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(54) Title: BOMBESIN RECEPTOR ANATOGONISTS WITH ANTI-CANCER ACTIVITY

(57) Abstract: Synthetic peptides that inhibit ligand-binding by bombesin receptors are provided. Methods for inhibiting growth and inducing apoptosis of neoplastic cells using these peptides are also provided. Methods for treating cancer by administering the peptides are also provided.



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BOMBESIN RECEPTOR ANTAGONISTS WITH ANTI-CANCER ACTIVITY**CROSS REFERENCES TO RELATED APPLICATION**

[001] This application claims priority from U.S. provisional application number 60/792,011, filed April 13, 2006, which is herein incorporated by reference.

FIELD

[002] This disclosure relates bombesin receptor antagonists and methods of using such antagonists.

ACKNOWLEDGMENT OF GOVERNMENT SUPPORT

[003] Aspects of this disclosure were made with the support of the United States Government pursuant to National Institutes of Health grant number 5 RO1 CA 79506. The Government of the United States has certain rights in this invention.

BACKGROUND

[004] The bombesin receptor is a growth factor receptor that has been shown to play a central role in the early events of pulmonary carcinogenesis. There are at least three human bombesin receptor types, the gastrin-releasing peptide (GRPR)- and neuromedin B (NMBR)-preferring types, and bombesin receptor subtype 3 (BB3). The three receptor types have been cloned, and are differentially expressed in the different histological types of human lung cancer cells (Fathi *et al.*, *J. Cellular Biochem. Suppl.* 24:237-246, 1996; Fathi *et al.*, *J. Biol. Chem.* 268:5979-5984, 1993).

[005] Bombesin and its mammalian homologue, gastrin releasing peptide, are natural ligands for these receptors, and are mitogens for lung cancer cells (Viallet and Minna, *Progress in Growth Factor Research* 1:89-97, 1989; Fong *et al.*, *J Thorac. Cardiovasc Surg.* 118:1136-1152, 1999). Bombesin-like peptides (BLPs) are produced by lung cancer cells, and are known to have autocrine growth-stimulatory effects on these cells (Cuttitta *et al.*, *Nature* 316:823-826, 1985). Antagonists for bombesin and its receptor have been shown to block small cell lung cancer growth *in vitro* (Schally *et al.*, *Front. Neuroendocrinol.* 22:248-291, 2001; Moody *et al.*, *Eur. J. Pharmacol.* 474:21-29, 2003). Early phase clinical trials using monoclonal antibodies directed against gastrin-releasing peptide have shown anti-cancer activity (Chaudhary *et al.*, *Clin. Cancer Res.* 5:3385-93, 1999). This receptor is also expressed in a variety of other cancer cells, such as breast, colon, gastric, pancreas,

prostate, melanoma, and it has limited distribution in normal human tissues (Jhou *et al.*, *Anticancer Drugs* 15:921-927, 2004).

[006] Opioids have been shown to significantly inhibit (> 90%) the growth of diverse histological types of lung cancer cells at nM concentrations (Maneckjee and Minna, *Proc. Natl. Acad. Sci. USA* 87:3294-3298, 1991; Maneckjee and Minna, *Mol. Biol. of the Cell* 3:613-619, 1992; Maneckjee and Minna, *Proc. Natl. Acad. Sci. USA* 89:1169-1173, 1992). These opioid effects involved the process of programmed cell death known as apoptosis (Maneckjee and Minna, *Cell Growth and Differentiation* 5:1033-1040, 1994), a vital mechanism for the prevention of tumor development (Kerr *et al.*, *Cancer* 73:2013-2026, 1994; Schwartzman and Cidlowski, *Endocrine Reviews* 14:133-151, 1993). Of the several opioids tested for their potential therapeutic value in the treatment of lung cancer, the long-acting synthetic opioid, methadone, used in the treatment of cancer pain, was found to be the most potent opioid-inducer of apoptosis in these cells. Furthermore, at non-toxic doses, it was found to significantly inhibit and retard *in vivo* growth of lung tumors in nude mouse xenografts (Maneckjee and Minna, *Proc. Natl. Acad. Sci. USA* 89:1169-1173, 1992).

[007] Opioid-induced apoptosis is blocked by nicotine in lung cancer cells (Maneckjee and Minna, *Cell Growth and Differentiation* 5:1033-1040, 1994). Similar to the effects of nicotine (Heusch and Maneckjee, *Carcinogenesis* 19:551-55, 1998), high concentrations of bombesin blocked methadone-induced apoptosis through activation of the MAP kinase signaling pathway, and increased expression of the apoptosis-blocking bcl-2 protein (Heusch and Maneckjee, *Cancer Letters* 136:177, 1999), suggesting that there may be endogenous pathways coupling the actions of methadone, nicotine and bombesin in lung cancer cells.

[008] The analgesic action of methadone is generally considered to be mediated through the μ -type opioid receptor (Knapp *et al.*, *FASEB J.* 9:516-525, 1995; Mestek *et al.*, *J. Neurosci.* 15:2396-2406, 1995; Yu *et al.*, *J. Biol. Chem.* 272:28869-28874, 1997). However, the growth-inhibitory effect of methadone in lung cancer cells does not appear to involve this receptor (Maneckjee and Minna, *Proc. Natl. Acad. Sci. USA* 89:1169-1173, 1992; Maneckjee and Minna, *Life Sciences* 61:PL333-338, 1998). For example, methadone binding to these cells can not be displaced by μ -specific ligands (Maneckjee and Minna, *Proc. Natl. Acad. Sci. USA* 89:1169-1173, 1992). In contrast, methadone binding to human brain membranes can be displaced by bombesin (Maneckjee and Minna, *Life Sciences* 61:PL333-338, 1998). Studies in the human breast cancer cell line T47D suggest that the anti-proliferative effects of morphine, another μ opioidreceptor, are mediated through interaction with the somatostatin receptor (Hatzoglou *et al.*, *Cancer Res.* 55:5632-5636,

1995), a member of the superfamily of G-protein coupled receptors that include opioid and bombesin receptors. This suggests that the growth inhibitory actions of therapeutic opioids, such as methadone and morphine, on cancer cells may not be through direct interaction with conventional opioid receptor subtypes.

[009] As described above, methadone can induce apoptosis in small cell lung carcinoma cells via a mechanism that involves one or more bombesin receptors expressed at high levels by neoplastic cells. Although promising results with respect to suppression of cancer growth have been achieved by administering methadone, long-term administration suffers several drawbacks. Methadone is a potent narcotic and treatment is associated with adverse effects of opiate administration. Additionally, the apoptotic effects of methadone are inhibited by nicotine, which is frequently found systemically in patients with lung cancer due to continued smoking.

SUMMARY

[010] Disclosed herein are domains within bombesin receptors that bind to methadone. These domains can be used to develop specific binding agents (including for example bombesin receptor antagonists) that bind to bombesin receptors. Accordingly, this disclosure also provides bombesin receptor antagonists that bind to bombesin receptors. In some examples, the bombesin receptor antagonists inhibit the growth of cells.

[011] The bombesin receptor antagonists disclosed herein include peptides and polypeptides, such as antibodies, that target the fifth transmembrane domain of the bombesin receptor. As such, the antagonists can be peptides such as peptides including at least one linear sequence of amino acids. In certain examples in which the antagonist includes a polypeptide, the antagonist can be an antibody. Antibody antagonists specifically bind to an epitope that is contained at least in part in the fifth transmembrane domain of the bombesin receptor. For example, an antibody antagonist of the bombesin receptor can specifically bind to an amino acid sequence consisting of a subsequence of SEQ ID NOs:1, 14, and 15.

[012] In other examples, the bombesin receptor antagonist is a peptide. Exemplary peptide antagonists are linear sequences of amino acids at least three amino acids in length that include a portion of the sequence YFVLISVFILSPIYVYSFYIA (SEQ ID NO:2), as well as certain variants thereof. In an example, the antagonist is a peptide that includes at least three contiguous amino acids of YFVLISVFILSPIYVYSFYIA (SEQ ID NO:2). In other examples, the antagonists are peptides that are variants of SEQ ID NO:2 or subsequences thereof that are at least three amino acids in length.

[013] Peptide antagonists can contain subsequences of SEQ ID NO:2 that (when aligned with SEQ ID NO:2 or a portion thereof) include a linear sequence of amino acids in which the amino acids corresponding to positions 1-3, 7, 10-16, 18, 20 and 21 (if present) are identical to SEQ ID NO:2; the amino acids corresponding to positions 4 and 5 (if present) of SEQ ID NO:2 are leucine or isoleucine; the amino acids corresponding to position 6 (if present) of SEQ ID NO:2 is serine or isoleucine; the amino acids corresponding to positions 8 and 9 (if present) of SEQ ID NO:2 are phenylalanine or isoleucine; the amino acid corresponding to position 17 (if present) of SEQ ID NO:2 is serine or phenylalanine; and the amino acid corresponding to position 19 (if present) of SEQ ID NO:2 is tyrosine or valine. In specific examples, the peptide antagonist includes one of the following amino acid sequences: YFVLISVFIL (SEQ ID NO:3), YFVILI (SEQ ID NO:4), SPIYV (SEQ ID NO:5), SFVIA (SEQ ID NO:6), SVIFL (SEQ ID NO:7), YFVYIA (SEQ ID NO:8), YSFY (SEQ ID NO:9), or SPIYVYSFYIA (SEQ ID NO:10). In certain examples, the antagonist is a peptide that includes the sequence YFVLISVFILSPIYVYSFYIA (SEQ ID NO:2). In one specific example, the antagonist is a peptide that consists of the sequence YFVLISVFILSPIYVYSFYIA (SEQ ID NO:2).

[014] The bombesin receptor antagonists suppress growth of neoplastic cells (for example, cancer cells) that are contacted with or exposed to the antagonist. For example, the disclosed antagonists suppress growth of neoplastic cells by inducing apoptosis. Typically, neoplastic cells express one or more bombesin receptors at high levels; that is, they over-express a bombesin receptor. In contrast, these antagonists do not interfere with growth of non-neoplastic cells that do not over-express bombesin receptors.

[015] Thus, bombesin receptor antagonists disclosed herein are inhibitors of bombesin-receptor mediated neoplastic activity that suppress the growth of neoplastic cells that over-express bombesin receptors by inducing apoptosis.

[016] The bombesin receptor antagonists can be produced by any methods known in the art for the production of peptides and/or polypeptides. For example, peptide antagonists are commonly produced by solid phase synthesis. Alternatively, polypeptide and peptide antagonists can be produced by expressing a recombinant nucleic acid that includes a polynucleotide sequence encoding the antagonist. The antagonist can be all or a portion of the expressed polypeptide, such as a polypeptide produced by cleavage of a signal sequence or tag, or a peptide produced by cleavage of a recombinant polypeptide that includes one or more protease or other cleavage sites.

[017] The antagonists disclosed herein are useful as anti-neoplastic agents for treating lung and other cancers. Accordingly, pharmaceutical composition including the disclosed

bombesin receptor antagonists and a pharmaceutically acceptable carrier are an aspect of this disclosure. Suitable pharmaceutical compositions include aqueous formulations and atomized freeze-dried formulations. In some cases, the pharmaceutical compositions are extended-release formulations, such as biodegradable microspheres or water-in-oil-in-water double emulsions. In one specific example, the pharmaceutical composition includes a biodegradable microsphere containing poly (lactide-co-glycolide).

[018] Another aspect of this disclosure concerns methods for suppressing growth of neoplastic cells. The methods involve contacting neoplastic cells with a bombesin receptor antagonist (that is, with an anti-neoplastic agent, such as a pharmaceutical composition) as described herein. Exposure of neoplastic cells to such an agent suppresses growth of the neoplastic cell(s), *e.g.*, by inducing apoptosis. The methods disclosed herein are particularly appropriate for suppressing growth of neoplastic cells that over-express at least one bombesin receptor. The neoplastic cells can express a gastrin releasing peptide receptor (GRP-R), a neuromedin B receptor (NMB-R), or both a GRP-R and a NMB-R. Optionally the neoplastic cells also express a bombesin-receptor-3 (BBR3). In some cases, the neoplastic cells also express at least one bombesin-like peptide (BLP). The methods are suitable for suppressing growth of a wide variety of neoplastic (*e.g.*, malignant) cells, such as lung cancer cells, osteosarcoma cells, breast cancer cells, colon cancer cells, gastric cancer cells, pancreatic cancer cells, prostate cancer cells and melanoma cells. In some examples of the method, neoplastic cells are contacted with the agent *in vivo*.

