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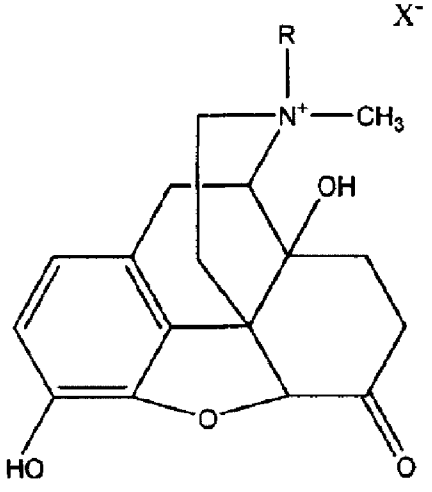
(54) **Titre : UTILISATION D'ANTAGONISTES DES OPIOIDES POUR ATTENUER LA PROLIFERATION ET LA MIGRATION DES CELLULES ENDOTHELIALES**

(54) **Title: USE OF OPIOID ANTAGONISTS TO ATTENUATE ENDOTHELIAL CELL PROLIFERATION AND MIGRATION**



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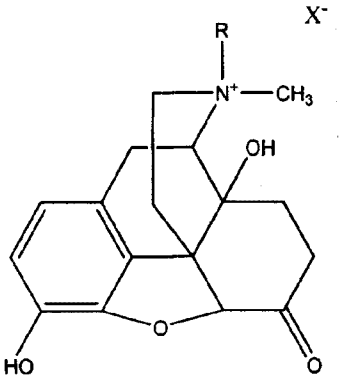
(57) **Abrégé/Abstract:**
The invention provides a use of a combination of a peripheral opioid antagonist of the Formula I such as methylnaltrexone and 5-fluorouracil (5-FU) for treating a disorder characterized by hyperproliferation of endothelial cells, hyperproliferation of cells overexpressing mu-opioid receptors, and for inhibiting proliferation of cancer cells:

(see formula I).

ABSTRACT

The invention provides a use of a combination of a peripheral opioid antagonist of the Formula I such as methylnaltrexone and 5-fluorouracil (5-FU) for treating a disorder characterized by hyperproliferation of endothelial cells, hyperproliferation of cells overexpressing mu-opioid receptors, and for inhibiting proliferation of cancer cells:



(I)

USE OF OPIOID ANTAGONISTS TO ATTENUATE ENDOTHELIAL CELL PROLIFERATION AND MIGRATION

Field of Invention

The invention relates to methods of attenuating migration and/or proliferation of endothelial cells, especially associated with tumors, utilizing opioid antagonists.

Introduction

Cellular proliferation is a normal ongoing process in all living organisms that involves numerous factors and signals that are delicately balanced to maintain regular cellular cycles. Whether or not mammalian cells will grow and divide is determined by a variety of feedback control mechanism, which includes the availability of space in which a cell can grow, and the secretion of specific stimulatory and inhibitory factors in the immediate environment.

Angiogenesis and angiogenesis-related diseases are affected by cellular proliferation. The process of angiogenesis results in the formation of new blood vessels. Under normal physiological conditions, animals, including humans, undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonic development, and formation of the corpus luteum, endometrium and placenta.

During the process of angiogenesis, endothelial cells, which normally exist in a quiescent state as part of an existing blood vessel, enter a migratory, proliferative state. This migratory, proliferative state of endothelial cells is eventually resolved when the cells return to the quiescent state as part of a functional new blood vessel. The generation of new capillaries involves a

complex process that requires a number of cellular and molecular events to occur in both a spatial and temporal pattern. Some of these activities include the degradation of the surrounding basement membrane of the originating vessel, the migration of the endothelial cells through the connective tissue stroma, cell proliferation, the formation of tube-like structures, and the maturation of these endothelial-lined tubes into new blood vessels. Cliff, WJ (1963) Phil. Trans. Roy. Soc. (Lond.) B. 246: 305-325; Schoefl, G.I. (1963) Virchows Arch. Pathol. Anat. 337: 97-141; Ausprunk DH and Folkman J Microvas. Res. (1977) 14: 53-65. Some essential angiogenic factors include fibroblast growth factor-basic, vascular endothelial growth factor (VEGF), angiopoietins, cytokines, extracellular matrix proteins, and matrix metalloproteases. These factors are produced locally by stromal cells and by activated leukocytes that are recruited to the area (Risau, W. (1997) Nature 386(6626): 671-674; Risau and Flamme (1995) Ann. Rev. Cell Dev. Biol. 11: 73-91). Unlike other angiogenic factors, VEGF acts as an endothelial cell specific mitogen during angiogenesis.

