 4,847,201, which is incorporated herein by reference.

Insertional variants of the present invention are those in which one or more amino acid residues are introduced into a predetermined site in the M-CSF. For instance, insertional variants can be fusions of heterologous proteins or polypeptides to the amino or carboxyl terminus of the subunits. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Non-natural amino acids (i.e., amino acids not normally found in native proteins), as well as isosteric analogs (amino acid or otherwise) are also suitable for use in this invention. Examples of suitable substitutions are well known in the art, such as the Glu->Asp, Ser->Cys, and Cys->Ser, His->alanine for example. Another class of variants are deletional variants, which are characterized by the removal of one or more amino acid residues from the M-CSF.

Other variants of the present invention may be produced by chemically modifying amino acids of the native protein (e.g., diethylpyrocarbonate treatment which modifies histidine residues). Preferred or chemical modifications which are specific for certain amino acid side chains. Specificity may also be achieved by blocking other side chains with antibodies directed to the side chains to be protected. Chemical modification includes such reactions as oxidation, reduction, amidation, deamidation, or substitution of bulky groups such as polysaccharides or polyethylene glycol (see e.g., U.S. Pat. No. 4,179,337 and WO91/21029 both of which are incorporated herein by reference).

Exemplary modifications include the modification of lysinyl and amino terminal residues by reaction with succinic or other carboxylic acid anhydrides. Modification with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for modifying amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea, 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate, and N-hydroxysuccinamide esters of polyethylenene glycol or other bulky substitutions.

Arginyl residues may be modified by reaction with a number of reagents, including phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Modification of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

Tyrosyl residues may also be modified with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Tyrosyl residues may also be iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R--N.dbd.C.dbd.N--R.sup.1), where R and R_1 are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Conversely, glutaminyl and asparaginyl residues may be deamidated to the corresponding glutamyl and aspartyl residues, respectively, under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins: Structure and Molecular Properties, W. H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Cystein caping may alos be employed to modify the M-CSF polypeptide. For example, sulfhydryl groups (Cysteins) may be selectively modified by reaction with Iodoacetamides (alkyl halide or haloacetamide) or Maleimides (N-ethyulmaeimide (NEM) or 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin CPM). Different alkyl groups are attached to the protein, resulting in a stable thioether form of the modified protein.

A number of methods can be used to determine the similarity of M-CSF muteins to the native M-CSF protein. For example, percent homology is calculated as the percentage of amino acid residues in the smaller of two sequences which align with identical amino acid residue in the sequence being compared, when four gaps in a length of 100 amino acids may be introduced to maximize alignment (Dayhoff, in Atlas of Protein Sequence and Structure, Vol. 5, p. 124, National Biochemical Research Foundation, Washington, D.C. (1972), incorporated herein by reference). Sequence alignment of polypeptides for purposes of sequence comparison also can be done using a variety of multiple alignment servers, most of which are presently available on the Internet, e.g., Clustal W, MAP, PIMA, Block Maker, MSA, MEME, and Match-Box. Preferably Clustal W (Higgins et al., Gene (1988) 73:237-244; Higgins et al., Meth. Enzymol.(1996) 266:383-402) is employed for sequence alignment of polypeptides (and also, polynucleotides). Similarly, the program BLASTP compares an amino acid query sequence against a protein database, and TBLASTN compares a protein query sequence against

a nucleotide sequence database dynamically translated in all six reading frames (both strands), and can be employed in the invention. Determinations of whether two amino acid sequences are substantially homologous (i.e., similar or identical) can also be based on FASTA searches in accordance with Pearson et al., Proc. Natl. Acad. Sci. USA 85:2444-2448 (1988).

In particular, preferred methods to determine identity and/or similarity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs (e.g., such as those previously described). Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux et al., Nucleic Acids Research (1984) 12(1):387; Genetics Computer Group, University of Wisconsin, Madison, WI), BLASTP, BLASTN, and FASTA (Altschul et al., J. Molec. Biol. (1990) 215:403-410). The BLAST X program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (Altschul et al., BLAST Manual, NCB NLM NIH Bethesda, MD 20894; Altschul et al., J. Mol. Biol. (1990) 215:403-410). The well known Smith Waterman algorithm may also be used to determine identity. In comparing polynucleotide sequences using the GAP program, the following default parameters are preferred: comparison matrix: match = +10, mismatch = 0, with a gap penalty of 50 and a gap length penalty of 3 (Needleman et al., J. Mol Biol. (1970) 48:443-453).

The relatedness of proteins can also be characterized through the relatedness of their encoding nucleic acids. Methods to determine identity and/or similarity of polynucleotide sequences are described above. In addition, methods to determine similarity of polynucleotide sequences through testing their ability to hybridize under moderately or highly stringent conditions may be determined as follows. Exemplary moderately stringent hybridization conditions are as follows: hybridization at 42°C in a hybridization solution comprising 50% formamide, 1% SDS, 1 M NaCl, 10% Dextran sulfate, and washing twice for 30 minutes at 60°C in a wash solution comprising 0.1x SSC and 1% SDS. Highly stringent conditions include washes at 68°C in a wash solution comprising 0.1x SSC and 1% SDS. It is understood in the art that conditions of equivalent stringency can be achieved through variation of temperature and buffer, or salt concentration as described in the art (Ausubel, et al. (Eds.), Protocols in Molecular Biology, John Wiley & Sons (1994), pp. 6.0.3 to 6.4.10). Modifications in hybridization conditions can be empirically determined or precisely calculated based on the length and the percentage of guanosine/cytosine (GC) base pairing of the probe. The hybridization conditions can be calculated as described in Sambrook et al., (Eds.), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47

to 9.51.

Gene Therapy

Delivery of a therapeutic protein to appropriate cells can be effected via gene therapy ex vivo, in situ, or in vivo by use of any suitable approach known in the art, including by use of physical DNA transfer methods (e.g., liposomes or chemical treatments) or by use of viral vectors (e.g., adenovirus, adeno-associated virus, or a retrovirus). For example, for in vivo therapy, a nucleic acid encoding the desired protein, either alone or in conjunction with a vector, liposome, or precipitate may be injected directly into the subject, and in some embodiments, may be injected at the site where the expression of the protein compound is desired. For ex vivo treatment, the subject's cells are removed, the nucleic acid is introduced into these cells, and the modified cells are returned to the subject either directly or, for example, encapsulated within porous membranes which are implanted into the patient. See, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187. There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, and calcium phosphate precipitation. A commonly used vector for ex vivo delivery of a nucleic acid is a retrovirus.

Other in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems. The nucleic acid and transfection agent are optionally associated with a microparticle. Exemplary transfection agents include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, quaternary ammonium amphiphile DOTMA ((dioleoyloxypropyl) trimethylammonium bromide, commercialized as Lipofectin by GIBCO-BRL))(Felgner et al, (1987) Proc. Natl. Acad. Sci. USA 84, 7413-7417; Malone et al. (1989) Proc. Natl Acad. Sci. USA 86 6077-6081); lipophilic glutamate diesters with pendent trimethylammonium heads (Ito et al. (1990) Biochem. Biophys. Acta 1023, 124-132); the metabolizable parent lipids such as the cationic lipid dioctadecylamido glycylspermine (DOGS, Transfectam, Promega) and dipalmitoylphosphatidyl ethanolamylspermine (DPPES)(J. P. Behr (1986) Tetrahedron Lett. 27, 5861-5864; J. P. Behr et al. (1989) Proc. Natl. Acad. Sci. USA 86, 6982-6986); metabolizable quaternary ammonium salts (DOTB, N-(1-[2,3-dioleoyloxy]propyl)-N,N,N-trimethylammonium methylsulfate (DOTAP)(Boehringer Mannheim), polyethyleneimine (PEI), dioleoyl esters, ChoTB, ChoSC, DOSC)(Leventis et al. (1990)

Biochim. Inter. 22, 235-241); 3beta[N-(N', N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), dioleoylphosphatidyl ethanolamine (DOPE)/3beta[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterolDC-Chol in one to one mixtures (Gao et al., (1991) Biochim. Biophys. Acta 1065, 8-14), spermine, spermidine, lipopolyamines (Behr et al., Bioconjugate Chem, 1994, 5: 382-389), lipophilic polylysines (LPLL) (Zhou et al., (1991) Biochim. Biophys. Acta 939, 8-18), [[(1,1,3,3-tetramethylbutyl)cre- soxy]ethoxy]ethyl]dimethylbe nzylammonium hydroxide (DEBDA hydroxide) with excess phosphatidylcholine/cholesterol (Ballas et al., (1988) Biochim. Biophys. Acta 939, 8-18), cetyltrimethylammonium bromide (CTAB)/DOPE mixtures (Pinnaduwage et al, (1989) Biochim. Biophys. Acta 985, 33-37), lipophilic diester of glutamic acid (TMAG) with DOPE, CTAB, DEBDA, didodecylammonium bromide (DDAB), and stearylamine in admixture with phosphatidylethanolamine (Rose et al., (1991) Biotechnique 10, 520-525), DDAB/DOPE (TransfectACE, GIBCO BRL), and oligogalactose bearing lipids. Exemplary transfection enhancer agents that increase the efficiency of transfer include, for example, DEAE-dextran, polybrene, lysosome-disruptive peptide (Ohmori N I et al, Biochem Biophys Res Commun Jun. 27, 1997;235(3):726-9), chondroitan-based proteoglycans, sulfated proteoglycans, polyethylenimine, polylysine (Pollard H et al. J Biol Chem, 1998 273 (13):7507-11), integrin-binding peptide CYGGRGDTP, linear dextran nonasaccharide, glycerol, cholesteryl groups tethered at the 3'-terminal internucleoside link of an oligonucleotide (Letsinger, R. L. 1989 Proc Natl Acad Sci USA 86: (17):6553-6), lysophosphatide, lysophosphatidylcholine, lysophosphatidylethanolamine, and 1-oleoyl lysophosphatidylcholine.

In some situations it may be desirable to deliver the nucleic acid with an agent that directs the nucleic acid-containing vector to target cells. Such "targeting" molecules include antibodies specific for a cell-surface membrane protein on the target cell, or a ligand for a receptor on the target cell. Where liposomes are employed, proteins which bind to a cell-surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake. Examples of such proteins include capsid proteins and fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. In other embodiments, receptor-mediated endocytosis can be used. Such methods are described, for example, in Wu et al., 1987 or Wagner et al., 1990. For review of the currently known gene marking and gene therapy protocols, see Anderson 1992. See also WO 93/25673 and the references cited therein. For additional reviews of gene therapy technology, see Friedmann, Science, 244: 1275-1281 (1989); Anderson, Nature, supplement to vol. 392, no 6679, pp. 25-30 (1998); Verma, Scientific American: 68-84 (1990); and Miller, Nature, 357: 455460 (1992).

Screening Methods

Effective therapeutics depend on identifying efficacious agents devoid of significant toxicity. Compounds potentially useful in preventing or treating bone loss associated with cancer metastasis may be screened using various assays. For instance, a candidate mutein may first be characterized in a cultured cell system to determine its ability to neutralize M-CSF in inducing osteoclastogenesis. Such a system may include the co-culture of mouse calvarial osteoblasts and spleen cells (Suda et al., Modulation of osteoclast differentiation. Endocr. Rev. 13: 66 80, 1992; Martin and Udagawa, Trends Endocrinol. Metab. 9: 6-12, 1998), the co-culture of mouse stromal cell lines (e.g., MC3T3-G2/PA6 and ST2) and mouse spleen cells (Udagawa et al., Endocrinology 125: 1805 13, 1989), and the co-culture of ST2 cells and bone marrow cells, peripheral blood mononuclear cells or alveolar macrophages (Udagawa et al., Proc. Natl. Acad. Sci. USA 87: 7260 4, 1990; Sasaki et al., Cancer Res. 58: 462 7, 1998; Mancino et al., J. Surg. Res.100: 18-24, 2001). In the absence of any M-CSF mutein, multinucleated cells formed in such co-cultures satisfy the major criteria of osteoclasts such as tartrate resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) activity, calcitonin receptors, p60C-STC, vitronectin receptors, and the ability to form resorption pits on bone and dentine slices. The presence of an effective M-CSF mutein inhibits the formation of such multinucleated cells.

In addition to the above co-culture systems, the ability of a candidate M-CSF mutein in inhibiting osteoclastogenesis may be assayed in a stromal cell-free or osteoblast-free system. The M-CSF required for osteoclastogenesis may be provided by co-cultured metastatic cancer cells (e.g., MDA 231) or conditioned medium from these cancer cells (Mancino et al., J. Surg. Res. 0: 18-24, 2001) or by addition of purified M-CSF.

Efficacy of a given M-CSF mutein in preventing or treating bone loss associated with cancer metastasis may also be tested in any of the animal bone metastasis model systems familiar to those skilled in the art. Such model systems include those involving direct injection of tumor cells into the medullary cavity of bones (Ingall, Proc. Soc. Exp. Biol. Med., 117: 819-22, 1964; Falasko, Clin. Orthop. 169: 20 7, 1982), into the rat abdominal aorta (Powles et al., Br. J. Cancer 28: 316 21, 1973), into the mouse lateral tail vein or into the mouse left ventricle (Auguello et al., Cancer Res. 48: 6876 81, 1988). In the absence of an effective M-CSF mutein, osteolytic bone metastases formed from injected tumor cells may be determined by radiographs (areas of osteolytic bone lesions) or histochemistry (bone and soft tissues). Sasaki et al., Cancer Res. 55: 3551 7, 1995; Yoneda et al., J. Clin. Invest. 99: 2509 17, 1997. Clohisy and Ramnaraine, Orthop Res. 16: 660 6, 1998. Yin et al., J. Clin. Invest. 103: 197 206, 1999. In the

presence of an effective M-CSF antigonist, osteolytic bone metastases may be prevented, or inhibited to result in fewer and/or smaller metastases.

The M-CSF muteins of the present invention may also be useful in preventing or treating cancer metastasis. The effectiveness of a candidate M-CSF mutein in preventing or treating cancer metastasis may be screened using a human amnionic basement membrane invasion model as described in Filderman et al., Cancer Res 52: 36616, 1992. In addition, any of the animal model systems for metastasis of various types of cancers may also be used. Such model systems include, but are not limited to, those described in Wenger et al., Clin. Exp. Metastasis 19: 169 73, 2002; Yi et al., Cancer Res. 62: 917 23, 2002; Tsutsumi et al., Cancer Lett 169: 77-85, 2001; Tsingotjidou et al., Anticancer Res. 21: 971 8, 2001; Wakabayashi et al., Oncology 59: 75 80, 2000; Culp and Kogerman, Front Biosci. 3:D672 83, 1998; Runge et al., Invest Radiol. 32: 212 7; Shioda et al., J. Surg. Oncol. 64: 122 6, 1997; Ma et al., Invest Ophthalmol Vis Sci. 37: 2293 301, 1996; Kuruppu et al., J Gastroenterol Hepatol. 11: 26 32, 1996. In the presence of an effective M-CSF antigonist, cancer metastases may be prevented, or inhibited to result in fewer and/or smaller metastases.