[019] Thus, the disclosed methods are applicable to the treatment of tumors, such as solid tumors, and are particularly useful in the treatment of cancers, such as lung cancer (*e.g.*, small cell and non-small cell lung carcinoma), osteosarcoma, breast cancer, colon cancer, gastric cancer, pancreatic cancer, prostate cancer and melanoma. The disclosure also provides methods for treating a subject with a neoplasm. The methods for treating subjects with neoplasms involve selecting a subject with at least one neoplastic cell and contacting the neoplastic cell(s) with a composition comprising one or more of the disclosed bombesin receptor antagonists. Such administration of a bombesin receptor antagonist suppresses growth of the neoplastic cell(s), thereby treating the subject with the neoplasm.

Administration of the anti-neoplastic bombesin receptor antagonist suppresses growth of the neoplastic cell(s) by inducing apoptosis of neoplastic cells, such as cancer cells. For example, administration of a bombesin receptor antagonist is useful for the treatment of cancers that over-express bombesin-receptors and/or a bombesin-like peptide (BLP). One benefit of these methods over conventional anti-neoplastic treatments is that bombesin receptor antagonists do not appreciably affect non-neoplastic cells. Additionally, unlike

many conventional anti-neoplastic treatments, growth suppression by bombesin receptor antagonists is typically resistant to inhibition by nicotine.

BRIEF DESCRIPTION OF THE DRAWINGS

[020] FIGS. 1A and B are line graphs illustrating saturation curves and Scatchard analyses (inset) of methadone binding to lung cancer cells (NCI-N417) displaced with excess Peptide 0 (A) and Peptide X (B). Cell membranes were incubated with various concentrations of (+)-[³H] methadone, in the presence and absence of excess (1 μM) non-radioactive displacer. Specific binding was calculated as the difference between total binding and binding in the presence of excess non-radioactive ligand. Data was analyzed using the KaleidaGraph.

[021] FIGS. 2A-C are line graphs illustrating the growth inhibitory effects of Peptides 0 and X, methadone and the chemotherapeutic drugs cisplatin and adriamycin on the *in vitro* growth of (A) NCI-N417 lung cancer cells, (B) SAEC normal lung cells, and (C) TE85 osteosarcoma cells, using a 4-day liquid culture AlamarBlue colorimetric growth assay. AlamarBlue is a non-destructive oxidation-reduction colorimetric indicator used to measure viable cell numbers.

SUMMARY OF THE SEQUENCE LISTING

[022] The amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for amino acids, as defined in 37 C.F.R. 1.822.

[023] SEQ ID NO:1 is the amino acid sequence of a peptide corresponding to the fifth transmembrane domain of the bombesin receptor (GRP-R).

[024] SEQ ID NOs:2-10 are the amino acid sequence of exemplary bombesin receptor antagonist peptides.

[025] SEQ ID NO:11 is the amino acid sequence of human gastrin-releasing peptide.

[026] SEQ ID NO:12 is the amino acid sequence of human neuromedin B.

[027] SEQ ID NO:13 is the amino acid sequence of bombesin.

[028] SEQ ID NO:14 is the amino acid sequence of a peptide corresponding to the fifth transmembrane domain of the NMB-R.

[029] SEQ ID NO:15 is the amino acid sequence of a peptide corresponding to the fifth transmembrane domain of the BB3-R

DETAILED DESCRIPTION

Terms

[030] Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

[031] The singular terms “a,” “an,” and “the” include plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Additionally, numerical limitations given with respect to concentrations or levels of a substance, such as a therapeutic peptide, are intended to be approximate. Thus, where a concentration is indicated to be at least (for example) 200 pg, it is intended that the concentration be understood to be at least approximately (or “about” or “~”) 200 pg.

[032] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term “comprises” means “includes.” The abbreviation, “*e.g.*” is derived from the Latin *exempli gratia*, and is used herein to indicate a non-limiting example. Thus, the abbreviation “*e.g.*” is synonymous with the term “for example.”

[033] In order to facilitate review of the various examples of this disclosure, the following explanations of specific terms are provided:

[034] The term “activity” as used herein with respect to a compound, composition or agent, refers to a biological effect of the compound. The activity or effect can be detected either qualitatively or quantitatively. Certain activities are detected in biological systems such as cells, tissues or organisms. For example, a therapeutic activity can be detected by administering a compound to a subject (such as a human or animal subject), and evaluating an effect on the organism. In some instances a biological activity is detected by contacting a cell with a compound and determining an effect of the compound on the cell. Alternatively, an activity of a compound can be detected in an acellular test system using biological

molecules. For example, binding activity of a compound can be detected via an interaction with a binding partner, such as an antibody, a receptor, a ligand, or a nucleic acid.

[035] The term “anti-neoplastic activity” indicates that a compound produces at least one effect on a neoplastic cell (such as a tumor cell, *e.g.*, a cancer cell), resulting in a measurable decrease in the physiological function of the neoplastic cell over time. For example, the decrease in physiological function can be a metabolic decrease or a decrease in growth (*e.g.*, DNA synthesis, cell division). Alternatively, the decrease in physiological function can be related to an increase in cell death, such as cell death resulting from apoptosis.

[036] The term “apoptosis” refers to a genetically programmed form of cell death (programmed cell death), characterized by cytoplasmic condensation and fragmentation of the nuclear chromatin. The term apoptosis is used in contradistinction to the term “necrosis.”

[037] The term “appreciable” or “appreciably,” with respect to an effect or result of an agent on a biological system (such as a cell), indicates that the agent produces a detectable or measurable effect on the biological system when the system is exposed to the agent. Thus, if an agent does not appreciably affect the system, contacting the system with the agent does not result in a measurable or detectable change in the system. For example, depending on method of detection, an agent does not appreciably affect the system if any change is within the margin of error for the assay system, or *e.g.*, is less than 5%, or less than 3%, or less than 2%, or less than 1%.

[038] The terms “bind” and “specifically bind” are used to indicate that two biological molecules are capable of associating via a reversible non-covalent interaction between the two molecules. Binding of biological molecules indicates a conformational complementarity between the two binding partners, and is generally accompanied by a lower thermodynamic energy state. Examples of biological molecules that bind to each other are an antigen and an antibody that possesses a complementarity determining region that recognizes, accommodates or conforms to the antigen and a ligand and its cognate receptor. Strength of binding (“affinity”) is measured as the ratio of receptor-ligand complex to free reactants at equilibrium. The affinity constant is equivalent to the association constant of the binding of a monovalent ligand to a single binding site on the receptor. Frequently, affinity is measured as the reciprocal of the dissociation constant (K_D). In some cases, multiple ligands specifically bind to a single receptor. Similarly, in some cases, a ligand can specifically bind to more than one receptor. In the context of the present disclosure, ligands of bombesin receptors include endogenous ligands, such as

gastrin releasing peptide (GRP), neuromedin B, and bombesin, as well as synthetic ligands, such as methadone.

[039] Mammalian “bombesin receptors” are members of a family of G-protein coupled receptors with seven transmembrane domains (Giladi *et al.*, *J Mol Neurosci.* 4:41-54, 1993). Three “bombesin receptor” subtypes are known in the art: gastrin releasing peptide receptor (GRP-R), neuromedin B receptor (NMB-R) and bombesin receptor subtype 3 (BB3). The first two receptor subtypes are defined in accordance with the ligand for which they exhibit the highest binding affinity. The nucleotide and amino acid sequences of human GRP-R and NMB-R are represented by GENBANK® Accession Nos. NM_005314 and NM_002511, respectively. The murine orthologues are represented by Accession Nos. NM_008177 and NM_008703, respectively. The human and mouse bombesin receptor 3 sequences are represented by Accession Nos. NM_001727 and NM_009766, respectively. The sequences represented by each of these Accession numbers, as of the filing date of this application, are incorporated herein by reference. The endogenous human ligand gastrin-releasing peptide is represented by the sequence:

VPLPAGGGTVLTKMYPRGNHWAVGHLM (SEQ ID NO:11), the endogenous human ligand neuromedin B is represented by the sequence GNLWATGHFM (SEQ ID NO:12), the peptide sequence of bombesin is represented by the sequence QRLGNQWAVGHLM (SEQ ID NO:13).

[040] The term “growth” in reference to a cell includes any physiological process required for cell survival and reproduction. For example, the term growth includes replication of nucleic acids (for example, nuclear or chromosomal DNA), the synthesis of new cellular materials (including transcription of RNA and translation of new proteins), and division of a parent or progenitor cell into progeny cells. In the context of a multicellular tissue, growth can be the result of accumulated cellular growth that may be measured as an increase in size (for example, an increase in volume and/or an increase in mass). For example, growth of a tumor (a benign or malignant neoplasm) is typically measured as an increase in volume and mass of the tumor. In the case of a metastatic malignant neoplasm (or cancer), the term growth can also be used to refer to an increase in number of tumors. Suppression of growth (*e.g.*, of a tumor) can be detected as a decrease in size (volume or mass) of a tumor or as a cessation of an increase in growth of a tumor. Alternatively, suppression of growth can be detected as a decrease in cellularity (cell number per volume) of a tumor, such as may be observed due to cell death (for example, due to apoptosis). In the case of metastatic tumors, suppression of growth can also be detected as a decrease in the number of tumors.

[041] The term “induces” means to initiate, promote or produce an effect. Thus, for example, a compound or agent (such as the synthetic peptides disclosed herein) that induces an effect on a cell, initiates, promotes, or produces the effect when the cell is contacted with the agent.

[042] The term “inhibits” means to reduce, diminish or prevent an event or effect. A compound or agent that inhibits an effect is referred to as an inhibitor or an antagonist. The event can be a molecular event, such as binding of a ligand to a receptor (such as a bombesin receptor). A compound or agent that inhibits binding of a ligand and a receptor is an agent that reduces the ratio of bound to unbound ligand at equilibrium. An agent that “competitively inhibits” ligand binding is an agent that reversibly binds to either the ligand or the receptor, typically at the ligand-receptor binding site, and interferes with the association of the ligand at the binding site of the receptor. Alternatively, the event can be a cellular event or effect, such as a cellular signaling event, a metabolic event, or a complex physiological effect representing an accumulation of genetic, biochemical and metabolic events, such as growth of a cell or tissue. For example, an inhibitor (or an antagonist) can reduce, diminish or prevent one or more events mediated by or induced by binding of a ligand to a receptor (*e.g.*, a bombesin receptor), such as cellular signaling events, increased DNA synthesis, cell division, *etc.* An effect or event is said to be “resistant to inhibition” if it is not substantially decreased by exposure to an agent. A substantial decrease is a decrease of more than about 50%.

[043] The term “neoplasia” refers to an abnormal, disorganized growth in a tissue or organ, usually forming a distinct mass. Such a growth is referred to as a “neoplasm” or “tumor.” The terms neoplasia, neoplasm and tumor refer to both benign and malignant growths, whereas the term “cancer” refers specifically to malignant neoplasia. With respect to cells, the term “neoplastic” indicates that the cell is a cell of a neoplasm.

[044] A “polypeptide” is any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation), such as a protein or a fragment or subsequence of a protein. The term “peptide” is typically used to refer to a chain of amino acids of between three and 30 amino acids in length. For example, various peptide antagonists of the bombesin receptor are at least three amino acids in length (such as at least 4, 5, 6, 7, 8, 9 or 10 amino acids in length). Other antagonists of a bombesin receptor are between 10 and 21 amino acids in length. For example, Peptide X is 21 amino acids in length. Optionally, a peptide is modified by the addition of one or more chemical moieties, such as labels (*e.g.*, fluorescent or radioisotopic labels) or bioactive agents (*e.g.*, chemotherapeutic agents or toxins).

[045] A “recombinant” nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, *e.g.*, by genetic engineering techniques. A recombinant protein is one that is encoded by a heterologous nucleic acid, which has been introduced into a cell, such as a microorganism. As used herein, the term “heterologous” refers to a nucleic acid or protein that does not naturally occur in host cell into which the nucleic acid was introduced.

[046] An “isolated” biological component (such as a nucleic acid molecule, protein or organelle) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, *i.e.*, other chromosomal and extra-chromosomal DNA and RNA, proteins and organelles. Nucleic acids and proteins that have been “isolated” include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

[047] The term “purified” does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified nucleic acid preparation is one in which the specified protein is more enriched than the nucleic acid is in its generative environment, for instance within a cell or in a biochemical reaction chamber. A preparation of substantially pure nucleic acid (or protein or peptide) can be purified such that the desired component represents at least 50% of the total content of the preparation. In certain examples, a substantially pure component will represent at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95% or more of the total content of the preparation.

[048] In the context of this disclosure, an agent (or ligand or antagonist or inhibitor, *etc.*) is said to “target” a receptor or a particular region or domain of a receptor if the agent’s activity can be localized to receptor or specified domain. The target region can be the site of a direct or indirect interaction between the agent and the receptor. For example, an antibody is said to target a domain if it binds specifically to an epitope contained at least partially in that domain. However, in some cases an agent (such as an antagonist) can target a domain by a means other than direct binding to the domain. For example, an agent can interact with a target domain by inducing an allosteric change that localizes at least in part to the specified domain.