Angiogenesis can be stimulated and harnessed by some neoplasms (e.g., tumors) to increase nutrient uptake. It has been found that angiogenesis is essential for the growth of solid tumors beyond 2-3 mm in diameter and for tumor metastasis. In contrast to normal angiogenesis, which leads to anastomoses and capillary maturation, angiogenesis associated with neoplasia is a continuous process. Endothelial cells are activated by nearby neoplastic cells to secrete not only VEGF which stimulates angiogenesis, but also matrix metalloproteases (MMP) which degrade the surrounding extracellular matrix. The endothelial cells then invade the extracellular matrix where they migrate, proliferate, and organize to form new blood vessels, which support neoplasm growth and survival.

The newly vascularized neoplasm continues to grow, leading to further nutrient deprivation and chronic pro-angiogenic signaling. The vasculature of neoplasms is characterized by the presence of lacunae and a low rate of anastomoses. This partially dysfunctional vasculature fuels the permanent requirement for angiogenesis. Additionally, this incomplete vasculature allows the shedding of neoplastic cells into the systemic circulation. Hence, the

angiogenic potential of a neoplasm correlates with metastatic potential. (Weidner et al. (1991) N. Engl. J. Med. 324(1):1-8; Folkman and Shing (1992) J. Biol. Chem. 267(16):10931-10934).

As a significant proportion of neoplasms are dependent on continued angiogenesis, inhibition of angiogenesis blocks neoplasm growth which often leads to complete necrosis of the neoplasm. (Weidner et al. (1991) N. Engl. J. Med. 324(1):1-8; Folkman and Shing (1992) J. Biol. Chem. 267(16):10931-10934).

Suppression of any one of the steps of and/or factors involved in angiogenesis could inhibit the formation of new vessels, and therefore, affect tumor growth and generation of metastases. Indeed, it has been estimated that the elimination of a single endothelial cell could inhibit the growth of 100 tumor cells. It has also been found that antibodies raised against the angiogenic factor VEGF have been shown to suppress tumor growth in vivo.

As part of treating and managing patients with cancer and many medical conditions, opioid agonists, such as morphine, are widely used for associated pain. For example, morphine is used in the terminal phase of care of approximately one-half of the patients that die of cancer each year in the United States. Opioid agonists, such as morphine comprise a group of compounds that act on a series of endogenous opioid receptors, such as mu-, kappa-, and delta-receptors in biological systems. Normally, these endogenous receptors bind endogenous opioids. Endogenous opioids are natively produced by mammalian cells. Endogenous opioids include beta-endorphins, enkephalins, and dynorphins. Beta-endorphins show a preference for mu receptors, enkephalins for delta receptors and dynorphins for kappa receptors. Opioid agonists are classified by their preferential effects on the endogenous opioid receptors. Generally, the mu-receptor is associated with pain relief, and chemical dependence (e.g., drug addiction and alcoholism). Morphine, for example, is a mu-opioid agonist. Opioid receptors are not limited to the brain and central nervous system (CNS),

e.g., to central receptors. Peripheral opioid receptors may be found in other tissues throughout the body, e.g., gastrointestinal tissue.

Despite wide use in pain management, morphine and other opioid medications can have severe side effects that may be caused by activation of the peripheral receptors. The side effects can be difficult to manage and can result in the patient refusing opioid-based pain management. Side effects of opioid treatment include nausea, constipation, inhibition of gastrointestinal motility, respiratory suppression and immunosuppression. Additionally, morphine and other opioid receptor agonists can stimulate human microvascular endothelial cell proliferation and angiogenesis in vitro and in vivo at typical morphine or morphine-equivalent blood concentrations. This pro-angiogenesis activity of the opioid agonists, while palliative for pain, may hasten tumor progression.