The anti-tumor activity of a particular M-CSF mutein, or combination of M-CSF antagonists, may be evaluated *in vivo* using a suitable animal model. For example, xenogenic lymphoma cancer models wherein human lymphoma cells are introduced into immune compromised animals, such as nude or SCID mice. Efficacy may be predicted using assays which measure inhibition of tumor formation, tumor regression or metastasis, and the like.

In one variation of an *in vitro* assay, the invention provides a method comprising the steps of (a) contacting an immobilized M-CSFR polypeptide with a candidate M-CSF mutein and (b) detecting binding of the candidate M-CSF mutein to the M-CSFR polypeptide. In an alternative embodiment, the candidate M-CSF mutein is immobilized and binding of M-CSFR polypeptide is detected. Immobilization is accomplished using any of the methods well known in the art, including covalent bonding to a support, a bead, or a chromatographic resin, as well as non-covalent, high affinity interaction such as antibody binding, or use of streptavidin/biotin binding wherein the immobilized compound includes a biotin moiety. Detection of binding can be accomplished (i) using a radioactive label on the compound that is not immobilized, (ii) using a fluorescent label on the non-immobilized compound, (iii) using an antibody immunospecific for the non-immobilized compound, (iv) using a label on the non-immobilized compound that excites a fluorescent support to which the immobilized compound is attached, as well as other techniques well known and routinely practiced in the art.

Methods of the invention to identify M-CSF muteins include variations on any of the methods described above, the variations including techniques wherein a M-CSF mutein is identified where binding between M-CSF and M-CSFR polypeptides changes in the presence of the candidate M-CSF mutein compared to binding in the absence of the candidate M-CSF mutein. A M-CSF mutein that increases binding between a M-CSF and M-CSFR polypeptide is described as an enhancer or activator, and a M-CSF mutein that decreases binding between a M-CSF and M-CSFR polypeptide is described as an inhibitor.

The invention also comprehends high throughput screening (HTS) assays to identify compounds that interact with or inhibit biological activity (i.e., inhibit enzymatic activity, binding activity, etc.) of a M-CSF polypeptide to a M-CSFR polypeptide. HTS assays permit screening of large numbers of compounds in an efficient manner. Cell-based HTS systems are contemplated to investigate the interaction between M-CSF and M-CSFR polypeptides. HTS assays are designed to identify "hits" or "lead compounds" having the desired property, from which modifications can be designed to improve the desired property. Chemical modification of the "hit" or "lead compound" is often based on an identifiable structure/activity relationship between the "hit" and the M-CSF–M-CSFR polypeptides.

Another aspect of the present invention is directed to methods of identifying M-CSF muteins that bind to a M-CSFR polypeptide, comprising contacting a M-CSFR polypeptide with a M-CSF mutein, and determining whether the M-CSF mutein binds the M-CSFR polypeptide. Binding can be determined by binding assays which are well known to the skilled artisan, including, but not limited to, gel-shift assays, Western blots, radiolabeled competition assay, phage-based expression cloning, co-fractionation by chromatography, co-precipitation, cross linking, interaction trap/two-hybrid analysis, southwestern analysis, ELISA, and the like, which are described in, for example, Current Protocols in Molecular Biology (1999) John Wiley & Sons, NY, which is incorporated herein by reference in its entirety. The M-CSFR polypeptide employed in such a test may either be free in solution, attached to a solid support, borne on a cell surface or located intracellularly or associated with a portion of a cell. One skilled in the art can, for example, measure the formation of complexes between a M-CSFR polypeptide and the M-CSF mutein being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between a M-CSF and M-CSFR polypeptide caused by the M-CSF mutein being tested.

Another aspect of the present invention is directed to methods of identifying compounds which modulate (i.e., decrease) activity of a M-CSFR polypeptide comprising

contacting a M-CSFR polypeptide with a M-CSF mutein, and determining whether the M-CSF mutein modifies activity of the M-CSFR polypeptide. The activity in the presence of the test M-CSF mutein is compared to the activity in the absence of the test M-CSF mutein. Where the activity of the sample containing the test M-CSF muteinis higher than the activity in the sample lacking the test M-CSF mutein, the compound will have increased activity. Similarly, where the activity of the sample containing the test M-CSF muteinis lower than the activity in the sample lacking the test M-CSF mutein, the compound will have inhibited activity.

The present invention is particularly useful for screening M-CSF muteins by using the M-CSF and/or M-CSFR polypeptides in any of a variety of drug screening techniques. The M-CSFR polypeptide employed in such a test may either be free in solution, attached to a solid support, borne on a cell surface or located intracellularly or associated with a portion of a cell. One skilled in the art can, for example, measure the formation of complexes between a M-CSFR polypeptide and the M-CSF mutein being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between a M-CSF and M-CSFR polypeptide caused by the M-CSF mutein being tested.

A variety of heterologous systems is available for functional expression of recombinant polypeptides that are well known to those skilled in the art. Such systems include bacteria (Strosberg, et al., Trends in Pharmacological Sciences (1992) 13:95-98), yeast (Pausch, Trends in Biotechnology (1997) 15:487-494), several kinds of insect cells (Vanden Broeck, Int. Rev. Cytology (1996) 164:189-268), amphibian cells (Jayawickreme et al., Current Opinion in Biotechnology (1997) 8: 629-634) and several mammalian cell lines (CHO, HEK293, COS, etc.; see Gerhardt, et al., Eur. J. Pharmacology (1997) 334:1-23). These examples do not preclude the use of other possible cell expression systems, including cell lines obtained from nematodes (PCT application WO 98/37177).

In preferred embodiments of the invention, methods of screening for compounds which modulate the activity M-CSFR polypeptides comprise contacting test M-CSF muteins with a M-CSFR polypeptide and assaying for the presence of a complex between the M-CSF mutein and the M-CSFR polypeptide. In such assays, the M-CSF mutein is typically labeled. After suitable incubation, free M-CSF mutein is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular M-CSF mutein to bind to the M-CSFR polypeptide.

In another embodiment of the invention, high throughput screening for M-CSF muteins having suitable binding affinity to a M-CSFR polypeptide is employed. Briefly, large

numbers of different test M-CSF muteins are immobilzed on a solid substrate. The test M-CSF muteins are contacted with a M-CSFR polypeptide and washed. Bound M-CSFR polypeptides are then detected by methods well known in the art. Purified M-CSF muteins of the invention can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the M-CSF mutein and immobilize it on the solid support.

Generally, an expressed M-CSF mutein can be used for HTS binding assays in conjunction with a substrate, ligand, adaptor or receptor molecule that is labeled with a suitable radioisotope, including, but not limited to, ^{125}I, ^{3}H, ^{35}S or ^{32}P, by methods that are well known t those skilled in the art. Alternatively, the substrate, ligand, adaptor or receptor molecule may b labeled by well-known methods with a suitable fluorescent derivative (Baindur, et al., Drug De Res., (1994) 33:373-398; Rogers, Drug Discovery Today (1997) 2:156-160). Radioactive ligan specifically bound to immobilized M-CSF mutein can be detected in HTS assays in one of several standard ways, including filtration of the M-CSF mutein-ligand complex to separate bound ligand from unbound ligand (Williams, Med. Res. Rev. (1991) 11, 147-184; Sweetnam, e al., J. Natural Products (1993) 56, 441-455). Alternative methods include a scintillation proximity assay (SPA) or a FlashPlate format in which such separation is unnecessary (Nakayama, Cur. Opinion Drug Disc. Dev. (1998) 1:85-91 Bossé, et al., J. Biomolecular Screening (1998) 3: 285-292.). Binding of fluorescent ligands can be detected in various ways, including fluorescence energy transfer (FRET), direct spectrophotofluorometric analysis of bound ligand, or fluorescence polarization (Rogers, Drug Discovery Today (1997) 2, 156-160; Hill, Cur. Opinion Drug Disc. Dev. (1998) 1, 92-97).

The invention contemplates a multitude of assays to screen and identify inhibitor of substrate, ligand, adaptor or receptor binding to M-CSFR. In one example, M-CSF or M-CSFR is immobilized and interaction with a binding partner is assessed in the presence and absence of a candidate M-CSF mutein. In another example, interaction between M-CSF and M-CSFR is assessed in a solution assay, both in the presence and absence of a candidate M-CSF mutein. In either assay, an inhibitor is identified as a M-CSF mutein that decreases binding between the M-CSF and M-CSFR. Another contemplated assay involves a variation of the di-hybrid assay wherein an inhibitor of protein/protein interactions is identified by detection of a positive signal in a transformed or transfected host cell, as described in PCT publication numbe WO 95/20652, published August 3, 1995.

Other assays may be used to identify specific ligands of M-CSFR, including

assays that identify ligands of the M-CSFR through measuring direct binding of test M-CSF muteins to the target protein, as well as assays that identify ligands of target proteins through affinity ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Alternatively, such binding interactions are evaluated indirectly using the yeast two-hybrid system described in Fields et al., Nature, 340:245-246 (1989), and Fields et al., Trends in Genetics, 10:286-292 (1994), both of which are incorporated herein by reference. The two-hybrid system is a genetic assay for detecting interactions between two proteins or polypeptides. It can be used to identify proteins that bind to a known protein of interest, or to delineate domains or residues critical for an interaction. Variations on this methodology have been developed to clone genes that encode DNA binding proteins, to identify peptides that bind to a protein, and to screen for drugs. The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA binding domain that binds to an upstream activation sequence (UAS) of a reporter gene, and is generally performed in yeast. The assay requires the construction of two hybrid genes encoding (1) a DNA-binding domain that is fused to a first protein and (2) an activation domain fused to a second protein. The DNA-binding domain targets the first hybrid protein to the UAS of the reporter gene; however, because most proteins lack an activation domain, this DNA-binding hybrid protein does not activate transcription of the reporter gene. The second hybrid protein, which contains the activation domain, cannot by itself activate expression of the reporter gene because it does not bind the UAS. However, when both hybrid proteins are present, the noncovalent interaction of the first and second proteins tethers the activation domain to the UAS, activating transcription of the reporter gene. For example, when the first protein is M-CSF or M-CSFR, or subunit or fragment thereof, that is known to interact with another protein or nucleic acid, this assay can be used to detect agents that interfere with the binding interaction. Expression of the reporter gene is monitored as different test agents are added to the system. The presence of an inhibitory agent results in lack of a reporter signal.

The yeast two-hybrid assay can also be used to identify proteins that bind to M-CSFR. In an assay to identify M-CSF muteins that bind to M-CSFR, or subunit or fragment thereof, a fusion polynucleotide encoding a M-CSFR (or subunit or fragment) and a UAS binding domain (i.e., a first protein) may be used. In addition, a large number of hybrid genes each encoding a different second protein (i.e., M-CSF mutein) fused to an activation domain are produced and screened in the assay. Typically, the second protein is encoded by one or more members of a total cDNA or genomic DNA fusion library, with each second protein-coding region being fused to the activation domain. This system is applicable to a wide variety of

proteins, and it is not even necessary to know the identity or function of the second binding protein. The system is highly sensitive and can detect interactions not revealed by other methods; even transient interactions may trigger transcription to produce a stable mRNA that can be repeatedly translated to yield the reporter protein.

Other assays may be used to search for agents that bind to the target protein. One such screening method to identify direct binding of test ligands to a target protein is described in U.S. Patent No. 5,585,277, incorporated herein by reference. This method relies on the principle that proteins generally exist as a mixture of folded and unfolded states, and continually alternate between the two states. When a test ligand binds to the folded form of a target protein (i.e., when the test ligand is a ligand of the target protein), the target protein molecule bound by the ligand remains in its folded state. Thus, the folded target protein is present to a greater extent in the presence of a test ligand which binds the target protein, than in the absence of a ligand. Binding of the ligand to the target protein can be determined by any method which distinguishes between the folded and unfolded states of the target protein. The function of the target protein need not be known in order for this assay to be performed. Virtually any agent can be assessed by this method as a test ligand, including, but not limited to, metals, polypeptides, proteins, lipids, polysaccharides, polynucleotides and small organic molecules.

Other embodiments of the invention comprise using competitive screening assays in which neutralizing antibodies capable of binding a M-CSFR polypeptide of the invention specifically compete with a test M-CSF mutein for binding to the M-CSFR polypeptide. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants with M-CSFR. Radiolabeled competitive binding studies are described in A.H. Lin et al. Antimicrobial Agents and Chemotherapy (1997) vol. 41, no. 10. pp. 2127-2131, the disclosure of which is incorporated herein by reference in its entirety.

In other embodiments of the invention, the M-CSF muteins of the invention are employed as a research tool for identification, characterization and purification of interacting, regulatory proteins. Appropriate labels are incorporated into the M-CSF muteins of the invention by various methods known in the art and the polypeptides are used to capture interacting molecules. For example, molecules are incubated with the labeled M-CSF muteins, washed to removed unbound M-CSF muteins, and the M-CSF mutein complex is quantified. Data obtained using different concentrations of M-CSF mutein are used to calculate values for the number, affinity, and association of M-CSF mutein with the protein complex.

Labeled M-CSFR polypeptides are also useful as reagents for the purification of

molecules with which the polypeptide interacts including, but not limited to, M-CSF muteins. In one embodiment of affinity purification, a polypeptide is covalently coupled to a chromatography column. Cells and their membranes are extracted, and various cellular subcomponents are passed over the column. Molecules bind to the column by virtue of their affinity to the polypeptide. The polypeptide-complex is recovered from the column, dissociated and the recovered molecule is subjected to protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate oligonucleotides for cloning the corresponding gene from an appropriate cDNA library.

Computer modeling can be used to develop a putative tertiary structure of the M-CSF muteins of the invention based on the available information of M-CSF or M-CSFR. Thus, novel ligands based on the predicted structure of M-CSF or M-CSFR can be designed.