Antagonists

[049] This disclosure concerns novel bombesin receptor antagonists that induce apoptosis in a nicotine resistant manner, and identifies a domain of the bombesin receptor that

mediates the apoptotic effects of such antagonists. A domain of the bombesin receptor involved in binding of the anti-neoplastic narcotic methadone was identified by competitive binding assays using a panel of synthetic peptides (20 amino acid residues in length) corresponding to specific sequence segments of the GRP type of the bombesin receptor (represented by GENBANK® Accession No. NM_005314). Of the panel, a single peptide with the sequence SFLVFYVIPLSIISVYYYFIA (SEQ ID NO:1), designated "Peptide O," was found to competitively inhibit binding of methadone to the bombesin receptor. Peptide O corresponds to the sequence of the fifth transmembrane domain of the bombesin receptor, and constitutes a target for antagonists that inhibit signaling events induced by binding of a BLP to the bombesin receptor. Additional experimental details regarding the identification of the antagonist target domain are provided in the Examples.

[050] Based on the identification of the antagonist target domain, exemplary antagonists were developed that interfere with bombesin receptor mediated signaling, and induce apoptosis and/or suppress growth of neoplastic cells *in vitro* and *in vivo*. The antagonists disclosed herein include peptides and polypeptides (and assemblies of polypeptides, such as antibodies) that target the fifth transmembrane domain of the bombesin receptor and/or interfere with bombesin receptor mediated signaling events. When cancer cells are contacted with one of the bombesin receptor antagonists described herein, apoptosis is induced suppressing growth of the neoplasm.

[051] In one specific example, the bombesin receptor antagonist is a peptide (a peptide antagonist) with a linear amino acid sequence of YFVLISVFILSPIYVYSFYIA (SEQ ID NO:2). This peptide antagonist shares certain characteristics with the fifth transmembrane domain of bombesin receptors (such as charge and size), but possesses a novel amino acid sequence that has not previously been described in any naturally occurring polypeptide or peptide. When cancer cells expressing high levels of bombesin receptors are exposed to this peptide *in vitro* or *in vivo*, it is believed that autocrine signaling maintained by binding of bombesin-like peptides to the bombesin receptor is interrupted, thereby interfering with the stimulatory signals involved in growth of such neoplastic cells. Thus, this peptide and related antagonists that target the fifth transmembrane domain of bombesin receptors are extremely useful in suppressing the growth of cancer cells, and can be used in the treatment of numerous neoplastic conditions that involve the growth of abnormal cells that express bombesin receptors.

[052] In addition to the peptide antagonist designated Peptide X (SEQ ID NO:2), numerous other related peptides that target the fifth transmembrane domain of bombesin receptors are features of this disclosure. Typically, bombesin receptor peptide antagonists

are peptides of at least three and no more than about 25 amino acids in length. In some examples, the peptide antagonists are four amino acids, five amino acids, six amino acids or larger, up to about 25 amino acids in length. In certain examples, the antagonists are approximately 20 amino acids (*e.g.*, 19 or 20 or 21 amino acids) in length.

[053] In certain examples, the peptide antagonists are subsequences of SEQ ID NO:2 or variants thereof with one or more amino acid substitutions. For example, the peptide antagonists are frequently subsequences of SEQ ID NO:2 including at least three amino acids. Commonly, the peptide antagonists are linear amino acids including a subsequence of SEQ ID NO:2 of at least three contiguous amino acids. Such variants are exemplified by SEQ ID NOs:3, 4, 6 and 10.

[054] In addition to SEQ ID NO:2 and subsequences thereof, certain variants of SEQ ID NO:2 including one (or two or three or four or five) amino acid substitutions that target the fifth transmembrane domain of bombesin receptors are also antagonists thereof. For example, included antagonists are peptides that can be aligned for purposes of sequence comparison with SEQ ID NO:2. When the antagonist peptides are aligned with at least a subsequence of SEQ ID NO:2, the aligned amino acids are said to correspond to the amino acid of SEQ ID NO:2 with which they are aligned. Thus, an antagonist peptide that is a subsequence of SEQ ID NO:2 includes amino acids corresponding to the aligned positions of SEQ ID NO:2 that are identical to SEQ ID NO:2. It will be appreciated, that an antagonist peptide of less than 21 amino acids will not include amino acids corresponding to all of the amino acids of SEQ ID NO:2, but rather will include amino acids corresponding to only a subset of SEQ ID NO:2. For example, a peptide antagonist that includes the first ten amino acids of SEQ ID NO:2 (that is, SEQ ID NO:3) includes amino acids corresponding to positions 1-10 of SEQ ID NO:2 that are identical to SEQ ID NO:2.

[055] More broadly, the antagonist peptides include variants of SEQ ID NO:2, in which amino acids corresponding to positions 1-3, 7, 10-16, 18, 20 and 21 (of the reference sequence of SEQ ID NO:2) are identical to SEQ ID NO:2; amino acids corresponding to positions 4 and 5 of SEQ ID NO:2 are leucine or isoleucine; the amino acids corresponding to position 6 of SEQ ID NO:2 is serine or isoleucine; the amino acids corresponding to positions 8 and 9 of SEQ ID NO:2 are phenylalanine or isoleucine; the amino acid corresponding to position 17 of SEQ ID NO:2 is serine or phenylalanine; and the amino acid corresponding to position 19 of SEQ ID NO:2 is tyrosine or valine. Such variants are specifically illustrated in SEQ ID NOs:5, 7, 8 and 9.

[056] Specific examples of antagonist peptides are provided in Table 1.

Table 1: Exemplary bombesin receptor antagonist peptides

Amino Acid Sequence	SEQUENCE ID NO.
YFVLISVFI LSPIYVYSFYIA	SEQ ID NO:2
YFVLISVFI L	SEQ ID NO:3
SPIYVYSFYIA	SEQ ID NO:4
YFVILI	SEQ ID NO:5
SPIYV	SEQ ID NO:6
SFVIA	SEQ ID NO:7
SVIFL	SEQ ID NO:8
YFVYIA	SEQ ID NO:9
YSFY	SEQ ID NO:10

[057] In additional examples, antagonists can include peptides with a linear sequence of amino acids, in which amino acids (if present) corresponding to positions 2, 7, 10, 11, 13, 15, 16, 20 and 21 of SEQ ID NO:2 are identical to SEQ ID NO:2; amino acids corresponding to positions 1, 6, 9, 12, 14, and 17 of SEQ ID NO:2 are amino acids with hydroxyl side chains; and amino acids corresponding to positions 3, 4, 5, 8, 18 and 19 of SEQ ID NO:2 are amino acids comprising a neutral side chain. For example, the peptide can include amino acids with hydroxyl side chains selected from S, Y and T and amino acids with neutral side chains selected from M, V, L, I, F, Y.

[058] Bombesin receptors possess a high degree of sequence similarity in the fifth transmembrane domain (Giladi *et al.*, *J. Mol. Neurosci.* 4:41-54, 1993; Corjay *et al.*, *J. Biol. Chem.* 266:18771-18779, 1991). Thus, peptides (and other antagonists) that target the fifth transmembrane domain of one bombesin receptor (*e.g.*, GRP-R) frequently also target the fifth transmembrane domain of other bombesin receptor(s) (*e.g.*, NMB-R). Where desired, bombesin receptor antagonists that specifically target one bombesin receptor, or that target two of the three known bombesin receptors (such as GRP-R and NMB-R) or that target all three known mammalian bombesin receptors can be identified and/or characterized in competitive binding assays using labeled ligands that are known to preferentially bind a particular bombesin receptors, such as [D-Phe6] bombesin-(6-13)-methyl ester for the GRP subtype, [Tyr4, D-Phe6] BN for the NMB subtype.

[059] In addition to the peptide antagonists described above, bombesin receptor antagonists can also be polypeptides (*e.g.*, longer than about 25 amino acids in length) that target the fifth transmembrane domain of a bombesin receptor. The polypeptide can be a fusion polypeptide including two (or more than two) domains originating (or corresponding to) different or heterologous proteins. For example, a fusion polypeptide antagonist can include a first domain corresponding to one of the peptide antagonists disclosed herein linked to a second protein domain, *e.g.*, selected from a protein other than the bombesin

receptor. The protein domain can be selected based on desired properties with respect to structure, function or both. For example, the second polypeptide domain can be selected to increase stability or enhance production or purification of the fusion polypeptide antagonist. Such a protein domain can also be a functional moiety, such as second or different growth inhibitory molecule or a toxin. When selecting appropriate fusion polypeptide domains, it is important to select domains that do not have an adverse effect on normal cells that express bombesin receptors albeit at lower levels than found on neoplastic cells.

[060] In certain examples, the bombesin receptor antagonists are antibodies that target the fifth transmembrane domain of a bombesin receptor. For example, the antibody-based antagonist can specifically bind to an epitope that includes at least a portion of SEQ ID NO:1, which represents the fifth transmembrane domain of the GRP-R. The antibody antagonist can alternatively bind to an epitope that includes at least a portion of the fifth transmembrane domain of the NMB-R represented by the amino acid sequence IFLVYFLIPLAIISIYYHIA (SEQ ID NO:14) or to an epitope that includes at least a portion of the fifth transmembrane domain of the BBR-3 represented by the amino acid sequence CFLVFYIPLSIISVYYSLIA (SEQ ID NO:15). In certain examples, the antibody antagonist binds to a cross-reactive epitope contained within the fifth transmembrane domains of two or more of the GRP-R, the NMB-R and the BBR-3. It will be appreciated by one of skill in the art that the fifth transmembrane domains of these two receptors share a high degree of sequence similarity and thus possess at least some epitopes that are specifically bound by the same antibody antagonists. In other instances, specific receptor antagonists based on antibodies that specifically bind to an epitope present within only one bombesin receptor are produced.

[061] The term “antibody” includes immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, that is, molecules that contain an antigen binding site that specifically binds (immunoreacts with) an epitope that is at least in part contained within the fifth transmembrane domain of a bombesin receptor. Bombesin receptor antagonists that are antibodies (either polyclonal or monoclonal, *e.g.*, IgG, IgM, IgD) typically include four polypeptide chains, two heavy (H) chains and two light (L) chains interconnected by disulfide bonds. In addition, the binding function of an antagonist antibody can be performed by fragments of a naturally occurring antibody. Thus, these antigen-binding fragments are also included within the term “antibody.” Specific, non-limiting examples of binding fragments encompassed within the term antibody include (i) a Fab fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) an F_d fragment consisting of the V_H and C_{H1} domains; (iii) an Fv fragment consisting of the V_L and V_H domains of a

single arm of an antibody, (iv) a dAb fragment (Ward *et al.*, *Nature* 341:544-546, 1989) which consists of a V_H domain; (v) an isolated complementarity determining region (CDR); and (vi) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region. Any such antibodies and/or antibody fragments are suitable as bombesin receptor antagonists so long as they bind to the bombesin receptor and interfere with ligand-induced signaling.

[062] In certain examples, the peptide and/or polypeptide and/or antibody antagonists can be used as diagnostic reagents for identifying cells that express high levels of bombesin receptors. In such applications, the bombesin receptor antagonist includes a detectable label or tag that facilitates detection of the diagnostic reagent. Numerous suitable, *e.g.*, optically detectable, tags and labels are known in the art, and can be conjugated to the bombesin receptor antagonists disclosed herein. For example, any compound or composition that is conjugated directly or indirectly to another molecule to facilitate detection of that molecule constitutes a label and can be used to produce diagnostic reagents based on the disclosed bombesin receptor antagonists. Specific, non-limiting examples of labels include fluorescent and other optically detectable labels (such as luminescent and phosphorescent labels), affinity tags, enzymatic linkages, and radioactive isotopes. An affinity tag is a peptide or polypeptide sequence capable of specifically binding to a specified substrate, for example, an organic, non-organic or enzymatic substrate or cofactor. A polypeptide including a peptide or polypeptide affinity tag can typically be recovered, for example, purified or isolated, by means of the specific interaction between the affinity tag and its substrate. An exemplary affinity tag is a poly-histidine (*e.g.*, six-histidine) affinity tag which can specifically bind to non-organic metals such as Nickel and/or cobalt. Additional affinity tags are well known in the art. In certain cases, the label is a fluorophore, that is, a luminescent chemical compound, which when excited by exposure to a particular stimulus such as a defined wavelength of light, emits light (luminesces or fluoresces), for example at a different wavelength. Numerous fluorophores including such commonly used agents as FITC, rhodamine, and various BODIPY dyes are well known in the art.