Opioid antagonists are similarly classified by their effects on the opioid receptors, e.g., by their ability to antagonize one receptor more effectively than another receptor. For example, the opioid antagonist naloxone acts as a competitive antagonist at all opioid receptors, but is approximately ten times more effective at mu-receptors than at kappa receptors, and is, therefore, classified as a mu-opioid antagonist. Opioid antagonists may antagonize central receptors, peripheral receptors or both. Opioid antagonists, and in particular peripheral opioid antagonists, have been used to lessen the side-effects of exogenously administered opioids, as well as to lessen the unwanted effects of excessive endogenous opioids. Opioid antagonists also have been examined for their potential use as anticancer agents for particular types of cancer, as described in US Patents No. 6,384,044 and 6,136,780 and in the scientific literature Gupta et al. *Cancer Research*, 62: 4491-98 (2002). The anticancer effects of opioid antagonists have been controversial and not well understood, but it has been held that the opioid antagonist anticancer effects, to the extent they have been shown at all, are unrelated to angiogenesis (Poonawala T, et al., Wound Repair Regen. 2005 Mar-Apr;13(2):165-74; Popov I., Acta Chir Iugosl. 2004;51(2):117-21; Blebea J, et al., J Vasc Surg. 2002 Mar;35(3):532-8; Balasubramanian S, et al., J Mol Cell Cardiol. 2001 Dec;33(12):2179-87; Zagon IS, et al., Int J Oncol. 2000

Nov;17(5):1053-61; Blebea J et al., J Vasc Surg. 2000 Aug;32(2):364-73; Pasi A, et al., Gen Pharmacol. (991;22(6):1077-9.) In fact, it has been reported that in xenograft tumor model in mice, the opioid antagonist naloxone did not exhibit a significant effect on morphine induced angiogenesis Gupta et al. *Cancer Research*, 62: 4491-98 (2002). Therefore, it is surprising that it is now discovered that opioid antagonists can inhibit endothelial proliferation and migration associated with angiogenesis.

Brief Description of the Invention

The invention provides methods of attenuating, e.g., inhibiting or reducing, cellular proliferation and migration, particularly endothelial cell proliferation and migration, including that associated with angiogenesis, using opioid antagonists, including, but not limited to, those that are peripherally restricted antagonists.

According to one aspect of the invention, a method of treatment is provided. The method involves administering to a subject with a disorder characterized by unwanted migration or proliferation of endothelial cells an effective amount of an opioid antagonist. The treatment may inhibit one or both of migration and proliferation. The unwanted migration or proliferation of endothelial cells can be unwanted migration or proliferation of vascular endothelial cells, including, but not limited to, unwanted neovascularization or angiogenesis. Examples of unwanted neovascularization include, but are not limited to, neovascularization associated with cancer and ocular neovascularization. The disorder can be any disorder characterized by unwanted migration or proliferation of endothelial cells. Important such disorders are cancer, sickle cell anemia, vascular wounds, proliferative retinopathies, and unwanted endothelial cell proliferation in the kidneys and the lung.

In important embodiments, the opioid antagonist is a peripheral opioid antagonist. Peripheral opioid antagonists include, but are not limited to, quaternary or tertiary morphinan derivatives, piperidine-N-alkylcarboxylates, and quaternary benzomorphans. One important such peripheral opioid antagonist is methylnaltrexone. Another opioid antagonist is alvimopan. In important

embodiments, the effective amount is such that the subject has effective circulating blood plasma levels of the opioid antagonist continuously for at least 1 week, at least 2 weeks, at least three weeks and, preferably, at least 4 weeks.

The invention also includes the coadministration of the opioid antagonists with agents that are not opioid antagonists, but which are nonetheless useful in treating disorders characterized by unwanted migration or proliferation of endothelial cells. Examples of such agents include anticancer agents, antineovascularization agents (for example, anti-VEGF monoclonal antibody), antidiabetes agents, anti-sickle cell agents, wound healing agents, and anti-endothelial cell proliferative agents.

It will be understood that the subjects may be, or may not be, on concurrent opioid therapy, depending on the particular disorder the subject has, the severity of the disorder, and the need the subject has for pain management. In some embodiments, the subject is taking concurrent opioid therapy. In some embodiments, the subject is not taking concurrent opioid therapy. In some embodiments, the subject is taking concurrent chronic opioid therapy. In some embodiments, the subject is not taking concurrent chronic opioid therapy.

According to another aspect of the invention, a method of inhibiting VEGF activity in endothelial cells is provided. The method involves contacting the cells with an effective amount of an opioid antagonist.

According to another aspect of the invention, a method of inhibiting exogenous opioid-induced cellular migration or proliferation in endothelial cells is provided. The method involves contacting the cells with an effective amount of an opioid antagonist.

According to another aspect of the invention, a method of inhibiting Rho A activation in endothelial cells is provided. The method involves contacting the cells with an effective amount of an opioid antagonist.

According to any of the foregoing embodiments, the opioid antagonist preferably is a peripheral opioid antagonist, and most preferably is methylnaltrexone.