Another aspect of the present invention is the use of the M-CSF or M-CSFR nucleotide sequences disclosed herein for identifying homologs in other animals. Any of the nucleotide sequences disclosed herein, or any portion thereof, can be used, for example, as probes to screen databases or nucleic acid libraries, such as, for example, genomic or cDNA libraries, to identify homologs, using screening procedures well known to those skilled in the art. Accordingly, homologs having at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95%, and most preferably at least 100% homology with M-CSF or M-CSFR sequences can be identified.

Combination Therapy

Having identified more than one M-CSF mutein that is effective in an animal model, it may be further advantageous to mix two or more such M-CSF antagonists together to provide still improved efficacy against cancer metastasis and/or bone loss associated with cancer metastasis. Compositions comprising one or more M-CSF antagonist may be administered to persons or mammals suffering from, or predisposed to suffer from, cancer metastasis and/or bone loss associated with cancer metastasis.

Although M-CSF antagonist therapy may be useful for all stages of cancers, M-CSF mutein therapy may be particularly appropriate in advanced or metastatic cancers. Combining the M-CSF mutein therapy method a chemotherapeutic or radiation regimen may be preferred in patients that have not received chemotherapeutic treatment, whereas treatment with the M-CSF mutein therapy may be indicated for patients who have received one or more chemotherapies. Additionally, M-CSF mutein therapy can also enable the use of reduced

dosages of concomitant chemotherapy, particularly in patients that do not tolerate the toxicity of the chemotherapeutic agent very well.

The method of the invention contemplate the administration of single M-CSF muteins, as well as combinations, or "cocktails", of different M-CSF muteins. Such M-CSF muteins in combination may exhibit synergistic therapeutic effects. In addition, the administration of M-CSF muteins may be combined with other therapeutic agents and/or procedures, including but not limited to various chemotherapeutic agents, androgen-blockers, and immune modulators (e.g., IL-2, GM-CSF), Bisphosphonate(s) (e.g., Aredia; Zometa; Clodronate), surgery, radiation, chemotherapy, hormone therapy (e.g., Tamoxifen; anti-Androgen therapy), antibody therapy (e.g., RANKL/RANK neutralizing antibodies; PTHrP neutralizing antibody, anti-Her2 antibody, VEGF neutralizing antibody), therapeutic protein therapy (e.g., soluble RANKL receptor; OPG, and PDGF and MMP inhibitors), small molecule drug therapy (e.g., Src-kinase inhibitor), kinase inhibitors of growth factor receptors; oligonucleotides therapy (e.g., RANKL or RANK or PTHrP Anti-sense), gene therapy (e.g., RANKL or RANK inhibitors), peptide therapy (e.g. muteins of RANKL) as well as those proteins, peptides, compounds, and small molecules described herein.

Cancer chemotherapeutic agents include, without limitation, alkylating agents, such as carboplatin and cisplatin; nitrogen mustard alkylating agents; nitrosourea alkylating agents, such as carmustine (BCNU); antimetabolites, such as methotrexate; purine analog antimetabolites, mercaptopurine; pyrimidine analog antimetabolites, such as fluorouracil (5-FU) and gemcitabine; hormonal antineoplastics, such as goserelin, leuprolide, and tamoxifen; natural antineoplastics, such as aldesleukin, interleukin-2, docetaxel, etoposide (VP-16), interferon alfa, paclitaxel, and tretinoin (ATRA); antibiotic natural antineoplastics, such as bleomycin, dactinomycin, daunorubicin, doxorubicin, and mitomycin; and vinca alkaloid natural antineoplastics, such as vinblastine, vincristine, vindesine; hydroxyurea; aceglatone, adriamycin, ifosfamide, enocitabine, epitiostanol, aclarubicin, ancitabine, nimustine, procarbazine hydrochloride, carboquone, carboplatin, carmofur, chromomycin A3, antitumor polysaccharides, antitumor platelet factors, cyclophosphamide, Schizophyllan, cytarabine, dacarbazine, thioinosine, thiotepa, tegafur, , neocarzinostatin, OK-432, bleomycin, furtulon, broxuridine, busulfan, honvan, peplomycin, , Bestatin (ubenimex), interferon- β, mepitiostane, mitobronitol, merphalan, laminin peptides, lentinan, Coriolus versicolor extract, tegafur/uracil, estramustine (estrogen/mechlorethamine).

Further, additional agents used as therapy for cancer patients include EPO, G-

CSF, ganciclovir; antibiotics, leuprolide; meperidine; zidovudine (AZT); interleukins 1 through 18, including mutants and analogues; interferons or cytokines, such as interferons α, β, and γ hormones, such as luteinizing hormone releasing hormone (LHRH) and analogues and, gonadotropin releasing hormone (GnRH); growth factors, such as transforming growth factor- β (TGF- β), fibroblast growth factor (FGF), nerve growth factor (NGF), growth hormone releasing factor (GHRF), epidermal growth factor (EGF), fibroblast growth factor homologous factor (FGFHF), hepatocyte growth factor (HGF), and insulin growth factor (IGF); tumor necrosis factor- α & β (TNF- α & β); invasion inhibiting factor-2 (IIF-2); bone morphogenetic proteins 1-7 (BMP 1-7); somatostatin; thymosin- α -1; γ-globulin; superoxide dismutase (SOD); complement factors; anti-angiogenesis factors; antigenic materials; and pro-drugs.

Administration and preparation

The present invention provides compounds, pharmaceutical formulations including the compounds, methods of preparing the pharmaceutical formulations, and methods of treating patients with the pharmaceutical formulations and compounds.

Such compositions can be in the form of, for example, granules, powders, tablets, capsules, syrup, suppositories, injections, emulsions, elixirs, suspensions or solutions. The instant compositions can be formulated for various routes of administration, for example, by oral administration, by nasal administration, by rectal administration, subcutaneous injection, intravenous injection, intramuscular injections, or intraperitoneal injection. The following dosage forms are given by way of example and should not be construed as limiting the instant invention.

For oral, buccal, and sublingual administration, powders, suspensions, granules, tablets, pills, capsules, gelcaps, and caplets are acceptable as solid dosage forms. These can be prepared, for example, by mixing one or more compounds of the instant invention, or pharmaceutically acceptable salts or tautomers thereof, with at least one additive such as a starch or other additive. Suitable additives are sucrose, lactose, cellulose sugar, mannitol, maltitol, dextran, starch, agar, alginates, chitins, chitosans, pectins, tragacanth gum, gum arabic, gelatins, collagens, casein, albumin, synthetic or semi-synthetic polymers or glycerides. Optionally, oral dosage forms can contain other ingredients to aid in administration, such as an inactive diluent, or lubricants such as magnesium stearate, or preservatives such as paraben or sorbic acid, or anti-oxidants such as ascorbic acid, tocopherol or cysteine, a disintegrating agent, binders, thickeners, buffers, sweeteners, flavoring agents or perfuming agents. Tablets and pills may be further treated with suitable coating materials known in the art.

Liquid dosage forms for oral administration may be in the form of pharmaceutically acceptable emulsions, syrups, elixirs, suspensions, and solutions, which may contain an inactive diluent, such as water. Pharmaceutical formulations and medicaments may be prepared as liquid suspensions or solutions using a sterile liquid, such as, but not limited to, an oil, water, an alcohol, and combinations of these. Pharmaceutically suitable surfactants, suspending agents, emulsifying agents, may be added for oral or parenteral administration.

As noted above, suspensions may include oils. Such oil include, but are not limited to, peanut oil, sesame oil, cottonseed oil, corn oil and olive oil. Suspension preparation may also contain esters of fatty acids such as ethyl oleate, isopropyl myristate, fatty acid glycerides and acetylated fatty acid glycerides. Suspension formulations may include alcohols, such as, but not limited to, ethanol, isopropyl alcohol, hexadecyl alcohol, glycerol and propylene glycol. Ethers, such as but not limited to, poly(ethyleneglycol), petroleum hydrocarbons such as mineral oil and petrolatum; and water may also be used in suspension formulations.

For nasal administration, the pharmaceutical formulations and medicaments may be a spray or aerosol containing an appropriate solvent(s) and optionally other compounds such as, but not limited to, stabilizers, antimicrobial agents, antioxidants, pH modifiers, surfactants, bioavailability modifiers and combinations of these. A propellant for an aerosol formulation may include compressed air, nitrogen, carbon dioxide, or a hydrocarbon based low boiling solvent.

Injectable dosage forms generally include aqueous suspensions or oil suspensions which may be prepared using a suitable dispersant or wetting agent and a suspending agent. Injectable forms may be in solution phase or in the form of a suspension, which is prepared with a solvent or diluent. Acceptable solvents or vehicles include sterilized water, Ringer's solution, or an isotonic aqueous saline solution. Alternatively, sterile oils may be employed as solvents or suspending agents. Preferably, the oil or fatty acid is non-volatile, including natural or synthetic oils, fatty acids, mono-, di- or tri-glycerides.

For injection, the pharmaceutical formulation and/or medicament may be a powder suitable for reconstitution with an appropriate solution as described above. Examples of these include, but are not limited to, freeze dried, rotary dried or spray dried powders, amorphous powders, granules, precipitates, or particulates. For injection, the formulations may optionally contain stabilizers, pH modifiers, surfactants, bioavailability modifiers and combinations of these.

For rectal administration, the pharmaceutical formulations and medicaments may

be in the form of a suppository, an ointment, an enema, a tablet or a cream for release of compound in the intestines, sigmoid flexure and/or rectum. Rectal suppositories are prepared by mixing one or more compounds of the instant invention, or pharmaceutically acceptable salts or tautomers of the compound, with acceptable vehicles, for example, cocoa butter or polyethylene glycol, which is present in a solid phase at normal storing temperatures, and present in a liquid phase at those temperatures suitable to release a drug inside the body, such as in the rectum. Oils may also be employed in the preparation of formulations of the soft gelatin type and suppositories. Water, saline, aqueous dextrose and related sugar solutions, and glycerols may be employed in the preparation of suspension formulations which may also contain suspending agents such as pectins, carbomers, methyl cellulose, hydroxypropyl cellulose or carboxymethyl cellulose, as well as buffers and preservatives.

Besides those representative dosage forms described above, pharmaceutically acceptable excipients and carries are generally known to those skilled in the art and are thus included in the instant invention. Such excipients and carriers are described, for example, in "Remingtons Pharmaceutical Sciences" Mack Pub. Co., New Jersey (1991), which is incorporated herein by reference.

The formulations of the invention may be designed to be short-acting, fast-releasing, long-acting, and sustained-releasing as described below. Thus, the pharmaceutical formulations may also be formulated for controlled release or for slow release.

The instant compositions may also comprise, for example, micelles or liposomes, or some other encapsulated form, or may be administered in an extended release form to provide a prolonged storage and/or delivery effect. Therefore, the pharmaceutical formulations and medicaments may be compressed into pellets or cylinders and implanted intramuscularly or subcutaneously as depot injections or as implants such as stents. Such implants may employ known inert materials such as silicones and biodegradable polymers.

Specific dosages may be adjusted depending on conditions of disease, the age, body weight, general health conditions, sex, and diet of the subject, dose intervals, administration routes, excretion rate, and combinations of drugs. Any of the above dosage forms containing effective amounts are well within the bounds of routine experimentation and therefore, well within the scope of the instant invention.

By the present methods, compositions comprising M-CSF muteins may be administered parenterally, topically, orally or locally for therapeutic treatment. Preferably, the compositions are administered orally or parenterally, i.e., intravenously, intraperitoneally,

intradermally or intramuscularly. Thus, this invention provides methods which employ compositions for administration which comprise one or more M-CSF antagonists in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like, and may include other proteins for enhanced stability, such as albumin, lipoprotein, globulin, etc., subjected to mild chemical modifications or the like.

M-CSF muteins useful as therapeutics for cancer metastasis or bone loss associated with cancer metastasis will often be prepared substantially free of other naturally occurring immunoglobulins or other biological molecules. Preferred M-CSF muteins will also exhibit minimal toxicity when administered to a mammal afflicted with, or predisposed to suffer from, cancer metastasis and/or bone loss associated with cancer metastasis.

The compositions of the invention may be sterilized by conventional, well known sterilization techniques. The resulting solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride and stabilizers (e.g., 1 20% maltose, etc.).

The M-CSF muteins of the present invention may also be administered via liposomes. Liposomes, which include emulsions, foams, micelles, insoluble monolayers, phospholipid dispersions, lamellar layers and the like, can serve as vehicles to target the M-CSF muteins to a particular tissue as well as to increase the half life of the composition. A variety of methods are available for preparing liposomes, as described in, e.g., U.S. Patent Nos. 4,837,028 and 5,019,369, which patents are incorporated herein by reference.

The concentration of the M-CSF mutein in these compositions can vary widely, i.e., from less than about 10%, usually at least about 25% to as much as 75% or 90% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Actual methods for preparing orally, topically and parenterally administrable compositions will be known or apparent to those skilled in the art and are described in detail in, for example, Remington's Pharmaceutical Science, 19th ed., Mack Publishing Co., Easton, PA (1995), which is incorporated herein by reference.

Determination of an effective amount of a composition of the invention to treat

cancer metastasis and/or bone loss associated with cancer metastasis in a patient can be accomplished through standard empirical methods which are well known in the art. For example, the in vivo neutralizing activity of sera from a subject treated with a given dosage of M-CSF mutein may be evaluated using an assay that determines the ability of the sera to block M-CSF induced proliferation and survival of murine monocytes (CD11b+ cell, a subset of CD11 cells, which expresses high levels of receptor to M-CSF) in vitro as described in Cenci et al., J Clin. Invest. 1055: 1279-87, 2000.

Compositions of the invention are administered to a mammal already suffering from, or predisposed to, cancer metastasis and/or bone loss associated with cancer metastasis in an amount sufficient to prevent or at least partially arrest the development of cancer metastasis and/or bone loss associated with cancer metastasis. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Effective amounts of a M-CSF mutein will vary and depend on the severity of the disease and the weight and general state of the patient being treated, but generally range from about 1.0 μg/kg to about 100 mg/kg body weight, with dosages of from about 10 μg/kg to about 10 mg/kg per application being more commonly used. Administration is daily, weekly or less frequently, as necessary depending on the response to the disease and the patient's tolerance of the therapy. Maintenance dosages over a prolonged period of time may be needed, and dosages may be adjusted as necessary.

Single or multiple administrations of the compositions can be carried out with the dose levels and pattern being selected by the treating physician. In any event, the formulations should provide a quantity of M-CSF mutein sufficient to effectively prevent or minimize the severity of cancer metastasis and/or bone loss associated with cancer metastasis. The compositions of the present invention may be administered alone or as an adjunct therapy in conjunction with other therapeutics known in the art for the treatment of cancer metastasis and/or bone loss associated with cancer metastasis.