Production of bombesin receptor antagonists

[063] Methods for producing peptides and polypeptides (including antibodies) are well known in the art, and can be used to produce bombesin receptor antagonists. For example, both chemical synthesis and recombinant methods can be adapted for the production of the bombesin receptor antagonists disclosed herein. The particular method chosen depends on the nature of the antagonist (for example, whether the antagonist is a peptide, a polypeptide, such as a fusion protein or an antibody). Selection of an appropriate method based on the

composition of the antagonist is well within the knowledge of one of ordinary skill in the art. The following description is provided as guidance in the selection of a suitable method for producing exemplary bombesin receptor antagonists.

Production of synthetic peptide antagonists

[064] Any of the peptide antagonists disclosed herein (such as SEQ ID NOs:2-10) can be produced using solid phase peptide synthesis methods. Numerous references sufficient to guide one of skill in the art through the process of solid phase synthesis are available. For example, Fields (ed.) *Solid-Phase Peptide Synthesis* in the series *Methods in Enzymology*, Academic Press, San Diego, 1997, describes this methodology. Typically, peptides are synthesized using an automated or semi-automated programmable system, in which a representation of the desired peptide sequence is entered and converted to instructions for synthesizing the physical peptide. Examples of suitable solid phase synthesis systems include the Applied Biosystems 432A peptide synthesizer, also known as the Synergy, and the Rainin PS3 automated peptide synthesizer, also known as the Symphony. Additionally, the peptides disclosed herein can be obtained from commercial sources, such as Global Peptide Services (Fort Collins, CO).

[065] Peptide synthesis relies on two functional attributes of amino acids, the amino and carboxyl groups attached to the same carbon molecule. Functional moieties are also present in the side chains of many amino acids. During synthesis, these functionalities must be protected so that they do not interfere with the formation of the peptide bond.

[066] The process of peptide bond formation involves four steps: protection, activation, coupling and selective deprotection. Synthesis starts with the C-terminal amino acid, which is coupled to a solid phase resin. The initial Fmoc amino acid can be esterified to the support using a coupling reagent, typically a carbodiimide. Racemization can be minimized using an equimolar quantity of 1-hydroxybenzotriazole. The reaction can be catalyzed using a small quantity of 4-dimethylaminopyridine (DMAP). Typically, the loading procedure is followed by treatment with acetic anhydride to ensure that any residual resin bound hydroxyl groups are capped. Alternatively, synthesis can be carried out using a variety of preloaded resins (such as the CLEAR Resins available from Peptides International, Louisville, KY).

[067] Amino acids are then added one at a time until the N-terminus of the desired peptide is reached. Three steps are repeated each time an amino acid is added: (1) Deprotection of the N-terminal amino acid of the peptide bound to the resin; (2) Activation and addition of the next amino acid; and (3) Deprotection of the new N-terminal amino acid. Typically, the

resin is washed between subsequent steps of the synthesis process to remove unbound reagents.

[068] Various protection schemes are known in the art. Most commonly, the α -amino group of each amino acid is protected with a 9-fluorenylmethoxycarbonyl (Fmoc) group. Sidechains of Cysteine, Asparagine, Histidine and Glutamine can be protected with a Trityl (trt) group; sidechains of Threonine and Serine can be protected with a *tert*-Butyl (tBu) group; lysine sidechains can be protected with a *tert*-Butyloxycarbonyl (Boc) group, the sidechains of proline, leucine, isoleucine and glycine are left unprotected. Additionally, the phosphate groups of phosphorylated Serines, Threonines and Tyrosines can be protected with a Benzyl ester (OBzl) group where desired.

[069] All of the protecting groups used to protect functional sidechains of individual amino acids are acid labile, while the N-terminal amino function of the amino acid is protected by the Fmoc group which is base labile. New amino acids are added to an Fmoc amino acid that is attached to a resin by contacting the resin-attached amino acid with base (for example, 20% piperidine/DMF), and adding the next Fmoc amino acid ester along with the appropriate activator.

[070] Peptides can be synthesized as either the free carboxyl or as the C-terminal amide. Similarly, the N-terminus can be free or acetylated. Optionally, one or both peptide termini can be modified chemically (for example, by the addition of a label) or by the inclusion of a modified amino acid (for example, norleucine). Incorporation of unusual amino acid derivatives are only restricted by the availability of the Fmoc activated esters.

[071] Fmoc protection chemistry is compatible with a variety of activation chemistries. For example, successive amino acids can be activated using N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethanaminium tetrafluoroborate N-Oxide (TBTU) and 1H-Benzotriazolium 1[*bis*(dimethylamino)methylene]-Scholorohexafluorophosphate (1-),3-oxide (HCTU), although other chemistries can be used instead (for example, according to the manufacturer's instructions when using an automated synthesizer). Using the TBTU/HCTU chemistry, each protected amino acid is coupled as a free acid.

[072] Following synthesis, the peptide is cleaved from the resin. Typically, the resin is treated with trifluoroacetic acid (TFA) in the presence of appropriate scavengers. These scavengers include phenol, water, and triisopropylsilane. For example, the completed peptide can be cleaved from the resin using 92% TFA/2% triisopropylsilane/2% ethanedithiol/2% anisole/2% water. When the cleavage is performed after releasing the resin from the synthesizer, the resin can be removed by filtration (*e.g.*, using Whatman #4

filter paper or glass wool), and the peptide can be precipitated using methyl t-butyl ether (MTBE) or diethyl ether. Once precipitated, the peptide can be collected by centrifugation, and if desired, lyophilized for storage.

[073] Typically, the synthesized peptide is purified after cleavage from the resin. For example, the peptide can be purified by High Performance Liquid Chromatography (HPLC). Under typical synthetic conditions, where the product of interest constitutes the majority of the preparation, yields are on the order of 50% of the crude preparation. However, this is highly dependent upon the complexity and amount of impurities found in the crude peptide preparation, as well as of the purification conditions themselves. A typical preparative run can handle 100-150 mg of crude peptide, and can be accomplished using a variety of commercially available columns, such as those available from Higgins Analytical (Mountain View, CA) and Millipore (Billerica, MA). Conditions vary and are dependent on the analytical HPLC chromatogram profile. Both isocratic and gradient conditions can be used. For example, using a Higgins Analytical 15x20 mm 100 column, a Waters 600e gradient system using 0.1% TFA/water (A) and 0.1% TFA/ACN (B) is used to create a gradient that proceeds from 99:1 A:B to 70:30 A:B over approximately 40 minutes. UV absorbance of the HPLC fractions is measured with a Waters 5487 UV detector, and the fractions are collected using a Waters fraction collector II, or manually.

[074] The synthesized peptides can be analyzed by reverse phase HPLC and Mass Spectrometry to confirm their identity. Exemplary HPLC conditions are as follows: 0.4 x 25 cm Vydac C18 analytical column; 10-50% Acetonitrile (0.1% TFA)/water 40 minute linear gradient with a flow rate 1.0 ml/min; UV detector--220 nm Beckman Diode Array Detector Model 168 with a chart speed of 0.5 cm/min. Conditions can be adapted to specific devices in accordance with the manufacturer's directions.

[075] If desired the peptides can include one or more modification, such as the addition of an amide at the C-terminus of the peptide, or the addition of an acetyl group at the N-terminus. Additionally or alternatively a label, such as biotin or a fluorescent moiety or dye can be added to the peptide, *e.g.*, at the N terminus.

[076] Common solid phase synthetic procedures yield upwards of 20-50 μ M peptide. For a 20 amino acid peptide, this is approximately 40 mg of peptide. However, due to the number of steps involved including the chemistry, cleavage and deprotection, and extraction and washing the final recovery of the peptide is in the range 25-30 mg which is enough for most purposes. In the event that larger scale synthesis is desired solution or liquid phase peptide synthesis can be employed to produce antagonist peptides. Procedures for increasing the scale of production are well known to those of ordinary skill in the art.

[077] Alternatively, the peptides and polypeptides (including antibodies and fusion polypeptides) disclosed herein can be produced using procedures for the expression and production of recombinant proteins. The expression and purification of proteins, such as the bombesin receptor antagonists (including peptide antagonists, polypeptide antagonists, such as antibody and fusion protein antagonists) disclosed herein, can be performed using standard laboratory techniques. Examples of such methods are discussed and/or referenced herein. Methods for expressing large amounts of protein from recombinant nucleic acids introduced into bacterial (*e.g.*, *Escherichia coli*) or eukaryotic (*e.g.*, baculovirus/Sf9) cells can be utilized for the production and purification of bombesin receptor antagonists. For example, fusion polypeptides including the sequence of a peptide antagonist can be produced as described in US Patent No. 5,366,871, incorporated herein by reference.

[078] For example, fusion proteins including the peptide (or polypeptide) antagonists linked to one or more peptide (or polypeptide) domains that facilitate expression and/or purification, such as a signal sequence, a linker sequence and/or a tag sequence can be expressed in cells and purified using various procedures (for example, based on the included tag). Methods and plasmid vectors for producing fusion proteins in cultured cells are well known in the art, and specific methods are described in Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, CSHL, New York, 2001) and in Brent *et al.*, *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, 2003). Such fusion proteins may be made in large amounts, are easy to purify, and can be used for functional assays and/or as therapeutic molecules. Recombinant proteins can be produced in bacteria by placing a strong, regulated promoter and an efficient ribosome-binding site upstream of the polynucleotide sequence that encodes the desired protein product. If low levels of protein are produced, additional steps may be taken to increase protein production; if high levels of protein are produced, purification is relatively easy. Suitable methods are presented in Sambrook *et al.* (In *Molecular Cloning: A Laboratory Manual*, CSHL, New York, 2001) and are well known in the art.

[079] Vector systems suitable for the expression of fusion proteins include the pUR series of vectors (Ruther and Muller-Hill, *EMBO J.* 2:1791, 1983), pEX1-3 (Stanley and Luzio, *EMBO J.* 3:1429, 1984), pMR100 (Gray *et al.*, *Proc. Natl. Acad. Sci. USA* 79:6598, 1982), and pET series vectors (Studier and Moffatt, *J. Mol. Biol.* 189:113, 1986).

[080] The polynucleotide sequence can also be transferred from its existing context to other cloning vehicles, such as other plasmids, bacteriophages, cosmids, animal viruses and yeast artificial chromosomes (YACs) (Burke *et al.*, *Science* 236:806-812, 1987). These vectors may then be introduced into a variety of hosts including somatic cells, and simple or

complex organisms, such as bacteria, fungi (Timberlake and Marshall, *Science* 244:1313-1317, 1989), invertebrates, plants (Gasser and Fraley, *Science* 244:1293, 1989), and animals (Pursel *et al.*, *Science* 244:1281-1288, 1989), which cell or organisms are rendered transgenic by the introduction of the heterologous nucleic acid.

[081] DNA sequences can be manipulated with standard procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-alteration via single-stranded bacteriophage intermediate or with the use of specific oligonucleotides in combination with PCR or other *in vitro* amplification.

[082] A polynucleotide sequence that encodes a bombesin receptor antagonist can be introduced into eukaryotic expression vectors by conventional techniques. These vectors are designed to permit the transcription of the polynucleotide sequence in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the coding sequence and ensure its proper splicing and polyadenylation. Vectors containing the promoter and enhancer regions of the SV40 or long terminal repeat (LTR) of the Rous Sarcoma virus and polyadenylation and splicing signal from SV40 are readily available (Mulligan *et al.*, *Proc. Natl. Acad. Sci. USA* 78:1078-2076, 1981; Gorman *et al.*, *Proc. Natl. Acad. Sci. USA* 78:6777-6781, 1982). The level of expression of the polynucleotide sequence can be manipulated with this type of vector, either by using promoters that have different activities (for example, the baculovirus pAC373 can express cDNAs at high levels in *S. frugiperda* cells (Summers and Smith, In *Genetically Altered Viruses and the Environment*, Fields *et al.* (Eds.) 22:319-328, CSHL Press, Cold Spring Harbor, New York, 1985) or by using vectors that contain promoters amenable to modulation, for example, the glucocorticoid-responsive promoter from the mouse mammary tumor virus (Lee *et al.*, *Nature* 294:228, 1982). The expression of the polynucleotide sequence can be monitored in the recipient cells 24 to 72 hours after introduction (transient expression).

[083] In addition, some vectors contain selectable markers such as the *gpt* (Mulligan and Berg, *Proc. Natl. Acad. Sci. USA* 78:2072-2076, 1981) or *neo* (Southern and Berg, *J. Mol. Appl. Genet.* 1:327-341, 1982) bacterial genes. These selectable markers permit selection of transfected cells that exhibit stable, long-term expression of the vectors (and therefore the desired polynucleotide sequence). The vectors can be maintained in the cells as episomal, freely replicating entities by using regulatory elements of viruses such as papilloma (Sarver *et al.*, *Mol. Cell Biol.* 1:486, 1981) or Epstein-Barr (Sugden *et al.*, *Mol. Cell Biol.* 5:410, 1985). Alternatively, one can also produce cell lines that have integrated the vector into genomic DNA. Both of these types of cell lines produce the gene product on a continuous

basis. One can also produce cell lines that have amplified the number of copies of the vector (and therefore of the polynucleotide sequence) to create cell lines that can produce high levels of the gene product (Alt *et al.*, *J. Biol. Chem.* 253:1357, 1978).