The invention provides methods of attenuating migration and/or proliferation of endothelial cells of a tumor or cancer, comprising contacting the cells with an antimigratory or antiproliferative amount of an opioid antagonist. In another aspect, the invention provides methods of attenuating angiogenesis associated with cancer. Thus, the invention contemplates treating a human cancer patient, for example, by a method of attenuating angiogenesis in a cancerous tissue of a patient, comprising administering to the cancer tissue of the patient an effective amount of an opioid antagonist.

The invention also provides a method of treating abnormal neovascularization, comprising administering to a patient in need of such treatment, an amount of an opioid antagonist effective to inhibit the formation of blood vessels.

The invention also includes a method of attenuating tumor progression and metastasis in animal tissues, comprising contacting tumor cells or tissues with a growth-inhibiting amount of an opioid antagonist, and a method of attenuating proliferation of hyperproliferative cells in a subject, comprising administering to the subject at least one opioid antagonist, in an amount which is effective to attenuate proliferation of the hyperproliferative cells. In one embodiment, the method involves administering a peripheral opioid antagonist, and, in particular, a quaternary derivative of noroxymorphone, to a subject with cancer, whether or not the cancer involves angiogenesis, to treat or inhibit the development or recurrence of the cancer. Cancers not involving angiogenesis include those that do not involve the formation of a solid tumor fed by neovasculature. Certain blood cell cancers fall into this category, for example: leukemias (cancer of the leukocytes or white cells), lymphomas (arising in the lymph nodes or lymphocytes), and some cancers of the bone marrow elements. Thus, in one aspect of the invention, a method of treatment is provided. The method involves administering to a subject with a disorder characterized by hyperproliferation of cells an effective amount of a peripheral opioid antagonist. In one embodiment, the cells are cancer cells. The cancer cells may be cancer cells associated with angiogenesis or they may be

unassociated with angiogenesis. In one embodiment, the peripheral opioid antagonist is methylnaltrexone.

In further embodiments, the invention provides methods of treating cancer, wherein a peripheral opioid antagonist and at least one other therapeutic agent that is not an opioid or opioid antagonist are co-administered to the patient. Suitable therapeutic agents include anticancer agents (including chemotherapeutic agents and antineoplastic agents), as well as other antiangiogenesis agents. It has been discovered that opioid antagonists co-administered with various anticancer drugs, radiotherapy or other antiangiogenic drugs can give rise to a significantly enhanced antiproliferative effect on cancerous cells, thus providing an increased therapeutic effect, e.g., employing peripheral opioid antagonists to certain tumors can potentiate their response to other therapeutic regimens. Specifically, a significantly increased antiproliferative effect, including but not limited to a significantly increased antiangiogenic effect, is obtained with co-administered combinations as described in more detail below. Not only can an existing regimen be enhanced, but new regimens are possible, resulting, for example, in lower concentrations of the anticancer compound, a lower dosing of radiation, or lower concentration of other antiangiogenic drugs, compared to the treatment regimes in which the drugs or radiation are used alone. There is the potential, therefore, to provide therapy wherein adverse side effects associated with the anticancer or other antiangiogenic drugs or radiotherapy are considerably reduced than normally observed with the anticancer or other antiangiogenic drugs or radiotherapy when used alone. Thus, in one aspect of the invention, a method of treatment is provided. The method involves administering to a subject with a disorder characterized by hyperproliferation of cells an effective amount of an opioid antagonist and an anticancer agent, radiation, or an antiangiogenic agent. In one embodiment, the cells are cancer cells. In one embodiment, the opioid antagonist is a peripheral opioid antagonist. In one embodiment, the peripheral opioid antagonist is methylnaltrexone. In another aspect of the invention, a method of reducing the risk of recurrence of a cancer in a subject after medical intervention is provided. The method involves administering to the subject

before, during or after the medical intervention an effective amount of an opioid antagonist and an anticancer agent, radiation, or an antiangiogenic agent. In one embodiment, the opioid antagonist is a peripheral opioid antagonist. In one embodiment, the peripheral opioid antagonist is methylnaltrexone.

In another aspect of the invention, the opioid antagonists are used peri-operatively. By "peri-operatively," it is meant immediately before (e.g., in preparation for), during, and/or immediately after a surgery or a surgical or endoscopic procedure, e.g. colonoscopy, gastrolaparoscopy, and especially a surgery or surgical procedure involving the removal of a tumor. The opioid antagonists act to attenuate the recurrence of and/or the metastasis of the tumor, especially that arising from angiogenesis associated therewith.