The invention is illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

EXAMPLE 1

This example shows that highly metastatic breast cancer cell lines express high

levels of M-CSF. Using microarrays, the M-CSF gene expression by the highly metastatic cell line, MDA 231, was compared with that of the cell lines MCF7 and ZR751. There was a 6.9 fold increase when the M-CSF expression level in MDA 231 was compared with that in MCF7, and a 5.2 fold increase when the M-CSF expression level in MDA 231 was compared with that in ZR751.

EXAMPLE 2

This example shows that purified M-CSF can be replaced by conditioned media (CM) from the metastatic cell line MDA 231 but not from the cell line MCF7 in *in vitro* assays of osteoclast formation (Figure 3).

Production of conditioned media (CM): MDA 231 or MCF7 cells were plated at a density of 1 x 10^6 cells/10 cm dish in 8 mls of 50% DMEM/ 50% HAMs F12 containing 1 x ITS, (BD Biosciences, Lexington, Ky), a culture supplement containing insulin, human transferrin, and selenous acid. After 48 hours of incubation at 37°C in 5% CO_2, the media were collected and centrifuged for 10 minutes at 1500 RPM to remove any suspended cells. The supernatant was collected, filtered through a 0.2 nM filter, and used as CM.

Osteoclast assay: Bone marrow $CD34^+$ cells were plated at a density of 15,000 cells/ 96 well in 100 µl of Alpha MEM containing 10% FCS, 1 X Pen/Strep and 1 x fungizone. The next day, 50 µl of media was removed from each well and replaced with 25 µl of Alpha MEM media and 75 µl of CM or 50% DMEM/ 50% HAMs F12 containing 1 x ITS. RANKL was added to each well at a final concentration of 100 ng/ml and 30 ng/ml M-CSF was added to the appropriate wells. The cells were incubated at 37°C in 5% CO_2 for 11 days. During that time fresh RANKL was added again after 6 days. After 11 days the cells were fixed and stained for tartrate resistant acid phosphatase using the Leukocyte acid phosphatase kit from Sigma.

Results: As shown in Figure 3, purified M-CSF can be replaced by conditioned media (CM) from the metastatic cell line MDA 231 but not from the cell line MCF7 in *in vitro* assays of osteoclast formation.

EXAMPLE 3

The following example provides a method for the design and production of M-CSF muteins.

X-ray crystallographic data described in U.S. Patent No. 6,025,146 provides sufficient structural information regarding M-CSF to be able to identify a limited subset of the amino acids in the protein that are likely to be crucial for M-CSF receptor binding and biological activity and thus which represented likely candidates for mutagenesis with the ultimate goal of providing M-CSF muteins having altered biological activity (i.e., agonists or antagonists). Based on this information, several criteria are used to generate a list of possible target amino acids for substitution.

The first criterion is solvent exposure or solvent accessibility, which refers to amino acids residues at the surface of the protein. Residues having a solvent accessible surface area of greater than about 0.25 and preferably greater than about 0.4 are preferred based on normalization of the surface area of the amino acid accessible when in the trypeptide gly-x-gly (Kabsch, W. et al., Biopolymers 22:2577 (1983)). Residues are chosen which do not interact with other parts of the protein such as the dimer interface in order to maintain the relative orientation of monomers and to avoid disturbing the process of protein folding. Still another criterion used in certain instances in selecting candidate amino acid substances is the relationship of the residues to corresponding residues in mouse M-CSF. Another important selection criterion is that the substitutions be non-conservative so as to attempt to disrupt possible hydrogen bonding or hydrophobic interactions with M-CSF receptor residues.

Preparation of M-CSF muteins can be carried essentially as described in U.S. Patent No. 6,025,146. Briefly, a variety of M-CSF muteins with altered solvent-accessible residues from regions of the M-CSF mature N terminus and helices A, C, and D are constructed using techniques known in the art. For example, two histidines in the N-terminal/A helix region are changed to alanine through site-directed mutagenesis of a truncated form of M-CSFα (encoded by pLCSF158A). Involvement of one of three M-CSF histidine residues in M-CSF receptor interaction is implicated by the observation that diethylpyrocarbonate (DEPC) modification of histidines in M-CSF at a 1:100 DEPC:histidine ratio (as described in Meth. in Enzymol. 47:431 (1977)) significantly reduced bioactivity.

Plasmid DNA pLCSF158A is prepared from the E. coli strain HW22 carrying the plasmid pLCSF158A (U.S. Pat. No. 4,929,700, Example 6, "E. coli strain HW22 transformed with pJN653 containing the asp.sub.59 SCSF/N.DELTA.3C.DELTA.158 gene"). The strain is grown in 350 ml R2 media (2x Luria Broth containing 1% sodium chloride and no glucose, J. Bact., 74:461 (1957)) containing 50 micrograms/ml ampicillin at 30 °C with shaking overnight. Plasmid DNA is prepared from the cells using a Qiagen-tip 100 column according to the

manufacturer's directions.

Twenty micrograms of pLCSF158A DNA is digested with 66 units of HindIII and 66 units of StuI at 37 °C for 3 hr. 20 min. in 200 microliters 1x New England Biolabs NEBuffer 2 (New England Biolabs, Beverly, Mass.). The DNA is extracted with phenol and chloroform, then ethanol precipitated. The DNA is treated with one unit of Calf Intestinal Alkaline Phosphatase in 100 microliters of 1x Dephosphorylation Buffer, supplied by Boehringer Mannheim (Indianapolis, Ind.), at 37 °C for 30 min. An additional unit of Calf Intestinal Alkaline Phosphatase is added to the reaction and incubation was continued at 50 °C for 1 hr. The resulting DNA is then run on a 1% FMC Bioproducts (Rockland, Me.) Sea KEM.RTM. GTG.RTM. agarose gel. The 5.7 kb pLCSF158A fragment is cut from the gel and purified on Qiagen (Chatsworth, Calif.) Qiaex beads according to the manufacturer's directions.

Polymerase chain reaction (PCR) is then performed and a PCR product is produced that contained a mutagenized M-CSF sequence in which, for example, histidines 9 and 15 (counting from the mature N-terminus) were altered to alanine (generating an H9A, H15A PCR fragment).

Five microliters of each reaction is run on a 3% agarose gel (1.5% FMC Bioproducts SeaKem.RTM. GTG.RTM. agarose, 1.5% FMC Bioproducts NuSeive.RTM. GTG.RTM. agarose in Tris-Borate buffer) (FMC Bioproducts, Rockland, Me.). Gels are then stained with ethidium bromide. Positive reactions are pooled, extracted with phenol and chloroform, precipitated with ethanol, resuspended and digested with 250 units of StuI in a final volume of 500 microliters 1x NEBuffer 2 at 37 °C for 2 hr., 500 units of HindIII are added to the reaction, the volume increased to 1 ml in 1x NEBuffer 2 and digestion was continued at 37 °C for an additional 2.5 hr. The DNA is electrophoresed on a 3% agarose gel. The digested product is cut from the gel and purified on Qiagen Qiaex beads according to the manufacturer's directions.

The PCR product is then ligated to pLCSF158A vector DNA at an insert-to-vector ratio of approximately 5:1. Ligation is carried out with 1 unit of Boehringer Mannheim T4 DNA ligase in 1x ligation buffer, supplied by the manufacturer, in a 20-microliter volume at 16 °C overnight.

Half of each ligation mixture is used to transform competent E. coli DG116 (ATCC#53606) cells using a protocol similar to the calcium chloride procedure described in Molecular Cloning a Laboratory Manual Maniatis et al., Cold Spring Harbor Laboratory (1982). Transformed cells are allowed to express at 30 °C with no selection for 90 min., plated on R2-4

(10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 drops antifoam A, 4 ml 50% glucose and 15 g agar in 1 liter) plates containing 50 micrograms/ml ampicillin. The plates are incubated at room temperature 72 hr. One fourth of each transformation is plated.

Transformants are then cultured and sequenced to verify the presence of the plasmid containing the M-CSF mutein sequence.

The M-CSF muteins can be expressed, purified, refolded to form dimeric protein and assayed essentially as described in U.S. Pat. No. 4,929,700, Example 5, using 8M urea as a denaturant and in the DEAE purification step.

EXAMPLE 4

This example provides a method for testing M-CSF muteins for their ability to bind M-CSFR.

An essential step in the biological function of M-CSF in vivo is the binding to the M-CSF receptor, also referred to as the c-fms gene product. Recombinant human soluble M-CSF receptor (rhsM-CSFR), representing amino acids 20 to 511 of SEQ ID NO: 8 (Coussens, L et al., Nature, 320:277 (1986)) is used as an in vitro assay reagent to test the receptor-binding ability of M-CSF proteins. To generate a soluble form of the transmembrane receptor, only the extracellular domain of the human M-CSF receptor is expressed in a baculovirus/insect cell recombinant expression system. In order to purify the soluble receptor without adversely effecting tertiary or quaternary structure, non-denaturing chromatographic methods are chosen. Other choices exist for the purification of the recombinant receptor. Affinity chromatography may be employed when either a suitable antibody to or ligand for the receptor are available. Alternatively, "tags" may be added to the C-terminus of the recombinant receptor, i.e., KT3 antibody recognition sequence, and purified by an anti-tag antibody, i.e., KT3, column, for use in affinity chromatography. In expression systems in which the rhsM-CSFR is glycosylated, lectin chromatography can be used to enrich for specific glycoproteins.

The rhsM-CSFR can be used to study ligand/receptor interactions as well as ligand-induced receptor dimerization. The assay used to detect ligand/receptor binding employs the use of size exclusion-HPLC, essentially as described in European Patent Application WO92/21029, C. Cunningham, et al., with the following modifications: the column used is a Superose 6 (Pharmacia LKB Biotechnology, Inc.) and the mobile phase is PBS at 0.5 ml/min and a M-CSF to rhsM-CSFR ratio of 1:2. At this ratio, the M-CSF/rhsM-CSFR complex chromatographs with an expected hydrodynamic radius of 190,00 molecular weight. Other

assays may be employed to measure ligand/receptor binding or receptor dimerization such as chemical crosslinking and SDS-PAGE or immunoprecipitation and SDS-PAGE. Molecules that inhibit receptor dimerization but not ligand binding provide another method to antagonize M-CSF actions.

5

EXAMPLE 5

The following example provides a method for testing M-CSF muteins for their ability to interfere with activation of M-CSFR by native M-CSF.

Competition for binding M-CSFR between native M-CSF and a candidate M-
10 CSF mutein is accomplished with a modification of the binding assays described in Example 4. Briefly, the size-exclusion HPLC method described above is carried out in the presence and absence of a candidate M-CSF mutein. M-CSF muteins that have the ability to interefere with the M-CSFR/M-CSF intereaction are then identified.

15 EXAMPLE 6

This example provides a method for testing M-CSF muteins for in vitro activity.

To measure the neutralizing ability of the M-CSF muteins against the activity of human M-CSF on murine M NFS 60 cells (American Type Culture Collection Accession No. CRL-1838, available from ATCC in Rockville, MD, USA, derived from a myelogenous
20 leukemia induced with the Cas-Br-MuLV wild mouse ecotropic retrovirous, responsive to both interleukin 3 and M-CSF and which contain a truncated c-myb proto-oncogene caused by the integration of a retrovirus), recombinant human CSF-1 (at 10 ng/ml final concentration) is pre-incubated with various concentrations (1 ng/ml to 1mg/ml) of a candidate M-CSF mutein for 1 hour at 37°C in 5% CO_2 in an incubator. Following the incubation, the mixture is added to the
25 M NFS 60 culture in 96 well microtiter plates. The total assay volume per well is 100µl, with 10 ng/ml rhM-CSF, and cell density at 5,000 cells/well. After 72 hours culture in a CO_2 incubator at 37°C, cell proliferation is assayed by CeIITiter Glo Kit (Promega).

It is expected that mixtures containing M-CSF mutein will inhibit M-CSF-induced M NFS 60 cell proliferation.

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EXAMPLE 7

The following example provides a method for testing muteins for in vivo activity.

Experimental Design. To evaluate the efficacy of M-CSF muteins as a therapeutic agent for the treatment of osteolysis, the highly metastatic human breast cancer cell line MDA-231 (3×10^5) was injected into the tibia bone marrow cavity of female nude mice. The mice were at the age of 4-7 weeks old, and had an average weight of ~20g. The mice were chipped for identification and underwent an acclimation period of at least 7 days prior to the start of the 8-week study.

The mice received a total dose of 1.5mpk (0.03mg per mouse) Buprenorphine subcutaneously at both flank 30 minutes before intra-tibia injection. Mice were anesthetized by isoflurane inhalation and the right hind leg was cleaned with 70% ethanol. Tumor cells (MDA-MB-231-luc, $3x10^5$) suspended in 10 µl of saline was injected into the right tibia bone marrow cavity using 50 or 100 µl micro-syringe.

Dosing. Treatment with M-CSF mutein begins the day following the injection of the tumor cells. The dosing will be a range from 0.1 mg/kg to 50 mg/kg. An example of the highest dosing value, 50 mg/kg, is as follows. The dosing solutions are provided at a pre-diluted concentration (=10 mg/ml) such that 100 uL injected IP will deliver the target dose for a 20 gram mouse (=1000 µg). For weight adjustment, the volume injected is increased or decreased by 5 µl per gram of weight difference. For example, a 23 gram mouse receives 115 µl, while an 18 gram mouse receives 90 µl.

Measurements. To assess the severity of osteolysis, each mouse receives a baseline Faxitron image taken the day following injection of tumor cells. Additionally, a Faxitron image is taken at the end of the study (8 weeks). Tumor growth is simultaneously measured using the Xenogen system since the tumor cells stably express luciferase.

It is contemplated that the number of animals with a mean osteolysis score of $\geq$ 2.5 is lowest in the group that received the M-CSF mutein treatment. Osteolytic bone damage is evaluated on a scale from 0-4, with 0 equal to no bone damage; 1-2 equal to some bone damage, such that scores of 2.25 or greater is indicative of severe bone damage.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by

reference, in their entirety.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

What is claimed:

1. A method of preventing bone metastases comprising administering to a subject afflicted with metastatic cancer a therapeutically effective amount of a M-CSF mutein or mutein product thereby preventing bone loss associated with the metastatic cancer.

2. A method of treating a subject afflicted with a metastatic cancer to bone comprising administering to said subject a therapeutically effective amount of a M-CSF mutein or mutein product thereby reducing the severity of bone loss associated with the metastatic cancer.