[084] The transfer of nucleic acids into eukaryotic, in particular human or other mammalian cells can be accomplished by a number of well-known procedures. The vectors are introduced into the recipient cells as pure DNA (transfection) by, for example, precipitation with calcium phosphate (Graham and vander Eb, *Virology* 52:466, 1973) or strontium phosphate (Brash *et al.*, *Mol. Cell Biol.* 7:2013, 1987), electroporation (Neumann *et al.*, *EMBO J* 1:841, 1982), lipofection (Felgner *et al.*, *Proc. Natl. Acad. Sci USA* 84:7413, 1987), DEAE dextran (McCuthan *et al.*, *J. Natl. Cancer Inst.* 41:351, 1968), microinjection (Mueller *et al.*, *Cell* 15:579, 1978), protoplast fusion (Schafner, *Proc. Natl. Acad. Sci. USA* 77:2163-2167, 1980), or pellet guns (Klein *et al.*, *Nature* 327:70, 1987). Alternatively, the nucleic acid can be introduced by infection with virus vectors. Systems are developed that use, for example, retroviruses (Bernstein *et al.*, *Gen. Engr'g* 7:235, 1985), adenoviruses (Ahmad *et al.*, *J. Virol.* 57:267, 1986), or Herpes virus (Spaete *et al.*, *Cell* 30:295, 1982). Protein, such as bombesin receptor antagonist, encoding sequences can also be delivered to target cells *in vitro* via non-infectious systems, for instance liposomes.

[085] Using the above techniques, the expression vectors encoding a bombesin receptor antagonist can be introduced into human cells, mammalian cells from other species or non-mammalian cells as desired. For example, monkey COS cells (Gluzman, *Cell* 23:175-182, 1981) that produce high levels of the SV40 T antigen and permit the replication of vectors containing the SV40 origin of replication may be used. Similarly, Chinese hamster ovary (CHO), mouse NIH 3T3 fibroblasts or human fibroblasts or lymphoblasts may be used.

[086] A host cell, which can be transfected with the vector of this disclosure, can be selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus subtilis*, *Bacillus stearothermophilus* or other bacilli; other bacteria; yeast; fungi; insect; mouse or other animal; plant hosts; or human tissue cells.

[087] Bombesin receptor antagonists that are antibodies can be produced using any method known in the art for producing antibodies with desired binding characteristics. Both polyclonal and monoclonal antibodies that specifically bind to the fifth transmembrane domain of a bombesin receptor can be used as bombesin receptor antagonists. Methods of producing polyclonal and monoclonal antibodies are known to those of ordinary skill in the art, *see, e.g.*, Coligan, *Current Protocols in Immunology* Wiley/Greene, NY, 1991; and Harlow and Lane, *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY, 1989; Stites *et al.*, (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications,

Los Altos, CA, and references cited therein; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY 1986; and Kohler and Milstein, *Nature* 256: 495-497, 1975. Other suitable techniques for antibody preparation include selection of libraries of recombinant antibodies in phage or similar vectors. *See*, Huse *et al.*, *Science* 246: 1275-1281, 1989; and Ward *et al.*, *Nature* 341: 544-546, 1989. "Specific" monoclonal and polyclonal antibodies and antisera (or antiserum) will usually bind with a K_D of at least about 0.1 μM , preferably at least about 0.01 μM or better, and most typically and preferably, 0.001 μM or better.

[088] For example, polyclonal serum including antibodies specific for an epitope contained at least partially within the fifth transmembrane domain of a bombesin receptor can be produced (for example) by conjugating a peptide corresponding to the fifth transmembrane domain of the receptor, such as the exemplary peptide Peptide O (SEQ ID NO:1) to keyhole limpet hemocyanin (KLH) using glutaraldehyde. The KLH-peptide conjugate is emulsified with 0.5 ml Freund's complete adjuvant and injected subcutaneously into a rabbit at multiple (*e.g.*, four) sites. Several (for example, four) booster injections are given over a period of several weeks, and the animals are bled periodically. Titer is typically determined using an ELISA assay. For example, plates are coated with free or conjugated antigen/peptide at an optimal concentration of approximately 5 $\mu\text{g/ml}$. Goat anti-rabbit HRP conjugate is used for detection with the OPD (o-phenylenediamine dihydrochloride) peroxidase substrate. The results are read at a wavelength of 490-650 nm.

[089] In addition to the production of polyclonal sera outlined above, and the production of monoclonal antibodies, immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture (*e.g.*, see U.S. Patent No. 4,745,055; U.S. Patent No. 4,444,487; WO 88/03565; EP 256,654; EP 120,694; EP 125,023; Faoukner *et al.*, *Nature* 298:286, 1982; Morrison, *J. Immunol.* 123:793, 1979; Morrison *et al.*, *Ann Rev. Immunol* 2:239, 1984). Detailed methods for preparation of chimeric (*e.g.*, humanized) antibodies can be found in U.S. Patent 5,482,856. Additional details on humanization and other antibody production and engineering techniques can be found in Borrebaeck (ed), *Antibody Engineering*, 2nd Edition Freeman and Company, NY, 1995; McCafferty *et al.*, *Antibody Engineering, A Practical Approach*, IRL at Oxford Press, Oxford, England, 1996, and Paul *Antibody Engineering Protocols* Humana Press, Towata, NJ, 1995.

[090] In some examples, it is beneficial to obtain isolated and purified bombesin receptor antagonist peptides or polypeptides, for instance for use as therapeutic agents in the treatment of lung or other cancers. One skilled in the art will understand that there are myriad ways to purify recombinant polypeptides, and such typical methods of protein

purification may be used to purify the disclosed antagonists. Such methods include, for instance, protein chromatographic methods including ion exchange, gel filtration, HPLC, monoclonal antibody affinity chromatography and isolation of insoluble protein inclusion bodies after over production. In addition, purification affinity-tags, for instance a six-histidine sequence, may be recombinantly fused to the protein and used to facilitate polypeptide purification (*e.g.*, in addition to another functionalizing portion of the fusion, such as a targeting domain or another tag, or a fluorescent protein, peptide, or other marker). A specific proteolytic site, for instance a thrombin-specific digestion site, can be engineered into the fusion protein between the tag and the remainder of the fusion to facilitate removal of the tag after purification, if such removal is desired.

[091] Commercially produced protein expression/purification kits provide tailored protocols for the purification of proteins made using each system. See, for instance, the *QIAexpress*TM expression system from QIAGEN (Chatsworth, CA) and various expression systems provided by INVITROGEN (Carlsbad, CA). Where a commercial kit is employed to produce an bombesin receptor antagonist fusion protein, the manufacturer's purification protocol is a preferred protocol for purification of that protein. For instance, proteins expressed with an amino-terminal hexa-histidine tag can be purified by binding to nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrix (*The QIAexpressionist*, QIAGEN, 1997).

[092] In the case of bombesin receptor antagonists that are antibodies, the binding specificities of the antibody can be exploited to facilitate specific purification of the antagonists. One example method of performing such specific purification would be column chromatography using column resin to which the target molecule, or an appropriate epitope or fragment (*e.g.*, corresponding to all or a portion of the fifth transmembrane domain of the bombesin receptor), has been attached.

[093] For example, antagonist antibodies can be affinity purified using the same peptides (antigen) that were used for the production of antibodies to build an affinity matrix. The immunoaffinity matrix is typically prepared by coupling the synthetic peptide to cyanogens bromide-activated sepharose beads. The serum is pre-filtered using a 0.45 μ m filter. After the matrix has been prepared, the filtered serum will be added to the matrix and incubated (*e.g.*, for 4 hours at room temperature) to allow the matrix to bind the free antibody in the serum. After incubation, the matrix-serum slurry is poured into a column, and the flowthrough fractions are collected. The column is attached to a UV monitor, which measures the absorbance at 280 nm, a direct indication of amount of protein. The matrix is then gently washed with PBS to remove any weakly bound proteins, and the strongly bound

antibody is eluted using a low pH (acidic) glycine buffer. The low-pH eluates is neutralized with pH 8.0 Tris-buffer, and dialyzed into PBSA (phosphate buffered saline solution with azide), and then concentrated.

Induction of apoptosis by synthetic peptide antagonists

[094] The bombesin receptor antagonists disclosed herein are useful for suppressing growth of neoplastic cells. Numerous neoplastic, *e.g.*, cancer, cells express one or more bombesin receptors at levels substantially higher than normal cells (for example, as compared to non-cancerous cells of the same tissue). For example, a cancer cell can express a bombesin receptor at a level more than twice (2x) that of a corresponding normal cell. In some cases, cancer cells express a bombesin receptor more than 5x a normal cell, in some instances cancer cells express a bombesin receptor more than 10x a corresponding normal cell. Such cells are said to “over-express” a bombesin receptor. Many such cells over-express more than one bombesin receptor (such as one or more of the GRP-R and NMB-R).

[095] Over-expression of one or more bombesin receptors is implicated in the promotion of neoplastic growth of cancer cells. Interrupting signaling activity initiated by binding of a ligand (*e.g.*, a BLP) to the bombesin receptor induces apoptosis of these cells and thus interferes with cancer growth. For example, some cancer cells that over-express bombesin receptors also express one or more BLP. Simultaneous expression of the bombesin receptor and ligand maintains an autocrine feedback loop that promotes continued growth and survival of the cells. Interference with this autocrine signaling system induces apoptosis and suppresses growth of the cells.

[096] The bombesin receptor antagonists (including both peptide and polypeptide antagonists) disclosed herein are useful for disrupting bombesin receptor mediated signaling activity and inducing apoptosis of cancer cells, *e.g.*, cancer cells that over-express one or more bombesin receptors, such as lung cancer (*e.g.*, small cell and non-small cell lung carcinoma), osteosarcoma, breast cancer, colon cancer, gastric cancer, pancreatic cancer, prostate cancer and melanoma (*see, e.g.*, Siegfried *et. al.*, *Pulm Pharmacol Ther* 12: 291-302, 1999 and Uchida *et. al.*, *J Cancer Res Clin Oncol.* 128: 633-640, 2002; Giacchetti *et. al.*, *Intl J Cancer* 46: 293-298, 1990. and Gugger *et. al.*, *Am J Path* 155: 2067-2076, 1999; Sun *et. al.*, *Prostate* 42: 295-303, 2000 and Markwalder *et. al.*, *Cancer Res* 59: 1152-1159, 1999; Halmos *et. al.*, *Cancer Lett* 85: 189-192, 1994; Carroll *et. al.*, *Mol Pharmacol* 58: 601-607, 2000; Ehler *et. al.*, *Ann Surg* 231: 838-848, 2000; Sun *et. al.*, *Reg Peptide* 90: 77-84, 2000; Pansky *et. al.*, *J Am Soc Nephrol* 11: 1409-1418, 2000). Accordingly, the disclosed bombesin receptor antagonists are useful in methods for suppressing growth and/or inducing apoptosis of neoplastic cells, such as cancer cells. Cells, *in vitro* and/or *in*

vivo are contacted with one or more bombesin receptor antagonist(s), and optionally with one or more additional agent. Without being bound by a mechanism, it is believed that the bombesin receptor antagonist interferes with bombesin receptor mediated signaling involved in maintaining and promoting neoplastic growth, thereby inducing apoptosis and suppressing growth of the neoplastic cells.

[097] The bombesin receptor antagonists disclosed herein possess at least two important advantages with respect to previously described agents that can interfere with bombesin receptor mediated signaling. Firstly, the disclosed bombesin receptor antagonists (peptide and polypeptide antagonists), suppress growth of cancer cells without appreciably affecting the growth or survival of normal cells of the same tissue. Secondly, the disclosed peptide and polypeptide (*e.g.*, antibody) antagonists are resistant to inhibition by nicotine.

[098] The ability of the antagonist to inhibit cancer cell growth (and conversely the ability to not appreciably affect growth of normal cells) can be evaluated and/or monitored by a variety of direct and or indirect methods for assessing apoptosis *in vitro* and *in vivo*. For example, apoptosis can be monitored in cells *in vitro* using the alamarBlue™ bioassay, according to the manufacturer's instructions. In brief, cells are plated in 24-well tissue culture plates (1×10^4 /well), and incubated for 24 hours at 37°C in an appropriate growth medium. For example, lung cancer cells can be cultured in RPMI-1640 medium with 5% fetal bovine serum, SAEC cells can be cultured in a specialized cell culture medium supplied by Clonetics Products, osteosarcoma cells and normal bone cells can be cultured in alpha-MEM medium with 5% fetal bovine serum.