It is anticipated that the opioid antagonist will preferably be given in a continuous dosing regimen, e.g., a regimen that maintains a minimum, and even more preferably relatively constant, blood level. It is further contemplated that the methods of the present invention may have prophylactic value in certain disorders associated with abnormal angiogenesis. Thus, the invention provides a method of preventing the appearance or re-appearance of a disorder in a mammal, the disorder being characterized by unwanted endothelial cell migration or proliferation, including abnormal angiogenesis, comprising administering to a mammal in need of such treatment, an effective amount of an opioid antagonist, wherein the disorder is a cancer, sickle cell anemia, ocular neovascular diseases, diabetes, ocular retinopathy, or other unwanted endothelial proliferation in kidneys, eye or lung. It will therefore be understood that, as used herein, treating a subject with a disorder characterized by unwanted endothelial cell proliferation or migration includes treating a subject with an active disorder to inhibit or cure the disorder and treating a subject to inhibit a disorder from reoccurring. For example, the subject may have had a solid tumor removed, and the subject may receive the treatment to inhibit the tumor from reoccurring.

In attenuating cell proliferation, the invention provides a method for the treatment of abnormal cell proliferation of a cell expressing vascular endothelial growth factor (VEGF) in a mammal which comprises administering to the

mammal a therapeutically effective amount of an opioid antagonist. The invention also includes a method of treating cancerous tissue in a subject comprising, administering to the subject an amount of an opioid antagonist sufficient to inhibit VEGF production in the cancerous tissue, as well as a method of treating angiogenic disease, the method comprising contacting a tissue or a population of endothelial cells with a composition comprising an amount of at least one of an opioid antagonist under conditions effective to inhibit VEGF-induced angiogenesis and to treat angiogenic disease.

In another aspect, the present invention provides a method of inhibiting or reducing angiogenesis, particularly opioid-induced angiogenesis, e.g., of tumor cells, by administrating or providing an opioid antagonist, particularly a peripheral opioid antagonist, to cells undergoing angiogenesis. In further aspect, the invention provides methods of treating opioid-induced angiogenesis in patients receiving opioid treatment or in patients where the angiogenesis is induced by endogenous opioids. The former group is typically cancer patients on opioid-based pain management. The methods comprise administering an opioid antagonist to a patient in an antiangiogenic amount, e.g., an amount sufficient to inhibit or reduce the opioid-induced angiogenesis. In those patients receiving opioid treatment, the opioid and the peripheral opioid antagonist may be co-administered. Peripheral opioid antagonists can, thus, be used to inhibit or reduce the angiogenic effects of opioids on tumor cells, and attenuate the growth of a tumor. Suitable opioid antagonists generally include heterocyclic amine compounds that belong to several different classes of compounds. For example, one class is suitably tertiary derivatives of morphinan, and in particular, tertiary derivatives of noroxymorphone. In one embodiment, the tertiary derivative of noroxymorphone is, e.g. naloxone or naltrexone.

Suitable peripheral opioid antagonists are also generally heterocyclic amine compounds that may belong to several different classes of compounds. For example, one class is suitably quaternary derivatives of morphinan, and in particular, quaternary derivatives of noroxymorphone. In one embodiment, the quaternary derivative of noroxymorphone is, e.g., N-methylnaltrexone (or simply

methylnaltrexone). Another class is N-substituted piperidines. In one embodiment, the N-piperidine is a piperidine-N- alkylcarbonylate, such as, e.g., alvimopan. Another class of compounds which may be of value in the methods of the present invention is quaternary derivatives of benzomorphans.

In some embodiments of the invention, the opioid antagonist may be a mu opioid antagonist. In other embodiments, the opioid antagonist may be a kappa opioid antagonist. The invention also encompasses administration of more than one opioid antagonist, including combinations of mu antagonists, combinations of kappa antagonists and combinations of mu and kappa antagonists, for example, a combination of methylnaltrexone and alvimopan, or a combination of naltrexone and methylnaltrexone.

In further embodiments, the invention provides methods of treating opioid-induced angiogenesis in patients receiving an opioid, wherein a peripheral opioid antagonist and at least one other therapeutic agent that is not an opioid or opioid antagonist are co-administered to the patient. Suitable therapeutic agents include anticancer agents (including chemotherapeutic agents and antineoplastic agents), as well as other antiangiogenesis agents.