3. The method according to claims 1 or 2 wherein said subject is a mammal.

4. The method according to claim 3 wherein said mammal is human.

5. The method according to claim 4 wherein said mutein or mutein product inhibits the interaction between M-CSF and its receptor (M-CSFR).

6. The method according to claim 5 wherein said M-CSF mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells.

7. The method according to claim 5 wherein the metastatic cancer is breast, lung, renal, multiple myeloma, thyroid, prostate, adenocarcinoma, blood cell malignancies, including leukemia and lymphoma; head and neck cancers; gastrointestinal cancers, including stomach cancer, colon cancer, colorectal cancer, pancreatic cancer, liver cancer; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers and cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; and skin cancer, including malignant melanoma or squamous cell cancer.

8. A method of screening for a M-CSF mutein comprising the steps of:

a) contacting metastatic tumor cell medium, osteoclasts and a candidate M-CSF mutein or mutein product;

b) detecting osteoclast formation, proliferation and/or differentiation; and

c) identifying said candidate as an M-CSF mutein or mutein product if a decrease in osteoclast formation, proliferation and/or differentiation is detected.

9. The method of claim 8 wherein said metastatic tumor cell medium includes tumor cells.

10. The method of claim 8 wherein said contacting step (a) occurs *in vivo*, said detecting step (b) comprises detecting size and/or number of bone metastases, and said candidate is identified as a M-CSF mutein or mutein product if a decrease in size and/or number of bone metastases is detected.

11. The method of claim 8 further comprising the step of determining if said candidate M-CSF mutein or mutein product inhibits interaction between M-CSF and its receptor M-CSFR.

12. A method of identifying a M-CSF mutein or mutein product that can prevent or treat metastatic cancer to bone, comprising the steps of:

(a) detecting binding of a candidate M-CSF mutein or mutein product to M-CSFR; and

(b) assaying the ability of said candidate M-CSF mutein or mutein product to prevent or treat metastatic cancer to bone *in vitro* or *in vivo*.

13. A method of identifying a M-CSF mutein or mutein product that can prevent or treat metastatic cancer to bone, comprising the steps of:

(a) identifying a candidate M-CSF mutein or mutein product that inhibits the interaction between M-CSF and M-CSFR; and

(b) assaying the ability of said candidate M-CSF mutein or mutein product to prevent or treat metastatic cancer to bone *in vitro* or *in vivo*.

14. A method of preventing bone metastases and tumor growth comprising administering to a subject afflicted with metastatic cancer therapeutically effective amounts of M-CSF mutein or mutein product and a therapeutic agent, thereby preventing bone loss associated with the metastatic cancer and preventing tumor growth.

15. A method of treating a subject afflicted with a metastatic cancer comprising administering to said subject therapeutically effective amounts of M-CSF mutein or mutein product and a therapeutic agent, thereby reducing the severity of bone loss associated with the metastatic cancer and inhibiting tumor growth.

16. The method according to claims 14 or 15 wherein said subject is a mammal.

17. The method according to claim 16 wherein said mammal is human.

18. The method according to claim 17 wherein said M-CSF mutein or mutein

product inhibits the interaction between M-CSF and its receptor M-CSFR.

19. The method according to claim 18 wherein said M-CSF mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells.

20. The methods according to claims 14or 15 wherein the therapeutic agent is a bisphosphonate.

21. The method according to claim 20 wherein the bisphonate is zeledronate, pamidronate, clodronate, etidronate, tilundronate, alendronate, or ibandronate.

22. The methods according to claims 14 or 15 wherin the therapeutic agent is a chemotherapeutic agent.

23. The method according to claim 22 wherein the subject is precluded from receiving bisphophonate treatment.

24. The methods according to claims 14 or 15 wherein the M-CSF mutein or mutein product is effective to reduce the dosage of therapeutic agent required to achieve a therapeutic effect.

25. The methods according to claims 14 or 15 further comprising the step of administering a non-M-CSF colony stimulating factor, for example G-CSF.

26. A pharmaceutical composition comprising a M-CSF mutein or mutein product and a cancer therapeutic agent.

27. A package, vial or container comprising a medicament comprising an M-CSF mutein or mutein product and instructions that the medicament should be used in combination with surgery or radiation therapy.

28. A method of preventing or treating metastatic cancer to bone comprising the steps of administering a M-CSF mutein or mutein product to a subject and treating said subject with surgery or radiation therapy.

29. A method of treating a subject suffering from a cancer, wherein the cells comprising said cancer do not secrete M-CSF, comprising the step of administering a M-CSF mutein or mutein product.

30. Use of a M-CSF mutein or mutein product in the manufacture of a medicament for preventing bone metastases in a subject afflicted with metastatic cancer.

31. Use of a M-CSF mutein or mutein product in the manufacture of a

medicament for preventing, in a subject afflicted with metastatic cancer, bone loss associated with the cancer.

32. Use of a M-CSF mutein or mutein product in the manufacture of a medicament for treating a subject afflicted with a metastatic cancer to bone.

33. Use of a M-CSF mutein or mutein product in the manufacture of a medicament for reducing, in a subject afflicted with a metastatic cancer to bone, the severity of bone loss associated with the cancer.

34. The use according to claims 30-33 wherein said subject is a mammal.

35. The use according to claim 34 wherein said mammal is human.

36. The use according to claim 35 wherein said mutein or mutein product inhibits the interaction between M-CSF and its receptor (M-CSFR).

37. The use according to claim 36 wherein said mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells.

38. The use according to claim 30 wherein the metastatic cancer is breast, lung, renal, multiple myeloma, thyroid, prostate, adenocarcinoma, blood cell malignancies, including leukemia and lymphoma; head or neck cancers; gastrointestinal cancers, including stomach cancer, colon cancer, colorectal cancer, pancreatic cancer, liver cancer; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers or cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; or skin cancer, including malignant melanoma or squamous cell cancer.

39. Use of a M-CSF mutein or mutein product and a second therapeutic agent in the manufacture of a medicament for preventing, in a subject afflicted with metastatic cancer, bone metastases and tumor growth.

40. Use of a M-CSF mutein or mutein product and a second therapeutic agent in the manufacture of a medicament for preventing, in a subject afflicted with metastatic cancer, bone loss associated with the cancer.

41. Use of a M-CSF mutein or mutein product and a second therapeutic agent in the manufacture of a medicament for treating a metastatic cancer to bone.

42. Use of a M-CSF mutein or mutein product and a second therapeutic agent in the manufacture of a medicament for reducing the severity of bone loss associated with the cancer and inhibiting tumor growth in a subject afflicted with metstatic cancer.

43. Product comprising a M-CSF mutein or mutein product and a second therapeutic agent as a combined preparation for use in treating cancer.

44. Use of a M-CSF mutein or mutein product in preparation of a medicament for preventing or treating metastatic cancer to bone, wherein the medicament is simultaneously separately or sequentially administered with a second therapeutic agent.

45. Use of a M-CSF mutein or mutein product in preparation of a medicament for preventing or treating metastatic cancer to bone,wherein said medicament is coordinated with treatment using a second therapeutic agent.

46. Use of a M-CSF mutein or mutein product in preparation of a medicament for treating a subject afflicted with a metastatic cancer to bone,wherein said subject has been pre-treated with the second therapeutic agent.

47. Use of a synergistic combination of a MCSF mutein or mutein product in the manufacture of a medicament for treating a patient having an osteolytic disease wherein said medicament is coordinated with treatment using an anti-MCSF antibody, anti-RANKL antibody, soluble RANKL receptor or bisphosphonate.

48. Use of a cancer therapeutic agent in preparation of a medicament for preventing or treating metastatic cancer to bone, wherein the medicament is simultaneously separately or sequentially administered with a M-CSF mutein or mutein product.

49. A package, vial or container comprising a medicament comprising a M-CSF mutein or mutein product and instructions that the medicament should be used in combination with surgery or radiation therapy.

50. The use according to claims 39-48 wherein said subject is a mammal.

51. The use according to claim 47 wherein said mammal is human.

52. The use according to claim 48 wherein said mutein or mutein product inhibits the interaction between M-CSF and its receptor M-CSFR.

53. The use according to claims 39-48 wherein said mutein or mutein product inhibits osteoclast proliferation and/or differentiation induced by tumor cells.

54. The use according to claims 39-48 wherein the second therapeutic agent is a bisphosphonate.

55. The use according to claim 54 wherein the bisphonate is zeledronate, pamidronate, clodronate, etidronate, tilundronate, alendronate, or ibandronate.

56. The use according to claims 39-48 wherin the second therapeutic agent is a chemotherapeutic agent.

57. The use according to claim 56 wherein the subject is precluded from receiving bisphophonate treatment.

58. The use according to claim 56 wherein the second therapeutic agent is a non-M-CSF colony stimulating factor.

59. The use according to claim 58 wherein the non-M-CSF colony stimulating factor is G-CSF.

60. Use of a M-CSF mutein or mutein product in the manufacture of a medicament for reducing the dose of a second therapeutic agent administered to a subject to treat or prevent bone metastases and tumor growth.

61. Use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, in the manufacture of a medicament for preventing bone metastases.

62. Use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, in the manufacture of a medicament for neutralizing M-CSF produced by a subject's cells.

63. Use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, in the manufacture of a medicament for treating a subject afflicted with a metastatic cancer to bone.

64. Use of a M-CSF mutein or mutein product, in an amount that is larger than the amount effective to neutralize M-CSF produced by cancer cells, in the manufacture of a medicament for treating cancer.