[099] Various (*e.g.*, 10 – 1000 nM) concentrations of the antagonist are added to the medium, and incubated at 37°C. Standard chemotherapeutic drugs (*e.g.*, cisplatin, adriamycin) or other known inhibitors of bombesin receptor signaling (*e.g.*, methadone), are typically used for comparison. Following a 4-day culture period, cell numbers are measured with the metabolic indicator alamarBlue™ (Biosource International, Camarillo, CA), which is a non-destructive oxidation-reduction colorimetric indicator used to measure viable cell numbers. A 500 μ L volume containing 10% alamarBlue in the tissue culture medium is added to each of the 24-well samples. Following a 2 hour incubation interval at 37°C, 200 μ L samples are collected, plated in a 96-well format in duplicate, and measured on a fluorescence measurement system (such as the Fluroskan Ascent FL, Thermo Labsystems, Helsinki, Finland), with 544 nm excitation and 590 nm emission. Control wells contain the 10% alamarBlue solution to provide the background level measurements for oxidation of alamarBlue. Absorbance values are converted into cell numbers extrapolated from established standard curves. Cell viability is typically determined relative to control (no

treatment). To determine statistical significance, (for example, between different dosages, or under different culture conditions, or in different cell types) multiple replicates (*e.g.*, 5) per analytical interval are performed to provide a 90% confidence level for the mean $\pm$ SD, and analysis of variance (ANOVA) is conducted.

[0100] The apoptotic effects of bombesin receptor antagonists can also be determined using methods that quantitatively measures soluble nuclear matrix proteins released from apoptotic nuclei (such as the Nuclear Matrix Protein (NMP) ELISA kit from Calbiochem, San Diego). In such methods, cells grown in culture are typically incubated (for example for 2 hours at 37°C) in serum free medium to which appropriate concentrations of bombesin receptor antagonists are added. After incubation, the culture media is removed and the cells are lysed. The cell lysates are centrifuged at 2000 rpm for 10 minutes and the supernatant is collected. 100 μ l of supernatant is assayed in 96 well plates, according to the manufacturer's instructions. Mouse monoclonal antibodies for the measurement of soluble nuclear matrix proteins are immobilized onto the surface of the plastic wells provided in the kit. The absorbance in each well is then measured in a spectrophotometric plate reader at a dual wavelength of 450/595 nm. Concentrations of NMP in the samples are determined by interpolation from the standard curve, derived using the NMP standard in the kit.

[0101] For analysis of DNA fragmentation on agarose gels, 2×10^6 cells are incubated at 37°C for 2 hours with various concentrations of antagonist, washed in PBS, centrifuged at 1000 rpm, and incubated at 37°C for 4 hours in digestion buffer (200 mM Tris, pH 8.5, 100 mM EDTA, 50 mg/ml proteinase K and 1% SDS). DNA is extracted with equal volume of phenol, and the aqueous phase dialyzed overnight in 10 mM Tris, pH 7.5, 1 mM EDTA. After dialysis, the DNA solution is incubated at 37°C with 50 mg/ml of RNase A for 4 hours, and with 120 mg of proteinase K for an additional 4 hours. DNA is extracted with an equal volume of phenol/chloroform. NaCl will be added to the aqueous phase (final concentration 150 mM), and precipitated with 2 volumes of ethanol. Pellets are resuspended in 50 μ l of distilled water. DNA concentration is calculated by determining the O.D. at 260 nm. Horizontal electrophoresis of the DNA is performed in 1% agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA), pH 8. DNA is then visualized by ethidium bromide staining. Fragmented DNA from apoptotic cells migrates more quickly than intact DNA on agarose gels.

[0102] Another method of evaluating (*e.g.*, monitoring) induction of apoptosis by the disclosed bombesin receptor antagonists is by Terminal dUTP Nick-End Labeling (TUNEL) ELISA Assay. In this method, cells treated with a bombesin receptor antagonist are harvested and lysed, and the cell lysates are analyzed to assess degenerative changes as

indicated by the extent of high-molecular weight DNA strand breaks. Strand breaks can be determined using commercially available reagents and kits, such as the Cell Death Detection ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's instructions.

[0103] To confirm the activation of caspase by CXCL10, 3 hours before the addition of CXCL10, a pan-caspase inhibitor [50 μ mol/L N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethy ketone (zVAD-fmk)] or a caspase-3-specific inhibitor [50 μ mol/L z-Asp-Glu-Val-Asp-fluoromethy ketone (DEVD-fmk)] can be added. Briefly, CXCL10-treated, control and antagonist-exposed cells are harvested and transferred to the streptavidin-coated microplates, followed by incubation with anti-histone-biotin and anti-DNA-POD complex. During the incubation period, the anti-histone antibody binds to the histone component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated MP via its biotinylation. Additionally, the anti-DNA-POD antibody reacts with the DNA component of the nucleosomes. After removing the unbound antibodies, the labeled nucleosomes are measured photometrically by ELISA. Positive controls (included in kit), as well as negative controls (untreated cell samples generally exhibit a 5 - 10% range of apoptosis), can be utilized to determine statistical significance, for example using a student's t-test.

[0104] A modified TUNEL staining protocol (*see, e.g., Gavrieli et al., J Cell Biol* 119:493-501, 1992; Hara *et al., Brain Res* 697:247-50, 1995) can be used to assess induction of apoptosis *in situ (in vivo)*. For example, desired tissue can be evaluated after excising the tissue (*e.g., after resecting the cancer*) and immersing the excised tissue in a fixative containing 4% paraformaldehyde. The tissue is then embedded and cut into 10- μ m-thick sections with a cryostat. Sections at desired intervals are mounted on precoated slides, and TUNEL staining is performed (*e.g., using commercially available apoptosis detection kits, such as from Roche Molecular Biochemicals, Indianapolis, IN*). Briefly, tissue sections are first incubated in a solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes on ice (4°C) to increase permeability. After washing twice in PBS, pH 7.4, the sections are immersed in the TUNEL reaction mixture, containing biotinylated dUTP and terminal deoxynucleotidyl transferase (TdT) conjugated with fluorochromes (tetramethylrhodamine red) for 60 minutes at 37°C in a dark, humidified atmosphere. The process is terminated by washing the sections twice in a blocking buffer (PBS, Triton X-100, and BSA). In each assay, negative controls are commonly included using the same incubation procedure but omitting TdT in the process, whereas positive controls are

performed by incubating the permeated sections with DNase (1 µg/ml) to induce DNA strand breakage. Slides are evaluated for breakage by fluorescence microscopy.

[0105] Another method of assessing apoptosis is using morphological parameters. Morphological analysis can be used alone or in conjunction with other methods (such as the alamarBlue bioassay described above). Apoptotic cells can be identified by direct staining of condensed nuclei or fragmented DNA in cells with bisbenzimidazole (Hoechst 33258, Sigma, St. Louis, MO). The bisbenzimidazole stock solution is typically added directly into the culture medium (at a final concentration of 0.05%) and incubated with the cells for 15 minutes at 37°C. The cells are then visualized by microscopic analysis with the use of an inverted phase-contrast fluorescence microscope (model DMIRB, Leica, Inc., Deerfield, MI). Image processing can be performed with the use of various software packages, such as ImagePro Plus (MediaCybernetics, Inc., Silver Spring, MD). For quantitative assessment of apoptosis, the condensed nuclei or fragmented DNA-positive cells are counted (for example, in 5 fields per sample), and expressed as the percentage of apoptotic cells. To assess statistical significance, each assay is typically performed at least three times, and the data is represented as means $\pm$ SD. Comparison between groups (*e.g.*, treated and untreated cells) can be assessed by a two-way ANOVA.

[0106] As described above, growth suppression by the disclosed bombesin receptor antagonists is resistant to inhibition by nicotine, which is frequently found in the system of subjects with cancer due to continued smoking. Resistance to inhibition by nicotine can be monitored using any of the growth and apoptosis assays described above. For example, cells can be treated with a fixed concentration (100 nM) of antagonist, and varying concentrations (10–1000 nM) of nicotine can be added to the growth medium. Apoptosis is then measured in the presence and absence of nicotine. Methadone, and the chemotherapeutic drugs, cisplatin and adriamycin, can also be used for comparison if desired.

[0107] Growth inhibition can also be measured *in vivo*, by administering a bombesin receptor antagonist to a subject with one or more naturally occurring or experimentally induced tumors, and monitoring the effect of the antagonist on the size and/or number of tumors.

[0108] For example, one experimental model for evaluating the effect of a bombesin receptor antagonist involves administering the antagonist to athymic rats into which cancer cells have been introduced, and monitoring the effects of the antagonist on tumor size and number. Eight week old male Harlan nude athymic rats (Hsd:RH-r nu) are housed in a sterile housing facility prior to surgery and subsequent evaluation. The animals are

withdrawn from food 12-16 hours prior to surgery. The animals are anesthetized with 1.5% isofluorane with 100% oxygen. Following the sterile preparation of the implant site by topical application of betadine and a sterile isopropal alcohol wipe, cancer cells (such as N417 human small cell lung carcinoma cells) mixed with BD Matrigel Matrix High Concentration (high proteins) are injected at a concentration of approximately $0.8 - 1 \times 10^6$ cells, in a subcutaneous position at the lower back. The cells are mixed with BD Matrigel high proteins to enhance the successful transplantation of the human tumor cells increase tumor growth rates *in vivo*. When the animals have recovered (are sternal and ambulatory), the rats are returned to individual cages. Each animal receives a veterinary analgesic for routine post-operative pain control.

[0109] Tumor growth is monitored, and is typically in the range of 3 - 4 mm per week in this model system. When the tumor reaches 1 cm in diameter, the animals are treated with a bombesin receptor antagonist or placebo control. For example, dual intratumoral tract injections of 50 μ L per tract evenly distributed within the tumor can be administered to deliver 1 μ g of a peptide antagonist per tract (appropriate concentrations of the various antagonists can be varied and determined empirically). Tumor growth then monitored (typically at least 3x per week).

[0110] Tumor mass is measured using a caliper and the length recorded. At the desired time point (for example after detection of tumor regression or growth), the tumor is excised en bloc and placed in 4% buffered paraformaldehyde. Following adequate fixation, the tumor is divided into smaller portions and processed for paraffin embedding. The embedded blocks are sectioned and stained with hematoxylin and eosin (H & E). Following location of the injection tracts, the stained histologic specimens can be quantitatively assessed for mitogenic indices at one or more sites adjacent to the injection tracts (within 1 mm) using a suitable optical imaging system, such as the Leica DC 300F digital imaging system with Image-Pro Plus interfaced with a Leica DMIRB Microscope (Leica Instruments, Inc.).

[0111] To insure adequate numbers for statistical analysis, at least 9 animals per treatment and/or control group are typically analyzed. Mean $\pm$ SD are determined and the treatment groups are compared using an ANOVA and post-hoc multiple comparison tests with time and treatments as repeated measures. Individual differences among the groups at each time period can be determined with the use of the Fisher protected least-significant difference test for multiple comparisons.

Treatment of cancer with synthetic peptide antagonists

[0112] Based on their ability to induce apoptosis and suppress growth of neoplastic cells, the bombesin receptor antagonists disclosed herein are useful for treating a variety of cancers. These antagonists are particularly useful for the treatment of cancers that over-express bombesin receptors and/or those that depend on an autocrine BLP-bombesin receptor mediated system for growth and/or survival, such as lung cancers (such as small cell lung carcinomas) as well as cancers of numerous other tissues, including bone, breast, colon, pancreas, prostate and stomach. Accordingly, the present disclosure provides methods for treating neoplastic conditions and diseases, such as cancer. Such methods involve administering to a subject with such a condition, an anti-neoplastic agent including a bombesin receptor antagonist under conditions that result in the neoplastic cell being contacted with an effective amount of the antagonist. Typically, the antagonist is delivered in a pharmaceutical composition or medicament as described in more detail below. Optionally, the subject is tested for over-expression of bombesin receptors before (and/or during treatment).

[0113] A pharmaceutical composition (for example, containing a bombesin receptor antagonist) can be administered by any means known to one of skill in the art, such as by systemic administration methods such as intravenous, subcutaneous, or intramuscular injection, but even oral, nasal and transdermal routes are contemplated. Alternatively, the pharmaceutical composition can be administered via a route that delivers the bombesin receptor antagonist directly to the desired site of action, *e.g.*, via an inhalation method using an aerosol to deliver the antagonist to the lung or injection directly into a tumor.