In yet another aspect, the invention provides a method of reducing the risk of recurrence of a cancer or tumor after medical intervention (such intervention to include but not be limited to surgery, e.g. pulmonary surgery, surgical and endoscopic procedures, e.g. colonoscopy, gastrolaparoscopy, chemotherapy, etc.), comprising co-administering to a cancer patient an opioid antagonist. Thus, the invention contemplates, for example, a method of minimizing the post-operative recurrence of, e.g., breast cancer in a patient, comprising administering to the patient an effective amount of an opioid antagonist. Peripheral opioid antagonists in accordance with the present invention, e.g., MNTX, can also inhibit VEGF, platelet-derived growth factor (PDGF), or sphingosine 1-phosphate (SIP) - stimulated or induced cell proliferation in the endothelial cells.

Brief Description of Drawings

The invention may be better understood and appreciated by reference to the detailed description of specific embodiments presented herein in conjunction with the accompanying drawings of which:

FIG. 1 is a bar graph of dose-dependent inhibition of human microvascular endothelial cell (HMVEC) migration, depicting the results from Example 1.

FIG. 2 is a bar graph of dose-dependent inhibition of human microvascular endothelial cell migration, depicting the results from Example 2.

FIG. 3 is a bar graph of dose-dependent inhibition of HMVEC migration using MNTX and MNTX + DAMGO.

FIG. 4 is a bar graph of dose-dependent inhibition of HMVEC migration using naloxone and naloxone + DAMGO.

FIG. 5 is a bar graph of dose-dependent effect of M3G and M6G on HMVEC migration.

FIG. 6 is a photomicrograph that shows morphine induced endothelial cell migration in the presence and absence of MNTX. Panel A = Control, Panel B = MS (morphine sulfate), Panel C = MNTX, and Panel D = MS + MNTX. Arrows are shown in Panel A to highlight several cells that have successfully migrated across the membrane.

FIG. 7 is a bar graph of percent proliferation (A) and migration (B) of human pulmonary microvascular endothelial cells in the presence of VEGF, morphine and DAMGO with or without MNTX.

FIG. 8 is a panel of immunoblots indicating the tyrosine phosphorylation (activation) of (A) of anti-VEGF R.1 (Flt-1) and 2 (Flk-1) using immunoprecipitated VEGF R.1 or 2 and anti-phospho-tyrosine in human pulmonary microvascular endothelial cells in the presence of VEGF, morphine and DAMGO with or without MNTX and a bar graph (B) of percent proliferation and migration of human pulmonary microvascular endothelial cells in the presence of VEGF, morphine and DAMGO with or without VEGF R. inhibitor.

FIG. 9 is a panel of immunoblots indicating RhoA activation using anti-RhoA in human pulmonary microvascular endothelial cells in the presence of

VEGF, morphine and DAMGO with or without MNTX (A) or VEGF R. Inhibitor (B).

FIG. 10 is a panel of immunoblots (A) of anti-RhoA of human pulmonary microvascular endothelial cells in the presence of scramble siRNA (targeting no known human mRNA sequence) or RhoA siRNA and a bar graph of percent proliferation (B) and migration (C) of human pulmonary microvascular endothelial cells in the presence of VEGF, morphine and DAMGO with or without scramble siRNA (targeting no known human mRNA sequence) or RhoA siRNA.

FIG. 11 is a schematic diagram summarizing the mechanism of MNTX effects on angiogenesis.

FIG. 12 is a bar graph of percent proliferation above control of pulmonary microvascular endothelial cells in the presence of S1 P, VEGF, PDGF, morphine and DAMGO with or without MNTX.

FIG. 13 is a bar graph of percent migration above control of pulmonary microvascular endothelial cells in the presence of S1P, VEGF, PDGF, morphine and DAMGO with or without MNTX.

FIG. 14 is a bar graph of percent proliferation above control of pulmonary microvascular endothelial cells in the presence of S1P, VEGF, PDGF, morphine and DAMGO with scramble (control) siRNA or with mu opioid receptor siRNA.

FIG. 15 is a bar graph of percent migration above control of pulmonary microvascular endothelial cells in the presence of S1P, VEGF, PDGF, morphine and DAMGO with scramble (control) siRNA or with mu opioid receptor siRNA.

FIG. 16 is a panel of immunoblots indicating phosphorylation (activation) of the mu opioid receptor using immunoprecipitated mu opioid receptor and (A, C) anti-phospho-serine, (B, D) anti-phospho-threonine of human pulmonary microvascular endothelial cells in the presence of morphine, DAMGO, S1P, VEGF, PDGF with MNTX (C, D) or without MNTX (A, B); (E) is an immunoblot of anti-mu opioid receptor.