FIG. 1

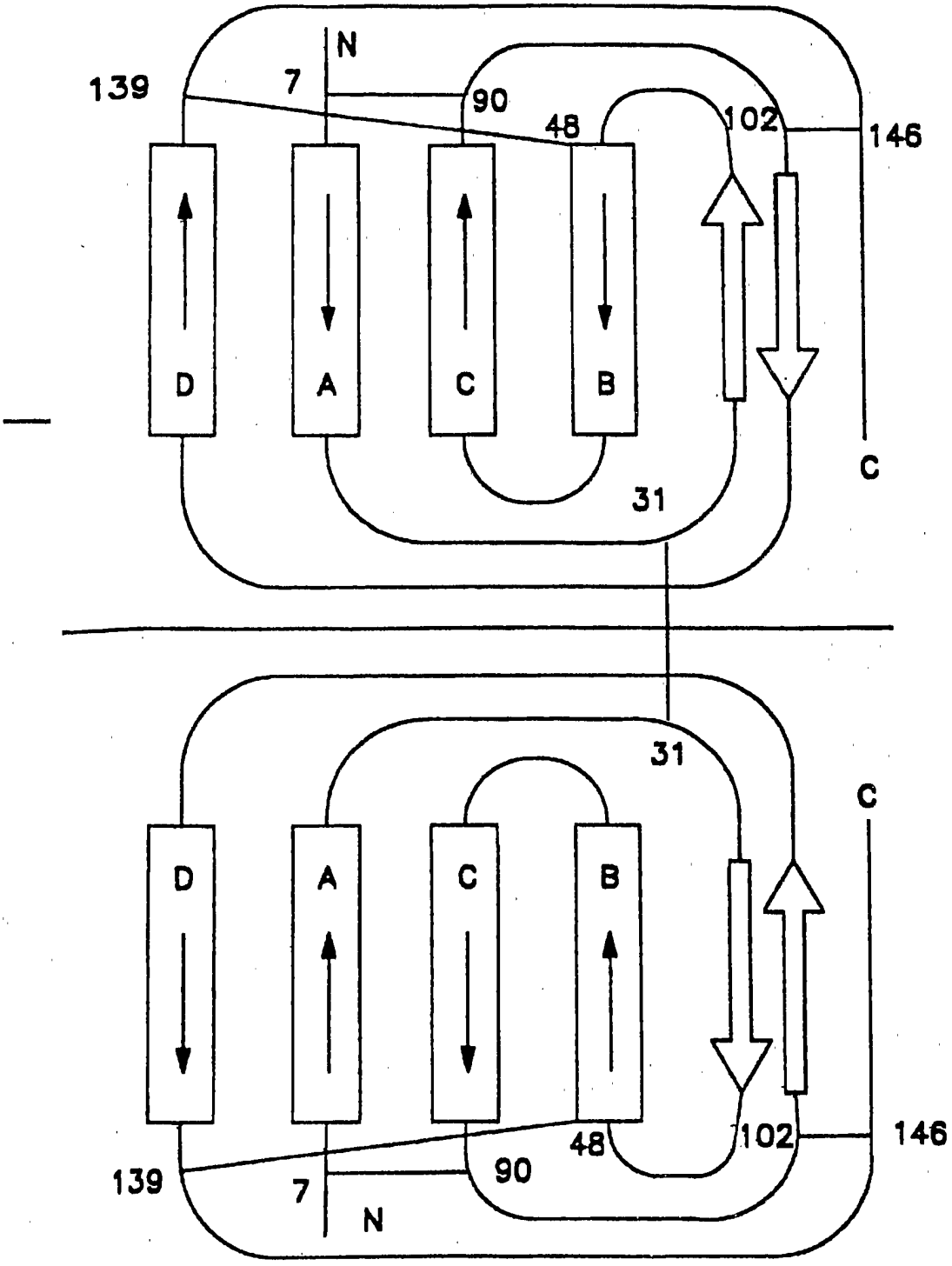


FIG. 2

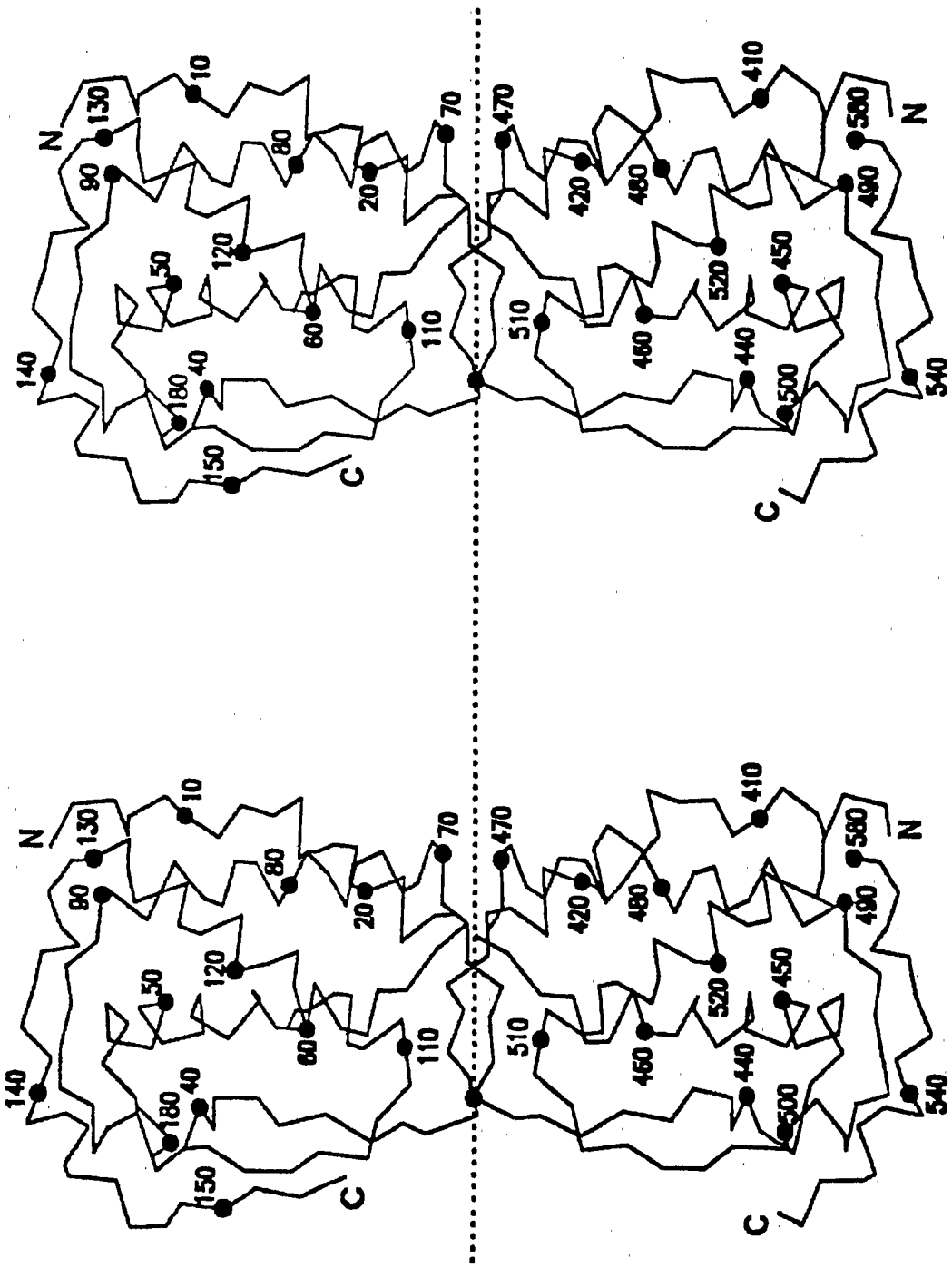

N
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FIG. 3

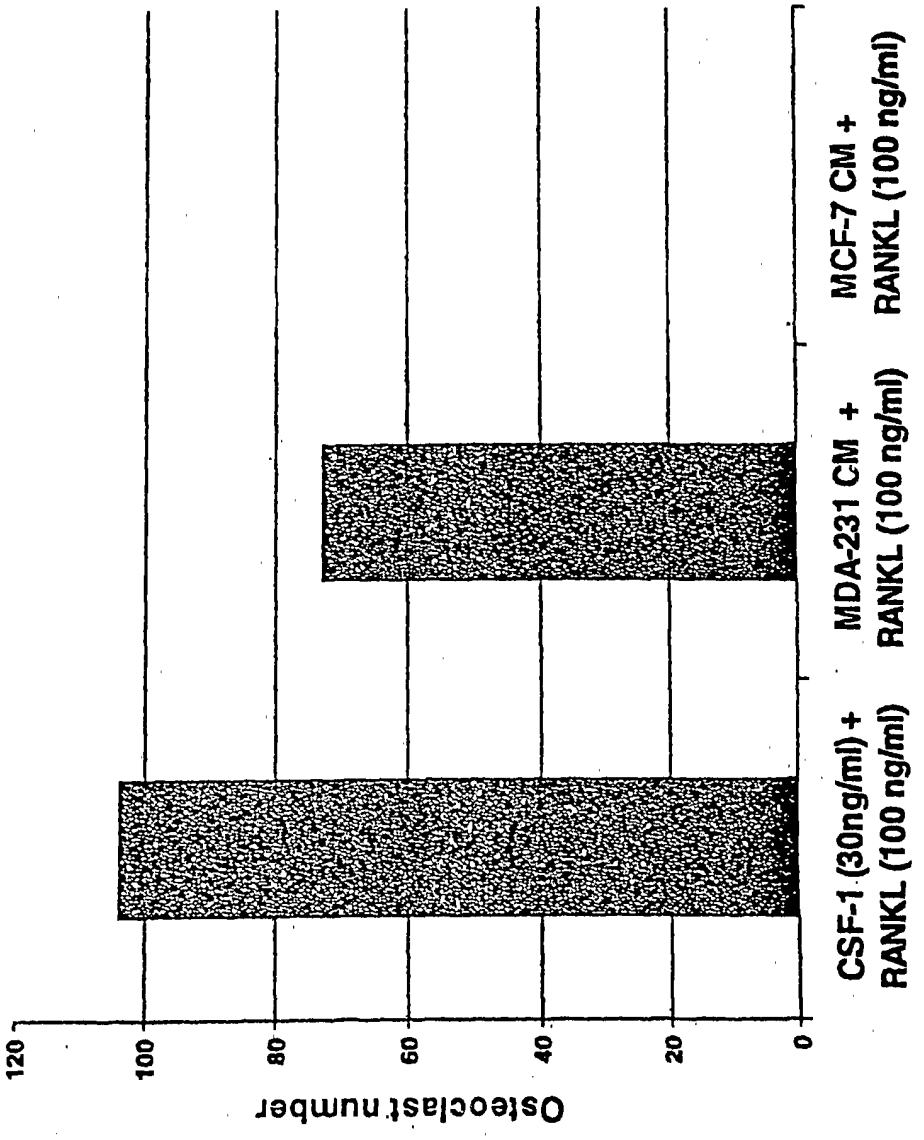

Osteoclast number
120
100
80
60
40
20
0
CSF-1 (30ng/ml) + RANKL (100 ng/ml)
MDA-231 CM + RANKL (100 ng/ml)
MCF-7 CM + RANKL (100 ng/ml)