[0114] As an alternative to liquid formulations, the bombesin receptor antagonist composition can be administered in solid form, *e.g.*, as a powder, pellet or tablet. For example, the bombesin receptor antagonist can be administered as a powder using a transdermal needleless injection device, such as the helium-powered POWDERJECT® injection device. This apparatus uses pressurized helium gas to propel a powder formulation of a bombesin receptor antagonist, *e.g.*, an antagonist peptide, at high speed so that the particles perforate the stratum corneum and contact cells in the epidermis.

[0115] The term “medicament” is used interchangeably with the term “pharmaceutical composition.” Such compositions are formulated for administration to human and/or animal (veterinary) subjects, and typically include a bombesin receptor antagonist as well as one or more additional components to facilitate administration to a subject, for the therapeutic or prophylactic treatment (prevention or reduction of recurrence or metastasis) of a neoplastic condition or disease, such as lung cancer. The additional components can include

pharmaceutically acceptable carriers, buffers or excipients. Pharmaceutically acceptable carriers, excipients, diluents, buffers and so forth, are well known in the art, and are described, *e.g.*, in *Remington's Pharmaceutical Sciences*, 19th Ed., Mack Publishing Company, Easton, Pennsylvania, 1995.

[0116] The pharmaceutical compositions (medicaments) containing a bombesin receptor antagonist disclosed herein can be administered to a subject to suppress the growth of neoplastic cells. Accordingly, the compositions are administered to a subject with a neoplastic condition or disease, or at risk of such a condition or disease, to ameliorate, prevent or reduce the deleterious effects of such a condition or disease. Optionally, the subject is evaluated prior and/or during treatment to assess the level of expression of one or more bombesin receptors.

[0117] The quantity of bombesin receptor antagonist included in the pharmaceutical composition is an amount determined to be sufficient to suppress growth (*e.g.*, to induce apoptosis) of the neoplastic cells. As such, the pharmaceutical compositions disclosed herein are anti-neoplastic agents (that is, anti-neoplastic agent that include at least one bombesin receptor antagonist). For example, when administered to a subject (such as a human subject) in one or more doses, an anti-neoplastic pharmaceutical composition can include an amount of a bombesin receptor antagonist sufficient to provide at least about 0.001 mg, such as about 0.005 mg, peptide antagonist per kg body weight of the subject (*e.g.*, about 0.005 mg/kg). Thus, exemplary compositions include an amount of a bombesin receptor antagonist peptide from about 0.008 mg/kg (for example, about 0.01 mg/kg, or about 0.02 mg/kg, or about 0.025 mg/kg, or about 0.05 mg/kg) to about 0.1 mg/kg.

Typically, the peptide antagonists are administered at a dose of at least about 5 µg/kg, and at no more than about 50 µg/kg body weight. Thus, for administration to an adult human, the composition can be formulated to include at least about 0.1 mg (100 µg) of a bombesin receptor antagonist peptide, to about 100 mg of the peptide antagonist, in a single dose. An antibody antagonist can be supplied in an amount sufficient to provide at least about 5µg, and generally no more than about 100 µg, per kg body weight of the subject. One of skill in the art will appreciate that due to the larger size and molecular weight of the antibody or other polypeptide antagonists, the weight of the antagonist in a formulation is increased to maintain a comparable molarity. Suitable dose ranges and dosage can be determined by one of skill in the art for any bombesin receptor antagonist.

[0118] The pharmaceutical composition typically includes one or more pharmaceutically acceptable constituents, such as a pharmaceutically acceptable carrier and/or pharmaceutically acceptable diluent. Typically, preparation of an anti-neoplastic

pharmaceutical composition (medicament) entails preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. Typically, the pharmaceutical composition contains appropriate salts and buffers to render the components of the composition stable and facilitate administration to a subject. Such components can be supplied in lyophilized form, or can be included in a diluent used for reconstitution of a lyophilized form into a liquid form suitable for administration. Alternatively, where the inactivated pathogen is prepared for administration in a solid state (*e.g.*, as a powder or pellet), a suitable solid carrier is included in the formulation.

[0119] Aqueous compositions typically include an effective amount of the bombesin receptor antagonist dispersed (for example, dissolved or suspended) in a pharmaceutically acceptable diluent or aqueous medium. Pharmaceutically acceptable molecular entities and compositions generally do not produce an adverse, allergic or other undesirable reaction when administered to a human or animal subject. As used herein, pharmaceutically acceptable carriers include any and all solvents, dispersion media, coatings, isotonic and absorption delaying agents, and the like. Optionally, a pharmaceutically acceptable carrier or diluent can include an antibacterial, antifungal or other preservative. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with production of an anti-neoplastic response, its use in the pharmaceutical compositions is contemplated. In some cases (for example, when liquid formulations are deemed desirable, or when the agent is reconstituted for multiple doses in a single receptacle), these preparations contain a preservative to prevent or inhibit the growth of microorganisms.

[0120] In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (*e.g.*, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example, sodium acetate or sorbitan monolaurate.

[0121] For example, the pharmaceutical compositions (medicaments) can include one or more of a stabilizing detergent, a micelle-forming agent, and an oil. Suitable stabilizing

detergents, micelle-forming agents, and oils are detailed in U.S. Patents No. 5,585,103; 5,709,860; 5,270,202; and 5,695,770. A stabilizing detergent is any detergent that allows the components of the emulsion to remain as a stable emulsion. Such detergents include polysorbate, 80 (TWEEN) (Sorbitan-mono-9-octadecenoate-poly(oxy-1,2-ethanediyl; manufactured by ICI Americas, Wilmington, DE), TWEEN 40TM, TWEEN 20TM, TWEEN 60TM, ZwittergentTM 3-12, TEEPOL HB7TM, and SPAN 85TM. These detergents are typically provided in an amount of approximately 0.05 to 0.5%, such as at about 0.2%. Micelle forming agents include polymer surfactants described by, *e.g.*, Schmolka, *J. Am. Oil. Chem. Soc.* 54:110, 1977, and Hunter *et al.*, *J. Immunol* 129:1244, 1981, and such agents as PLURONICTM L62LF, L101, and L64, PEG1000, and TETRONICTM 1501, 150R1, 701, 901, 1301, and 130R1. The chemical structures of such agents are well known in the art. In one example, the agent is chosen to have a hydrophile-lipophile balance (HLB) of between 0 and 2, as defined by Hunter and Bennett (*J. Immun.* 133:3167, 1984). The agent can be provided in an effective amount, for example between 0.5 and 10%, or in an amount between 1.25 and 5%.

[0122] An oil can be included in the composition to promote the retention of the antagonist in oil-in-water emulsion, and preferably has a melting temperature of less than 65 °C, such that emulsion is formed either at room temperature, or once the temperature of the emulsion is adjusted to room temperature. Examples of such oils include squalene, squalane, EICOSANETM, tetratetracontane, glycerol, and peanut oil or other vegetable oils. In one specific, non-limiting example, the oil is provided in an amount between 1 and 10%, or between 2.5 and 5%. The oil should be both biodegradable and biocompatible so that the subject can break down the oil over time, and so that no adverse affects, such as granulomas, are evident upon use of the oil.

[0123] In particular examples, the pharmaceutical compositions containing bombesin receptor antagonists are extended release formulations, such as injectable microspheres and water-in-oil-in-water (w/o/w) double emulsions. Polymers can also be used for controlled release. Various degradable and nondegradable polymeric matrices for use in controlled drug delivery are known in the art (Langer, *Accounts Chem. Res.* 26:537, 1993). For example, the block copolymer, polaxamer 407 exists as a viscous yet mobile liquid at low temperatures but forms a semisolid gel at body temperature (Johnston *et al.*, *Pharm. Res.* 9:425, 1992; and Pec, *J. Parent. Sci. Tech.* 44(2):58, 1990). Alternatively, hydroxyapatite has been used as a microcarrier for controlled release (Ijntema *et al.*, *Int. J. Pharm.* 112:215, 1994). In other examples, liposomes are used for controlled release as well as drug targeting of the lipid-encapsulated bombesin receptor antagonist (Betageri *et al.*, *Liposome*

Drug Delivery Systems, Technomic Publishing Co., Inc., Lancaster, PA, 1993). Numerous additional systems for controlled delivery of therapeutic compositions are known (*e.g.*, U.S. Patents No. 5,055,303; 5,188,837; 4,235,871; 4,501,728; 4,837,028; 4,957,735; 5,019,369; 5,055,303; 5,514,670; 5,413,797; 5,268,164; 5,004,697; 4,902,505; 5,506,206; 5,271,961; 5,254,342; and 5,534,496).

[0124] In one specific example, the controlled release formulation is a an injectable formulation containing biodegradable microspheres. In one exemplary example, the bombesin receptor antagonist is encapsulated into poly (lactide-co-glycolide) or "PLG" microspheres. For example, the bombesin receptor antagonist peptide or polypeptide can be encapsulated into a bioresorbable injectable microsphere, such as those made from poly (lactide-co-glycolide) (PLG). Encapsulating antagonists in such microspheres results in continued bioactivity for periods of several weeks or more following administration of the pharmaceutical composition. Bioresorbable microspheres made of a poly (lactide-co-glycolide) (PLG, Medisorb 50/50 DL, MW = 10,000, Alkermes, Inc., Wilmington, Ohio) are fabricated by a solvent evaporation method to provide a 4:1 ratio of a release modifier (zinc) to lyophilized antagonist using a coacervation process as previously described (Emerich *et al.*, *Pharm Res* 17:767-775, 2000). Initially, approximately 1.0 g of PLG is dissolved in 10 ml of methylene chloride. 110 mg of the 4:1 zinc:antagonist is added to this solution, sonicated for 5 minutes, followed by vigorous vortexing. Nine ml of poly (dimethylsiloxane) (Aldrich, 350 cst; Milwaukee, WI) is added and the resulting emulsion is mixed and stirred with 1 liter of heptane for 2 hours. The microspheres are collected by filtration and the solvent allowed to evaporate. The microspheres are washed twice with 0.8% triton X-100, suspended in a 1% polyvinyl acetate (PVA) solution and sieved through 70 and 40 μm cell strainers. The 40 - 70 μm sized fraction is collected and washed with distilled water. The microspheres are then frozen at -70°C and lyophilized under 50 μTorr for 2 days.

[0125] The water-in-oil-in-water (w/o/w) double emulsion approach can also be utilized to produce extended release formulations of bombesin receptor antagonists. Briefly, an aqueous solution of the antagonist (0.5 mL internal water phase (10% 4:1 zinc:antagonist) is emulsified into an organic solution (2 ml methylene chloride or ethyl acetate) containing 30 mg PLG by a homogenizer (8000 rpm for 20 seconds). This primary emulsion is immediately poured into 20 mL of an aqueous solution of physiologic saline containing 2% polyvinyl alcohol, w/v, and homogenized for 20 seconds at 6000 rpm to produce a w/o/w emulsion. Solidification of the double emulsion is carried out using evaporation for the methylene chloride solvent and solvent-diffusion method for ethyl acetate.

[0126] It will be apparent that the precise details of the methods or compositions described can be varied or modified without departing from the spirit of the described invention. The following examples are provided to illustrate certain particular features and/or examples. These examples should not be construed to limit the invention to the particular features or examples described. Each of the references cited below is incorporated by reference for all purposes.

Examples

Example 1: Identification of the methadone binding site of the bombesin receptor

[0127] Bombesin and bombesin-like peptides bind to both GRP-R and NMB-R with high affinity. Synthetic peptides based on linear sequences of amino acids selected from the GRP-R were used to evaluate the relationship between receptor structure and ligand binding. A panel of synthetic peptides (20 amino acid residues in length) corresponding to specific sequence segments of the GRP subtype of the bombesin receptor were used to identify specific binding sites for the opiate antagonist methadone. Peptides were evaluated for their ability to competitively inhibit specific binding of (+)-[³H] methadone to human lung cancer cell membranes (NCI-N417 and NCI H1299) that have been shown to have specific high affinity binding sites for both bombesin and methadone.

[0128] A specific region of the receptor was identified that constitutes the high-affinity binding site for methadone. The synthetic peptide corresponding to this region has been designated herein as Peptide O (NH₂-SFLVFYVIPLSIISVYYYFIA-COOH; SEQ ID NO:1). Synthetic peptides replicating other regions on the receptor were less effective in inhibiting methadone binding to these cells. Peptide O was able to competitively inhibit (+)-[³H] methadone binding to the lung cancer cell membranes. FIG. 1A shows specific, high-affinity methadone binding to NCI-N417 lung cancer cells, and the inset shows displacement of methadone in the presence of an excess of non-radioactive Peptide O ($K_d = 0.8$ nM, $B_{max} = 4029$ fmols/mg protein). This is similar to the high affinity ($K_d = 0.8$ nM) methadone binding that we have previously observed in lung cancer cells, in the presence of excess non-radioactive methadone. Similar results were obtained with NCI-1299 lung cancer cells.