FIG. 17 is an anti-RhoA immunoblot of (A, B) activated RhoA and (C) total RhoA of human pulmonary microvascular endothelial cells in the presence of

morphine, DAMGO, S1P, VEGF, PDGF with MNTX (B) and without MNTX (A).

FIG. 18 is a panel of immunoblots of top panel: (A, B) anti-phospho-tyrosine, (C) anti-VEGF R and bottom panel: (A, B) anti-phospho-tyrosine, (C) anti-PDGF R, of human pulmonary microvascular endothelial cells in the presence of morphine, DAMGO, VEGF (top panel) or PDGF (bottom panel) with MNTX (B in each panel) or without MNTX (A in each panel).

FIG. 19 is a panel of immunoblots indicating tyrosine phosphorylation (activation) of the $S1P_3$ receptor using immunoprecipitated $S1P_3$ receptor and (A, B) anti-phospho-tyrosine, (C) anti-$S1P_3$ R, of human pulmonary microvascular endothelial cells in the presence of morphine, DAMGO, and S1P with MNTX (B) or without MNTX (A).

FIG. 20 is a bar graph of percent proliferation above control of pulmonary microvascular endothelial cells in the presence of S1P, VEGF, PDGF, morphine and DAMGO with scramble (control) siRNA or with RhoA siRNA.

FIG. 21 is a bar graph of percent migration above control of pulmonary microvascular endothelial cells in the presence of S1P, VEGF, PDGF, morphine and DAMGO with scramble (control) siRNA or with RhoA siRNA.

FIG. 22 is an schematic diagram summarizing the mechanism of MNTX effects on RhoA activation and angiogenesis.

FIG. 23 is a graph of percent proliferation above control of microvascular endothelial cells in the presence of VEGF with MNTX, with 5-FU and with a combination of MNTX and 5-FU.

FIG. 24 is a graph of percent migration above control of microvascular endothelial cells in the presence of VEGF with MNTX, with Bevacizumab and with a combination of MNTX and Bevacizumab.

FIG. 25 is a bar graph of the effects of MNTX, 5-FU, and a combination of both on SW480 human colorectal cancer cell line.

FIG. 26 is a bar graph of the effects of MNTX, 5-FU, and a combination of both on HCT116 human colorectal cancer cell line.

FIG. 27 is a bar graph of the effects of MNTX, 5-FU, and a combination of both on MCF-7 human breast cancer cell line.

FIG. 28 is a bar graph of the effects of MNTX, 5-FU, and a combination of both on non-small lung cancer cell (NSLCC) line.

Detailed Description of the Invention

The present invention provides methods of attenuating abnormal or undesirable migration and/or proliferation of endothelial cells. As such, the invention provides methods for attenuating angiogenesis in a tissue or an organ of a subject by the use of opioid antagonists, and a novel approach for treating angiogenic related diseases and other hyperproliferative diseases in mammals. For example, as described above, solid tumors rely on the generation of new blood vessels for nutrients to reach the cells within the tumor. The growth factors required for angiogenesis can be produced by the tumor cells or alternatively, exogenous factors, such as opioids can stimulate new blood vessel growth. The present invention by the use of opioid antagonists provides a novel therapeutic approach to the treatment of such tumors, wherein the generation of new blood vessels within the tumor, rather than the tumor cells themselves, is the target. This treatment is not likely to lead to the development of resistant tumor cells.

Described herein are opioid antagonists inhibit proliferation and migration induced by opioids, endogenous or exogenous, and growth factors, such as VEGF, PDGF, SIP etc. Peripheral opioid antagonists, in particular, showed a substantial efficacy in inhibiting opioid and growth factor induced proliferation and migration of endothelial cells. The peripheral opioid antagonist methylnaltrexone (MNTX) inhibited both opioid and growth factor induced proliferation and migration in a concentration dependent manner. In addition, naloxone also inhibited opioid-induced endothelial migration. It should be noted, however, that the naloxone inhibition of DAMGO induced migration of endothelial cells occurred at a relatively high, micromolar-, concentration of naloxone. Furthermore, it has now been discovered that opioid antagonists, and the peripheral opioid antagonist MNTX in particular, inhibit agonist induced endothelial cell (EC) proliferation

and migration via inhibition of receptor phosphorylation and/or transactivation and subsequent inhibition of RhoA activation. The agonists can be opioids, exogenous and/or endogenous, angiogenic factors (VEGF), and other proliferation and/or migration stimulating factors (PDGF, S1P, $S1P_3$ receptor, RhoA, etc). These results suggest that inhibition of angiogenesis by opioid antagonists can be a useful therapeutic intervention for, among other disorders, cancer.