Fig. 4

```
Met Thr Ala Pro Gly Ala Ala Gly Arg Cys Pro Pro Thr Thr Trp Leu
1               5                  10                  15

Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
            20                  25                  30

Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
        35                  40                  45

Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
    50                  55                  60

Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
65                  70                  75                  80

Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
                85                  90                  95

Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
            100                 105                 110

Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
        115                 120                 125

Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
    130                 135                 140

Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
145                 150                 155                 160

Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
                165                 170                 175

Glu Cys Ser Ser Gln Gly His Glu Arg Gln Ser Glu Gly Ser Ser Ser
            180                 185                 190

Pro Gln Leu Gln Glu Ser Val Phe His Leu Leu Val Pro Ser Val Ile
        195                 200                 205

Leu Val Leu Leu Ala Val Gly Gly Leu Leu Phe Tyr Arg Trp Arg Arg
    210                 215                 220

Arg Ser His Gln Glu Pro Gln Arg Ala Asp Ser Pro Leu Glu Gln Pro
225                 230                 235                 240

Glu Gly Ser Pro Leu Thr Gln Asp Asp Arg Gln Val Glu Leu Pro Val
                245                 250                 255
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Fig. 5

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Met Thr Ala Pro Gly Ala Ala Gly Arg Cys Pro Pro Thr Thr Trp Leu
1               5                  10                  15
Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
            20                  25                  30
Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
        35                  40                  45
Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
    50                  55                  60
Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
65                  70                  75                  80
Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
                85                  90                  95
Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
            100                 105                 110
Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
        115                 120                 125
Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
    130                 135                 140
Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
145                 150                 155                 160
Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
                165                 170                 175
Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn Cys Leu
            180                 185                 190
Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser Pro His
        195                 200                 205
Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu
    210                 215                 220
Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu Gln Pro
225                 230                 235                 240
Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro Arg Ser
                245                 250                 255
Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys Asp Ser
            260                 265                 270
Thr Ile Gly Gly Ser Pro Gln Pro Arg Pro Ser Val Gly Ala Phe Asn
        275                 280                 285
Pro Gly Met Glu Asp Ile Leu Asp Ser Ala Met Gly Thr Asn Trp Val
    290                 295                 300
Pro Glu Glu Ala Ser Gly Glu Ala Ser Glu Ile Pro Val Pro Gln Gly
305                 310                 315                 320
Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln Thr Glu
                325                 330                 335
Pro Ala Arg Pro Ser Asn Phe Leu Ser Ala Ser Ser Pro Leu Pro Ala
            340                 345                 350
Ser Ala Lys Gly Gln Gln Pro Ala Asp Val Thr Gly Thr Ala Leu Pro
        355                 360                 365
Arg Val Gly Pro Val Arg Pro Thr Gly Gln Asp Trp Asn His Thr Pro
    370                 375                 380
Gln Lys Thr Asp His Pro Ser Ala Leu Leu Arg Asp Pro Pro Glu Pro
385                 390                 395                 400
Gly Ser Pro Arg Ile Ser Ser Leu Arg Pro Gln Gly Leu Ser Asn Pro
                405                 410                 415
Ser Thr Leu Ser Ala Gln Pro Gln Leu Ser Arg Ser His Ser Ser Gly
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Ser Val Leu Pro Leu Gly Glu Leu Glu Gly Arg Arg Ser Thr Arg Asp
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Arg Arg Ser Pro Ala Glu Pro Glu Gly Gly Pro Ala Ser Glu Gly Ala
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Ala Arg Pro Leu Pro Arg Phe Asn Ser Val Pro Leu Thr Asp Thr Gly
465                 470                 475                 480
His Glu Arg Gln Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser
                485                 490                 495
Val Phe His Leu Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val
            500                 505                 510
Gly Gly Leu Leu Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro
        515                 520                 525
Gln Arg Ala Asp Ser Pro Leu Glu Gln Pro Glu Gly Ser Pro Leu Thr
    530                 535                 540
Gln Asp Asp Arg Gln Val Glu Leu Pro Val
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Fig. 6

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1               5                  10                  15
Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
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Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
        35                  40                  45
Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
    50                  55                  60
Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
65                  70                  75                  80
Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
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Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
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Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
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Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
    130                 135                 140
Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
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Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
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Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn Cys Leu
            180                 185                 190
Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser Pro His
        195                 200                 205
Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu
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Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu Gln Pro
225                 230                 235                 240
Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro Arg Ser
                245                 250                 255
Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys Asp Ser
            260                 265                 270
Thr Ile Gly Gly Ser Pro Gln Pro Arg Pro Ser Val Gly Ala Phe Asn
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Pro Gly Met Glu Asp Ile Leu Asp Ser Ala Met Gly Thr Asn Trp Val
    290                 295                 300
Pro Glu Glu Ala Ser Gly Glu Ala Ser Glu Ile Pro Val Pro Gln Gly
305                 310                 315                 320
Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln Thr Glu
                325                 330                 335
Pro Ala Arg Pro Ser Asn Phe Leu Ser Ala Ser Ser Pro Leu Pro Ala
            340                 345                 350
Ser Ala Lys Gly Gln Gln Pro Ala Asp Val Thr Gly His Glu Arg Gln
        355                 360                 365
Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser Val Phe His Leu
    370                 375                 380
Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val Gly Gly Leu Leu
385                 390                 395                 400
Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro Gln Arg Ala Asp
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gac acc atg cgc ttc aga gat aac acc ccc aat gcc atc gcc att gtg      519
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                115                 120                 125

tat gaa gag cat gac aag gcc tgc gtc cga act ttc tat gag aca cct      615
Tyr Glu Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro
            130                 135                 140

ctc cag ttg ctg gag aag gtc aag aat gtc ttt aat gaa aca aag aat      663
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Pro Val
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195 200 205

Leu Val Leu Leu Ala Val Gly Gly Leu Leu Phe Tyr Arg Trp Arg Arg
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gtg tgc tac ctt aag aag gca ttt ctc ctg gta caa gac ata atg gag      471
Val Cys Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu
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gac acc atg cgc ttc aga gat aac acc ccc aat gcc atc gcc att gtg      519
Asp Thr Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val
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Gln Leu Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp
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Leu Leu Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser
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ttt gct gaa tgc tcc agc caa gat gtg gtg acc aag cct gat tgc aac      759
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tgc ctg tac ccc aaa gcc atc cct agc agt gac ccg gcc tct gtc tcc      807
Cys Leu Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser
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cct cat cag ccc ctc gcc ccc tcc atg gcc cct gtg gct ggc ttg acc      855
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Trp Glu Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu
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cag ccc ctg cac aca gtg gat cca ggc agt gcc aag cag cgg cca ccc      951
Gln Pro Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro
    240                 245                 250

agg agc acc tgc cag agc ttt gag ccg cca gag acc cca gtt gtc aag      999
Arg Ser Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys
255                 260                 265                 270

gac agc acc atc ggt ggc tca cca cag cct cgc ccc tct gtc ggg gcc     1047
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            290                 295                 300

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aca gag ccc gcc aga ccc agc aac ttc ctc tca gca tct tct cca ctc     1239
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335                 340                 345                 350

cct gca tca gca aag ggc caa cag ccg gca gat gta act ggt aca gcc     1287
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                355                 360                 365

ttg ccc agg gtg ggc ccc gtg agg ccc act ggc cag gac tgg aat cac     1335
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acc ccc cag aag aca gac cat cca tct gcc ctg ctc aga gac ccc ccg     1383
Thr Pro Gln Lys Thr Asp His Pro Ser Ala Leu Leu Arg Asp Pro Pro
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gag cca ggc tct ccc agg atc tca tca ctg cgc ccc cag ggc ctc agc     1431
Glu Pro Gly Ser Pro Arg Ile Ser Ser Leu Arg Pro Gln Gly Leu Ser
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415                 420                 425                 430

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aca ggc cat gag agg cag tcc gag gga tcc tcc agc ccg cag ctc cag     1671
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Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
100 105 110

Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu

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Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
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Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
145                 150                 155                 160

Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
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Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn Cys Leu
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Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser Pro His
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Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu
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305                 310                 315                 320

Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln Thr Glu
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Ser Val Leu Pro Leu Gly Glu Leu Glu Gly Arg Arg Ser Thr Arg Asp
        435                 440                 445

Arg Arg Ser Pro Ala Glu Pro Glu Gly Gly Pro Ala Ser Glu Gly Ala
    450                 455                 460

Ala Arg Pro Leu Pro Arg Phe Asn Ser Val Pro Leu Thr Asp Thr Gly
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His Glu Arg Gln Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser
                485                 490                 495

Val Phe His Leu Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val
            500                 505                 510

Gly Gly Leu Leu Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro
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          1               5                  10

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Trp Leu Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser
15                  20                  25                  30

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Ile Thr Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly
                35                  40                  45

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His Leu Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser
            50                  55                  60

tgc caa att aca ttt gag ttt gta gac cag gaa cag ttg aaa gat cca      423
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Val Cys Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu
    80                  85                  90

gac acc atg cgc ttc aga gat aac acc ccc aat gcc atc gcc att gtg      519
Asp Thr Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val
95                  100                 105                 110

cag ctg cag gaa ctc tct ttg agg ctg aag agc tgc ttc acc aag gat      567
Gln Leu Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp
                115                 120                 125

tat gaa gag cat gac aag gcc tgc gtc cga act ttc tat gag aca cct      615
Tyr Glu Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro
            130                 135                 140

ctc cag ttg ctg gag aag gtc aag aat gtc ttt aat gaa aca aag aat      663
Leu Gln Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn
        145                 150                 155

ctc ctt gac aag gac tgg aat att ttc agc aag aac tgc aac aac agc      711
Leu Leu Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser
    160                 165                 170

ttt gct gaa tgc tcc agc caa gat gtg gtg acc aag cct gat tgc aac      759
Phe Ala Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn
175                 180                 185                 190

tgc ctg tac ccc aaa gcc atc cct agc agt gac ccg gcc tct gtc tcc      807
Cys Leu Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser
                195                 200                 205

cct cat cag ccc ctc gcc ccc tcc atg gcc cct gtg gct ggc ttg acc      855
Pro His Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr
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            210                 215                 220

tgg gag gac tct gag gga act gag ggc agc tcc ctc ttg cct ggt gag      903
Trp Glu Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu
        225                 230                 235

cag ccc ctg cac aca gtg gat cca ggc agt gcc aag cag cgg cca ccc      951
Gln Pro Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro
    240                 245                 250

agg agc acc tgc cag agc ttt gag ccg cca gag acc cca gtt gtc aag      999
Arg Ser Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys
255                 260                 265                 270

gac agc acc atc ggt ggc tca cca cag cct cgc ccc tct gtc ggg gcc     1047
Asp Ser Thr Ile Gly Gly Ser Pro Gln Pro Arg Pro Ser Val Gly Ala
                275                 280                 285

ttc aac ccc ggg atg gag gat att ctt gac tct gca atg ggc act aat     1095
Phe Asn Pro Gly Met Glu Asp Ile Leu Asp Ser Ala Met Gly Thr Asn
            290                 295                 300

tgg gtc cca gaa gaa gcc tct gga gag gcc agt gag att ccc gta ccc     1143
Trp Val Pro Glu Glu Ala Ser Gly Glu Ala Ser Glu Ile Pro Val Pro
        305                 310                 315

caa ggg aca gag ctt tcc ccc tcc agg cca gga ggg ggc agc atg cag     1191
Gln Gly Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln
    320                 325                 330

aca gag ccc gcc aga ccc agc aac ttc ctc tca gca tct tct cca ctc     1239
Thr Glu Pro Ala Arg Pro Ser Asn Phe Leu Ser Ala Ser Ser Pro Leu
335                 340                 345                 350

cct gca tca gca aag ggc caa cag ccg gca gat gta act ggc cat gag     1287
Pro Ala Ser Ala Lys Gly Gln Gln Pro Ala Asp Val Thr Gly His Glu
                355                 360                 365

agg cag tcc gag gga tcc tcc agc ccg cag ctc cag gag tct gtc ttc     1335
Arg Gln Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser Val Phe
            370                 375                 380

cac ctg ctg gtg ccc agt gtc atc ctg gtc ttg ctg gcc gtc gga ggc     1383
His Leu Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val Gly Gly
        385                 390                 395

ctc ttg ttc tac agg tgg agg cgg cgg agc cat caa gag cct cag aga     1431
Leu Leu Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro Gln Arg
    400                 405                 410

gcg gat tct ccc ttg gag caa cca gag ggc agc ccc ctg act cag gat     1479
Ala Asp Ser Pro Leu Glu Gln Pro Glu Gly Ser Pro Leu Thr Gln Asp
415                 420                 425                 430

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Asp Arg Gln Val Glu Leu Pro Val
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Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
        35                  40                  45

Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
    50                  55                  60

Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
65                  70                  75                  80

Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
                85                  90                  95

Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
            100                 105                 110

Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
        115                 120                 125

Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
    130                 135                 140

Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
145                 150                 155                 160

Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
                165                 170                 175

Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn Cys Leu
            180                 185                 190

Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser Pro His
        195                 200                 205

Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu
    210                 215                 220

Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu Gln Pro
225                 230                 235                 240

Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro Arg Ser
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245 250 255

Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys Asp Ser
260 265 270

Thr Ile Gly Gly Ser Pro Gln Pro Arg Pro Ser Val Gly Ala Phe Asn
275 280 285

Pro Gly Met Glu Asp Ile Leu Asp Ser Ala Met Gly Thr Asn Trp Val
290 295 300

Pro Glu Glu Ala Ser Gly Glu Ala Ser Glu Ile Pro Val Pro Gln Gly
305 310 315 320

Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln Thr Glu
325 330 335

Pro Ala Arg Pro Ser Asn Phe Leu Ser Ala Ser Ser Pro Leu Pro Ala
340 345 350

Ser Ala Lys Gly Gln Gln Pro Ala Asp Val Thr Gly His Glu Arg Gln
355 360 365

Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser Val Phe His Leu
370 375 380

Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val Gly Gly Leu Leu
385 390 395 400

Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro Gln Arg Ala Asp
405 410 415

Ser Pro Leu Glu Gln Pro Glu Gly Ser Pro Leu Thr Gln Asp Asp Arg
420 425 430

Gln Val Glu Leu Pro Val
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ggaagaagag gatcagccca aggaggagga agaggaaaac aagacaaaca gccagtgcag 180

aggagaggaa cgtgtgtcca gtgtcccgat ccctgcggag ctagtagctg agagctctgt 240

gccctgggca ccttgcagcc ctgcacctgc ctgccacttc cccaccgagg cc atg ggc 298
Met Gly
1

cca gga gtt ctg ctg ctc ctg ctg gtg gcc aca gct tgg cat ggt cag 346
Pro Gly Val Leu Leu Leu Leu Leu Val Ala Thr Ala Trp His Gly Gln
5 10 15

gga atc cca gtg ata gag ccc agt gtc cct gag ctg gtc gtg aag cca 394
Gly Ile Pro Val Ile Glu Pro Ser Val Pro Glu Leu Val Val Lys Pro
20 25 30

gga gca acg gtg acc ttg cga tgt gtg ggc aat ggc agc gtg gaa tgg 442
Gly Ala Thr Val Thr Leu Arg Cys Val Gly Asn Gly Ser Val Glu Trp
35 40 45 50

gat ggc ccc cca tca cct cac tgg acc ctg tac tct gat ggc tcc agc 490
Asp Gly Pro Pro Ser Pro His Trp Thr Leu Tyr Ser Asp Gly Ser Ser
55 60 65

agc atc ctc agc acc aac aac gct acc ttc caa aac acg ggg acc tat 538
Ser Ile Leu Ser Thr Asn Asn Ala Thr Phe Gln Asn Thr Gly Thr Tyr
70 75 80

cgc tgc act gag cct gga gac ccc ctg gga ggc agc gcc gcc atc cac 586
Arg Cys Thr Glu Pro Gly Asp Pro Leu Gly Gly Ser Ala Ala Ile His
85 90 95

ctc tat gtc aaa gac cct gcc cgg ccc tgg aac gtg cta gca cag gag 634
Leu Tyr Val Lys Asp Pro Ala Arg Pro Trp Asn Val Leu Ala Gln Glu
100 105 110

gtg gtc gtg ttc gag gac cag gac gca cta ctg ccc tgt ctg ctc aca 682
Val Val Val Phe Glu Asp Gln Asp Ala Leu Leu Pro Cys Leu Leu Thr
115 120 125 130

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gac ccg gtg ctg gaa gca ggc gtc tcg ctg gtg cgt gtg cgt ggc cgg      730
Asp Pro Val Leu Glu Ala Gly Val Ser Leu Val Arg Val Arg Gly Arg
                135                 140                 145

ccc ctc atg cgc cac acc aac tac tcc ttc tcg ccc tgg cat ggc ttc      778
Pro Leu Met Arg His Thr Asn Tyr Ser Phe Ser Pro Trp His Gly Phe
            150                 155                 160

acc atc cac agg gcc aag ttc att cag agc cag gac tat caa tgc agt      826
Thr Ile His Arg Ala Lys Phe Ile Gln Ser Gln Asp Tyr Gln Cys Ser
        165                 170                 175

gcc ctg atg ggt ggc agg aag gtg atg tcc atc agc atc cgg ctg aaa      874
Ala Leu Met Gly Gly Arg Lys Val Met Ser Ile Ser Ile Arg Leu Lys
    180                 185                 190

gtg cag aaa gtc atc cca ggg ccc cca gcc ttg aca ctg gtg cct gca      922
Val Gln Lys Val Ile Pro Gly Pro Pro Ala Leu Thr Leu Val Pro Ala
195                 200                 205                 210

gag ctg gtg cgg att cga ggg gag gct gcc cag atc gtg tgc tca gcc      970
Glu Leu Val Arg Ile Arg Gly Glu Ala Ala Gln Ile Val Cys Ser Ala
                215                 220                 225

agc agc gtt gat gtt aac ttt gat gtc ttc ctc caa cac aac aac acc     1018
Ser Ser Val Asp Val Asn Phe Asp Val Phe Leu Gln His Asn Asn Thr
            230                 235                 240

aag ctc gca atc cct caa caa tct gac ttt cat aat aac cgt tac caa     1066
Lys Leu Ala Ile Pro Gln Gln Ser Asp Phe His Asn Asn Arg Tyr Gln
        245                 250                 255

aaa gtc ctg acc ctc aac ctc gat caa gta gat ttc caa cat gcc ggc     1114
Lys Val Leu Thr Leu Asn Leu Asp Gln Val Asp Phe Gln His Ala Gly
    260                 265                 270

aac tac tcc tgc gtg gcc agc aac gtg cag ggc aag cac tcc acc tcc     1162
Asn Tyr Ser Cys Val Ala Ser Asn Val Gln Gly Lys His Ser Thr Ser
275                 280                 285                 290

atg ttc ttc cgg gtg gta gag agt gcc tac ttg aac ttg agc tct gag     1210
Met Phe Phe Arg Val Val Glu Ser Ala Tyr Leu Asn Leu Ser Ser Glu
                295                 300                 305

cag aac ctc atc cag gag gtg acc gtg ggg gag ggg ctc aac ctc aaa     1258
Gln Asn Leu Ile Gln Glu Val Thr Val Gly Glu Gly Leu Asn Leu Lys
            310                 315                 320

gtc atg gtg gag gcc tac cca ggc ctg caa ggt ttt aac tgg acc tac     1306
Val Met Val Glu Ala Tyr Pro Gly Leu Gln Gly Phe Asn Trp Thr Tyr
        325                 330                 335

ctg gga ccc ttt tct gac cac cag cct gag ccc aag ctt gct aat gct     1354
Leu Gly Pro Phe Ser Asp His Gln Pro Glu Pro Lys Leu Ala Asn Ala
    340                 345                 350

acc acc aag gac aca tac agg cac acc ttc acc ctc tct ctg ccc cgc     1402
Thr Thr Lys Asp Thr Tyr Arg His Thr Phe Thr Leu Ser Leu Pro Arg
355                 360                 365                 370

ctg aag ccc tct gag gct ggc cgc tac tcc ttc ctg gcc aga aac cca     1450
Leu Lys Pro Ser Glu Ala Gly Arg Tyr Ser Phe Leu Ala Arg Asn Pro
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                375                 380                 385

gga ggc tgg aga gct ctg acg ttt gag ctc acc ctt cga tac ccc cca     1498
Gly Gly Trp Arg Ala Leu Thr Phe Glu Leu Thr Leu Arg Tyr Pro Pro
            390                 395                 400

gag gta agc gtc ata tgg aca ttc atc aac ggc tct ggc acc ctt ttg     1546
Glu Val Ser Val Ile Trp Thr Phe Ile Asn Gly Ser Gly Thr Leu Leu
        405                 410                 415

tgt gct gcc tct ggg tac ccc cag ccc aac gtg aca tgg ctg cag tgc     1594
Cys Ala Ala Ser Gly Tyr Pro Gln Pro Asn Val Thr Trp Leu Gln Cys
    420                 425                 430

agt ggc cac act gat agg tgt gat gag gcc caa gtg ctg cag gtc tgg     1642
Ser Gly His Thr Asp Arg Cys Asp Glu Ala Gln Val Leu Gln Val Trp
435                 440                 445                 450

gat gac cca tac cct gag gtc ctg agc cag gag ccc ttc cac aag gtg     1690
Asp Asp Pro Tyr Pro Glu Val Leu Ser Gln Glu Pro Phe His Lys Val
                455                 460                 465

acg gtg cag agc ctg ctg act gtt gag acc tta gag cac aac caa acc     1738
Thr Val Gln Ser Leu Leu Thr Val Glu Thr Leu Glu His Asn Gln Thr
            470                 475                 480

tac gag tgc agg gcc cac aac agc gtg ggg agt ggc tcc tgg gcc ttc     1786
Tyr Glu Cys Arg Ala His Asn Ser Val Gly Ser Gly Ser Trp Ala Phe
        485                 490                 495

ata ccc atc tct gca gga gcc cac acg cat ccc ccg gat gag ttc ctc     1834
Ile Pro Ile Ser Ala Gly Ala His Thr His Pro Pro Asp Glu Phe Leu
    500                 505                 510

ttc aca cca gtg gtg gtc gcc tgc atg tcc atc atg gcc ttg ctg ctg     1882
Phe Thr Pro Val Val Val Ala Cys Met Ser Ile Met Ala Leu Leu Leu
515                 520                 525                 530

ctg ctg ctc ctg ctg cta ttg tac aag tat aag cag aag ccc aag tac     1930
Leu Leu Leu Leu Leu Leu Leu Tyr Lys Tyr Lys Gln Lys Pro Lys Tyr
                535                 540                 545

cag gtc cgc tgg aag atc atc gag agc tat gag ggc aac agt tat act     1978
Gln Val Arg Trp Lys Ile Ile Glu Ser Tyr Glu Gly Asn Ser Tyr Thr
            550                 555                 560

ttc atc gac ccc acg cag ctg cct tac aac gag aag tgg gag ttc ccc     2026
Phe Ile Asp Pro Thr Gln Leu Pro Tyr Asn Glu Lys Trp Glu Phe Pro
        565                 570                 575

cgg aac aac ctg cag ttt ggt aag acc ctc gga gct gga gcc ttt ggg     2074
Arg Asn Asn Leu Gln Phe Gly Lys Thr Leu Gly Ala Gly Ala Phe Gly
    580                 585                 590

aag gtg gtg gag gcc acg gcc ttt ggt ctg ggc aag gag gat gct gtc     2122
Lys Val Val Glu Ala Thr Ala Phe Gly Leu Gly Lys Glu Asp Ala Val
595                 600                 605                 610

ctg aag gtg gct gtg aag atg ctg aag tcc acg gcc cat gct gat gag     2170
Leu Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala His Ala Asp Glu
                615                 620                 625

aag gag gcc ctc atg tcc gag ctg aag atc atg agc cac ctg ggc cag     2218
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Lys Glu Ala Leu Met Ser Glu Leu Lys Ile Met Ser His Leu Gly Gln
            630                 635                 640

cac gag aac atc gtc aac ctt ctg gga gcc tgt acc cat gga ggc cct     2266
His Glu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr His Gly Gly Pro
        645                 650                 655

gta ctg gtc atc acg gag tac tgt tgc tat ggc gac ctg ctc aac ttt     2314
Val Leu Val Ile Thr Glu Tyr Cys Cys Tyr Gly Asp Leu Leu Asn Phe
    660                 665                 670

ctg cga agg aag gct gag gcc atg ctg gga ccc agc ctg agc ccc ggc     2362
Leu Arg Arg Lys Ala Glu Ala Met Leu Gly Pro Ser Leu Ser Pro Gly
675                 680                 685                 690

cag gac ccc gag gga ggc gtc gac tat aag aac atc cac ctc gag aag     2410
Gln Asp Pro Glu Gly Gly Val Asp Tyr Lys Asn Ile His Leu Glu Lys
                695                 700                 705

aaa tat gtc cgc agg gac agt ggc ttc tcc agc cag ggt gtg gac acc     2458
Lys Tyr Val Arg Arg Asp Ser Gly Phe Ser Ser Gln Gly Val Asp Thr
            710                 715                 720

tat gtg gag atg agg cct gtc tcc act tct tca aat gac tcc ttc tct     2506
Tyr Val Glu Met Arg Pro Val Ser Thr Ser Ser Asn Asp Ser Phe Ser
        725                 730                 735

gag caa gac ctg gac aag gag gat gga cgg ccc ctg gag ctc cgg gac     2554
Glu Gln Asp Leu Asp Lys Glu Asp Gly Arg Pro Leu Glu Leu Arg Asp
    740                 745                 750

ctg ctt cac ttc tcc agc caa gta gcc cag ggc atg gcc ttc ctc gct     2602
Leu Leu His Phe Ser Ser Gln Val Ala Gln Gly Met Ala Phe Leu Ala
755                 760                 765                 770

tcc aag aat tgc atc cac cgg gac gtg gca gcg cgt aac gtg ctg ttg     2650
Ser Lys Asn Cys Ile His Arg Asp Val Ala Ala Arg Asn Val Leu Leu
                775                 780                 785

acc aat ggt cat gtg gcc aag att ggg gac ttc ggg ctg gct agg gac     2698
Thr Asn Gly His Val Ala Lys Ile Gly Asp Phe Gly Leu Ala Arg Asp
            790                 795                 800

atc atg aat gac tcc aac tac att gtc aag ggc aat gcc cgc ctg cct     2746
Ile Met Asn Asp Ser Asn Tyr Ile Val Lys Gly Asn Ala Arg Leu Pro
        805                 810                 815

gtg aag tgg atg gcc cca gag agc atc ttt gac tgt gtc tac acg gtt     2794
Val Lys Trp Met Ala Pro Glu Ser Ile Phe Asp Cys Val Tyr Thr Val
    820                 825                 830

cag agc gac gtc tgg tcc tat ggc atc ctc ctc tgg gag atc ttc tca     2842
Gln Ser Asp Val Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser
835                 840                 845                 850

ctt ggg ctg aat ccc tac cct ggc atc ctg gtg aac agc aag ttc tat     2890
Leu Gly Leu Asn Pro Tyr Pro Gly Ile Leu Val Asn Ser Lys Phe Tyr
                855                 860                 865

aaa ctg gtg aag gat gga tac caa atg gcc cag cct gca ttt gcc cca     2938
Lys Leu Val Lys Asp Gly Tyr Gln Met Ala Gln Pro Ala Phe Ala Pro
            870                 875                 880
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aag aat ata tac agc atc atg cag gcc tgc tgg gcc ttg gag ccc acc     2986
Lys Asn Ile Tyr Ser Ile Met Gln Ala Cys Trp Ala Leu Glu Pro Thr
        885                 890                 895

cac aga ccc acc ttc cag cag atc tgc tcc ttc ctt cag gag cag gcc     3034
His Arg Pro Thr Phe Gln Gln Ile Cys Ser Phe Leu Gln Glu Gln Ala
    900                 905                 910

caa gag gac agg aga gag cgg gac tat acc aat ctg ccg agc agc agc     3082
Gln Glu Asp Arg Arg Glu Arg Asp Tyr Thr Asn Leu Pro Ser Ser Ser
915                 920                 925                 930

aga agc ggt ggc agc ggc agc agc agc agt gag ctg gag gag gag agc     3130
Arg Ser Gly Gly Ser Gly Ser Ser Ser Ser Glu Leu Glu Glu Glu Ser
                935                 940                 945

tct agt gag cac ctg acc tgc tgc gag caa ggg gat atc gcc cag ccc     3178
Ser Ser Glu His Leu Thr Cys Cys Glu Gln Gly Asp Ile Ala Gln Pro
            950                 955                 960

ttg ctg cag ccc aac aac tat cag ttc tgc tga ggagttgacg acagggagta   3231
Leu Leu Gln Pro Asn Asn Tyr Gln Phe Cys
        965                 970

ccactctccc ctcctccaaa cttcaactcc tccatggatg gggcgacacg gggagaacat   3291

acaaactctg ccttcggtca tttcactcaa cagctcggcc cagctctgaa acttgggaag   3351

gtgagggatt caggggaggt cagaggatcc cacttcctga gcatgggcca tcactgccag   3411

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ccagtcccgt cattctgggc tagaaggcag gggaccttgg catgtggctg gccacaccaa   3891

gcaggaagca caaactcccc caagctgact catcctaact aacagtcacg ccgtgggatg   3951

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Lys Pro Gly Ala Thr Val Thr Leu Arg Cys Val Gly Asn Gly Ser Val
35 40 45

Glu Trp Asp Gly Pro Pro Ser Pro His Trp Thr Leu Tyr Ser Asp Gly
50 55 60

Ser Ser Ser Ile Leu Ser Thr Asn Asn Ala Thr Phe Gln Asn Thr Gly
65 70 75 80

Thr Tyr Arg Cys Thr Glu Pro Gly Asp Pro Leu Gly Gly Ser Ala Ala
85 90 95

Ile His Leu Tyr Val Lys Asp Pro Ala Arg Pro Trp Asn Val Leu Ala
100 105 110

Gln Glu Val Val Val Phe Glu Asp Gln Asp Ala Leu Leu Pro Cys Leu
115 120 125

Leu Thr Asp Pro Val Leu Glu Ala Gly Val Ser Leu Val Arg Val Arg
130 135 140

Gly Arg Pro Leu Met Arg His Thr Asn Tyr Ser Phe Ser Pro Trp His
145 150 155 160

Gly Phe Thr Ile His Arg Ala Lys Phe Ile Gln Ser Gln Asp Tyr Gln
165 170 175

Cys Ser Ala Leu Met Gly Gly Arg Lys Val Met Ser Ile Ser Ile Arg
180 185 190

Leu Lys Val Gln Lys Val Ile Pro Gly Pro Pro Ala Leu Thr Leu Val
195 200 205

Pro Ala Glu Leu Val Arg Ile Arg Gly Glu Ala Ala Gln Ile Val Cys
210 215 220

Ser Ala Ser Ser Val Asp Val Asn Phe Asp Val Phe Leu Gln His Asn
225 230 235 240

Asn Thr Lys Leu Ala Ile Pro Gln Gln Ser Asp Phe His Asn Asn Arg
245 250 255

Tyr Gln Lys Val Leu Thr Leu Asn Leu Asp Gln Val Asp Phe Gln His
260 265 270

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Ala Gly Asn Tyr Ser Cys Val Ala Ser Asn Val Gln Gly Lys His Ser
        275                 280                 285

Thr Ser Met Phe Phe Arg Val Val Glu Ser Ala Tyr Leu Asn Leu Ser
    290                 295                 300

Ser Glu Gln Asn Leu Ile Gln Glu Val Thr Val Gly Glu Gly Leu Asn
305                 310                 315                 320

Leu Lys Val Met Val Glu Ala Tyr Pro Gly Leu Gln Gly Phe Asn Trp
                325                 330                 335

Thr Tyr Leu Gly Pro Phe Ser Asp His Gln Pro Glu Pro Lys Leu Ala
            340                 345                 350

Asn Ala Thr Thr Lys Asp Thr Tyr Arg His Thr Phe Thr Leu Ser Leu
        355                 360                 365

Pro Arg Leu Lys Pro Ser Glu Ala Gly Arg Tyr Ser Phe Leu Ala Arg
    370                 375                 380

Asn Pro Gly Gly Trp Arg Ala Leu Thr Phe Glu Leu Thr Leu Arg Tyr
385                 390                 395                 400

Pro Pro Glu Val Ser Val Ile Trp Thr Phe Ile Asn Gly Ser Gly Thr
                405                 410                 415

Leu Leu Cys Ala Ala Ser Gly Tyr Pro Gln Pro Asn Val Thr Trp Leu
            420                 425                 430

Gln Cys Ser Gly His Thr Asp Arg Cys Asp Glu Ala Gln Val Leu Gln
        435                 440                 445

Val Trp Asp Asp Pro Tyr Pro Glu Val Leu Ser Gln Glu Pro Phe His
    450                 455                 460

Lys Val Thr Val Gln Ser Leu Leu Thr Val Glu Thr Leu Glu His Asn
465                 470                 475                 480

Gln Thr Tyr Glu Cys Arg Ala His Asn Ser Val Gly Ser Gly Ser Trp
                485                 490                 495

Ala Phe Ile Pro Ile Ser Ala Gly Ala His Thr His Pro Pro Asp Glu
            500                 505                 510

Phe Leu Phe Thr Pro Val Val Val Ala Cys Met Ser Ile Met Ala Leu
        515                 520                 525
```