Example 2: Design of an exemplary peptide antagonists of the bombesin receptor

[0129] Because binding of methadone is known to influence signaling pathways mediated by bombesin receptors, localization of the specific binding site for methadone on the receptor provided a specific domain within the receptor that was likely to constitute a target for agents that interfere with bombesin receptor mediated signaling. By altering the primary

amino acid sequence of Peptide O, a synthetic antagonist of the bombesin receptor was developed. This peptide antagonist has been designated Peptide X (NH₂-YFVLISVFILSPIYVYSFYIA-COOH; SEQ ID NO:2). Peptide X exhibited similar inhibitory activity with respect to methadone binding as the wild type Peptide O to these membranes. FIG. 1B illustrates displacement of methadone binding to NCI-N417 lung cancer cells in the presence of an excess of non-radioactive Peptide X ($K_d = 0.74$ nM, $B_{max} = 4588$ fmols/mg protein).

[0130] The biological activity of Peptide X in cancer cells was then evaluated. Peptide X was found to significantly induce apoptosis and suppress the growth of lung cancer cells (NCI-N417), as well as an osteosarcoma cells (TE 85). Using a 4-day liquid culture AlamarBlue growth assay, Peptide X significantly inhibited (> 90%) the growth of both cell lines, at concentrations of 10-50 nM (FIG. 2A & 2C). Percent growth inhibition was similar to that observed with similar concentrations of methadone, and conventional chemotherapeutic drugs (cisplatin and adriamycin) used in the treatment of both lung cancer and osteosarcoma cells. However, it was significantly more effective than cisplatin in both the cell lines that were tested. Peptide X did not inhibit the growth of non-malignant lung (SEAC) cells (FIG. 2B). Thus, the growth suppressing activity of this peptide antagonist is selectively for malignant cells, and does not appreciably inhibit the growth of non-malignant (normal) cells.

Example 3: Induction of apoptosis by Peptide X

[0131] Cell death through apoptosis is an important mechanism for the inhibition of tumor development, and agents that induce this process play an important role in controlling cancer cell growth. Methadone and the chemotherapeutic drugs, cisplatin and adriamycin, have been shown to inhibit growth of human lung cancer and osteosarcoma cells by inducing apoptosis. Therefore, the ability of Peptide X to induce apoptosis of lung cancer cells (NCI-N417) and osteosarcoma cells (TE 85) was evaluated.

[0132] Treatment of these cells with nanomolar concentrations of Peptide X resulted in morphological changes, and cleavage of DNA into nucleosome-sized fragments, characteristic of apoptosis. Thus, Peptide X is a potent inducer of apoptosis of neoplastic cells, such a lung cancer cells and osteosarcoma cells.

[0133] In view of the many possible examples to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated examples are only preferred examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

We claim:

1. A bombesin receptor antagonist wherein the antagonist targets the fifth transmembrane domain of the bombesin receptor.
2. The bombesin receptor antagonist of claim 1, wherein the antagonist is a peptide.
3. The antagonist of claim 1, wherein antagonist competitively inhibits binding of methadone to the bombesin receptor.
4. The antagonist of claim 1, wherein the antagonist is an antibody.
5. The antagonist of claim 4, wherein the antagonist specifically binds to an epitope contained at least in part in the fifth transmembrane domain of the bombesin receptor.
6. The antagonist of claim 5, wherein the antagonist specifically binds to an amino acid sequence consisting of SFLVFYVIPLSIISVYYYFIA (Peptide O; SEQ ID NO:1) or fragments thereof.
7. The antagonist of claim 2, wherein the antagonist comprises at least 3 contiguous amino acids of YFVLISVFILSPIYVYSFYIA (Peptide X; SEQ ID NO:2).
8. The antagonist of claim 7, wherein the antagonist is selected from peptides that when aligned with SEQ ID NO:2 comprise:
 - amino acids corresponding to positions 1-3, 7, 10-16, 18, 20 and 21 that are identical to SEQ ID NO:2;
 - amino acids corresponding to positions 4 and 5 of SEQ ID NO:2 that are leucine or isoleucine;
 - amino acids corresponding to position 6 of SEQ ID NO:2 is serine or isoleucine;
 - amino acids corresponding to positions 8 and 9 of SEQ ID NO:2 are phenylalanine or isoleucine;
 - amino acid corresponding to position 17 of SEQ ID NO:2 is serine or phenylalanine; or
 - amino acid corresponding to position 19 of SEQ ID NO:2 is tyrosine or valine.
9. The antagonist of claim 2, wherein the peptide comprises a subsequence of SEQ ID NO:2, wherein the subsequence is selected from YFVLISVFIL (SEQ ID NO:3), YFVILI (SEQ ID NO:4), SPIYV (SEQ ID NO:5), SFVIA (SEQ ID NO:6), SVIFL (SEQ ID NO:7), YFVYIA (SEQ ID NO:8), YSFY (SEQ ID NO:9), and SPIYVYSFYIA (SEQ ID NO:10).

10. The antagonist of claim 2, wherein the peptide comprises the sequence YFVLISVFILSPIYVYSFYIA (Peptide X; SEQ ID NO:2).
11. The antagonist of claim 2, wherein the peptide consists of the sequence YFVLISVFILSPIYVYSFYIA (Peptide X; SEQ ID NO:2).
12. The antagonist of claim 1, wherein the antagonist of the bombesin receptor suppresses growth of a cell contacted with the antagonist.
13. The antagonist of claim 12, wherein the antagonist suppresses growth by inducing apoptosis of the cell.
14. The antagonist of claim 12, wherein the cell over-expresses a bombesin receptor.
15. The antagonist of claim 12, wherein the cell that over-expresses the bombesin receptor is a neoplastic cell.
16. The antagonist of claim 12, wherein suppression of growth by the antagonist is resistant to inhibition by nicotine.
17. The antagonist of claim 1, wherein the antagonist is a peptide produced by solid phase synthesis.
18. The antagonist of claim 1, wherein the antagonist is a recombinant polypeptide or subsequence thereof.
19. The antagonist of claim 18, wherein the antagonist is a peptide produced by cleavage of a recombinant polypeptide.
20. A bombesin receptor antagonist, wherein the antagonist is an inhibitor of bombesin-receptor mediated neoplastic activity comprising at least one linear sequence of amino acids, which at least one linear sequence of amino acids targets the fifth transmembrane domain of the bombesin receptor.
21. The antagonist of claim 20, wherein the antagonist suppresses growth of a neoplastic cell over-expressing a bombesin receptor when brought into contact with the neoplastic cell.
22. The antagonist of claim 20, wherein the antagonist induces apoptosis of the neoplastic cell, thereby suppressing growth of the neoplastic cell.
23. An anti-neoplastic agent comprising at least one linear sequence of amino acids, wherein the at least one linear sequence of amino acids targets the fifth transmembrane domain of the bombesin receptor.
24. A pharmaceutical composition comprising the antagonist of any of the preceding claims and a pharmaceutically acceptable carrier.

25. The pharmaceutical composition of claim 24, wherein the composition comprises an aqueous formulation or an atomized freeze-dried formulation.

26. The pharmaceutical composition of claim 24, wherein the composition comprises a extended-release formulation.

27. The pharmaceutical composition of claim 24, wherein the extended-release formulation comprises a biodegradable microsphere or a water-in-oil-in-water double emulsion.

28. The pharmaceutical composition of claim 27, wherein the biodegradable microsphere comprises a poly (lactide-co-glycolide) microsphere.

29. A method for suppressing growth of a neoplastic cell, the method comprising:

contacting the neoplastic cell with the antagonist of any one of claims 1-22, the anti-neoplastic agent of claim 23 or the pharmaceutical composition of any one of claims 24-28, thereby suppressing growth of the neoplastic cell.

30. The method of claim 29, wherein the antagonist, anti-neoplastic agent and/or pharmaceutical composition suppresses growth by inducing apoptosis of the neoplastic cell.

31. The method of claim 29, wherein the neoplastic cell is a cell of a tumor.

32. The method of claim 29, wherein the neoplastic cell is a cancer cell.

33. The method of claim 32, wherein the cancer cell is a lung cancer cell, an osteosarcoma cell, a breast cancer cell, a colon cancer cell, a gastric cancer cell, a pancreatic cancer cell, a prostate cancer cell or a melanoma cell.

34. The method of claim 29, wherein the neoplastic cell over-expresses a bombesin receptor.

35. The method of claim 34, wherein the bombesin receptor is a gastrin releasing peptide receptor (GRP-R) or a neuromedin B receptor (NMB-R).

36. The method of claim 34, wherein the neoplastic cell further expresses at least one bombesin-like peptide (BLP).

37. The method of claim 29, wherein the neoplastic cell is contacted *in vivo*.

38. A method for treating a subject with a neoplasm, the method comprising: selecting a subject with at least one neoplastic cell; and

contacting the at least one neoplastic cell with a composition comprising the antagonist of any one of claims 1-22, the anti-neoplastic agent of claim 23 or the pharmaceutical composition of any one of claims 24-28, which composition suppresses growth of the at least one neoplastic cell, thereby treating the subject with the neoplasm.

39. The method of claim 38, wherein the composition suppresses growth of the at least one neoplastic cell by inducing apoptosis of the at least one neoplastic cell.

40. The method of claim 38, wherein the neoplastic cell is a cancer cell.

41. The method of claim 38, wherein the neoplastic cell over-expresses a bombesin receptor.

42. The method of claim 41, wherein the neoplastic cell further expresses a bombesin-like peptide (BLP).

43. The method of claim 38, wherein the antagonist does not appreciably affect a non-neoplastic cell.

44. The method of claim 38, wherein suppression of growth by the composition is resistant to inhibition by nicotine.

FIGS. 1A and 1B

FIG. 1B

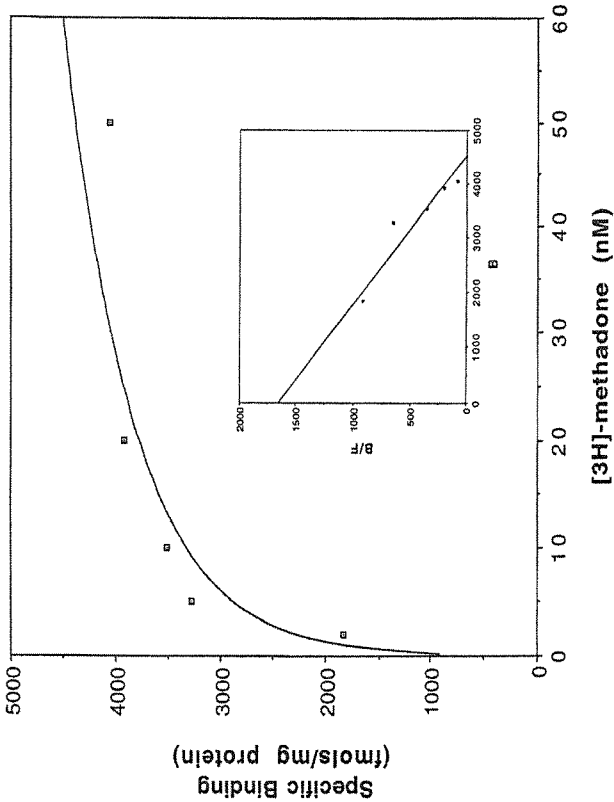
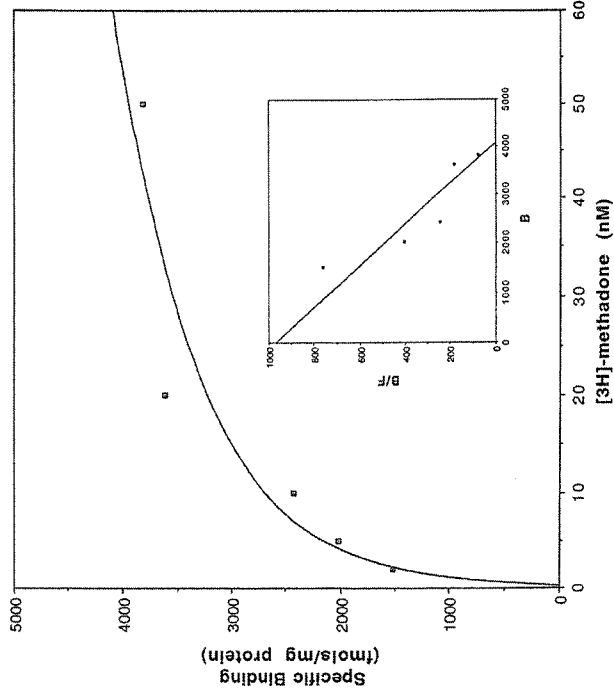


FIG. 1A



FIGS. 2A-2C