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Leu Leu Leu Leu Leu Leu Leu Leu Leu Tyr Lys Tyr Lys Gln Lys Pro
    530                 535                 540

Lys Tyr Gln Val Arg Trp Lys Ile Ile Glu Ser Tyr Glu Gly Asn Ser
545                 550                 555                 560

Tyr Thr Phe Ile Asp Pro Thr Gln Leu Pro Tyr Asn Glu Lys Trp Glu
                565                 570                 575

Phe Pro Arg Asn Asn Leu Gln Phe Gly Lys Thr Leu Gly Ala Gly Ala
            580                 585                 590

Phe Gly Lys Val Val Glu Ala Thr Ala Phe Gly Leu Gly Lys Glu Asp
        595                 600                 605

Ala Val Leu Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala His Ala
    610                 615                 620

Asp Glu Lys Glu Ala Leu Met Ser Glu Leu Lys Ile Met Ser His Leu
625                 630                 635                 640

Gly Gln His Glu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr His Gly
                645                 650                 655

Gly Pro Val Leu Val Ile Thr Glu Tyr Cys Cys Tyr Gly Asp Leu Leu
            660                 665                 670

Asn Phe Leu Arg Arg Lys Ala Glu Ala Met Leu Gly Pro Ser Leu Ser
        675                 680                 685

Pro Gly Gln Asp Pro Glu Gly Gly Val Asp Tyr Lys Asn Ile His Leu
    690                 695                 700

Glu Lys Lys Tyr Val Arg Arg Asp Ser Gly Phe Ser Ser Gln Gly Val
705                 710                 715                 720

Asp Thr Tyr Val Glu Met Arg Pro Val Ser Thr Ser Ser Asn Asp Ser
                725                 730                 735

Phe Ser Glu Gln Asp Leu Asp Lys Glu Asp Gly Arg Pro Leu Glu Leu
            740                 745                 750

Arg Asp Leu Leu His Phe Ser Ser Gln Val Ala Gln Gly Met Ala Phe
        755                 760                 765

Leu Ala Ser Lys Asn Cys Ile His Arg Asp Val Ala Ala Arg Asn Val
    770                 775                 780
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Leu Leu Thr Asn Gly His Val Ala Lys Ile Gly Asp Phe Gly Leu Ala
785                 790                 795                 800

Arg Asp Ile Met Asn Asp Ser Asn Tyr Ile Val Lys Gly Asn Ala Arg
                805                 810                 815

Leu Pro Val Lys Trp Met Ala Pro Glu Ser Ile Phe Asp Cys Val Tyr
            820                 825                 830

Thr Val Gln Ser Asp Val Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile
        835                 840                 845

Phe Ser Leu Gly Leu Asn Pro Tyr Pro Gly Ile Leu Val Asn Ser Lys
    850                 855                 860

Phe Tyr Lys Leu Val Lys Asp Gly Tyr Gln Met Ala Gln Pro Ala Phe
865                 870                 875                 880

Ala Pro Lys Asn Ile Tyr Ser Ile Met Gln Ala Cys Trp Ala Leu Glu
                885                 890                 895

Pro Thr His Arg Pro Thr Phe Gln Gln Ile Cys Ser Phe Leu Gln Glu
            900                 905                 910

Gln Ala Gln Glu Asp Arg Arg Glu Arg Asp Tyr Thr Asn Leu Pro Ser
        915                 920                 925

Ser Ser Arg Ser Gly Gly Ser Gly Ser Ser Ser Ser Glu Leu Glu Glu
    930                 935                 940

Glu Ser Ser Ser Glu His Leu Thr Cys Cys Glu Gln Gly Asp Ile Ala
945                 950                 955                 960

Gln Pro Leu Leu Gln Pro Asn Asn Tyr Gln Phe Cys
                965                 970
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