The present invention also provides methods of attenuating abnormal or undesirable proliferation of cancer cells *per se*. This aspect of the invention is useful in situations involving the presence or absence of angiogenic activity. The absence of angiogenic activity is evidenced by one or more of the following characteristics: nonsolid tumors or tumors where there is repulsion of existing blood vessels and/or absence of microvessels within the tumor, limited growth, for example up to about 1 mm in diameter in vivo, at which time further expansion is stopped, harmless to the host until it switches to an angiogenic phenotype, etc. Nonangiogenic tumors can be completely avascular or they can contain empty lumen without red blood cells. The gross difference between the nonangiogenic and angiogenic tumors (i.e. white vs. red tumors) is most likely due to the reactive hyperemia that accompanies the onset of blood flow after the angiogenic switch is completed in a previously hypoxic tumor. Examples of nonangiogenic tumor lineages include but are not limited to breast adenocarcinoma, osteosarcoma, glioblastoma, embryonic kidney tumors etc. There are many factors that could play a role in tumor dormancy and the rate-determining step for tumor expansion of nonangiogenic tumors could be governed by lack of angiogenesis and/or differentiation programs, tumor cell survival, immune response to the host etc. Although some nonagiogenic tumors never switch to an angiogenic phenotype, many undergo spontaneous transformation into an angiogenic and harmful phenotype. Therefore, treatment of nonangiogenic tumors is of therapeutic significance.

Cancers not involving angiogenesis include those that do not involve the formation of a solid tumor fed by neovasculature. Certain blood cell cancers can fall into this category, for example: leukemias, including acute lymphocytic

leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), and hairy cell leukemia; lymphomas (arising in the lymph nodes or lymphocytes) including Hodgkin lymphoma, Burkitt's lymphoma, cutaneous lymphoma, cutaneous T-cell lymphoma, follicular lymphoma, lymphoblastic lymphoma, MALT lymphoma, mantle cell lymphoma, Waldenstrom's macroglobulinemia, primary central nervous system lymphoma; and some cancers of the bone marrow elements including Ewing's sarcoma and osteosarcoma.

Before any embodiments of the invention are explained in detail, it is to be understood that the invention is not limited in its application to the details of the structure and function of the invention set forth in the following description or illustrated in the appended figures of the drawing. The invention is capable of other embodiments and of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of terms such as "including," "comprising," or "having" and variations thereof herein is meant to encompass the item listed thereafter and equivalents thereof as well as additional items.

Unless otherwise noted, technical terms are used according to conventional usage. As used herein, however, the following definitions may be useful in aiding the skilled practitioner in understanding the invention:

"Subject" refers to humans, dogs, cats, and horses.

"Chronic opioid use" refers to and is characterized by the need for substantially higher levels of opioid to produce the therapeutic benefit as a result of prior opioid use, as is well known in the art. Chronic opioid use as used herein includes daily opioid treatment for a week or more or intermittent opioid use for at least two weeks.

"Alkyl" refers to an aliphatic hydrocarbon group which is saturated and which may be straight, branched or cyclic having from 1 to about 10 carbon atoms in the chain, and all combinations and subcombinations of chains therein.

Exemplary alkyl groups include methyl, ethyl, n-propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, pentyl, hexyl, heptyl, octyl, nonyl and decyl.

"Lower alkyl" refers to an alkyl group having 1 to about 6 carbon atoms.

"Alkenyl" refers to an aliphatic hydrocarbon group containing at least one carbon-carbon double bond and having from 2 to about 10 carbon atoms in the chain, and all combinations and sub combinations of chains therein. Exemplary alkenyl groups include vinyl, propenyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl and decenyl groups.

"Alkynyl" refers to an aliphatic hydrocarbon group containing at least one carbon-carbon triple bond and having from 2 to about 10 carbon atoms in the chain, and combinations and sub combinations of chains therein. Exemplary alkynyl groups include ethynyl, propenyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl and decenyl groups.

"Alkylene" refers to a bivalent aliphatic hydrocarbon group having from 1 to about 6 carbon atoms, and all combinations and subcombinations of chains therein. The alkylene group may be straight, branched or cyclic. There may be optionally inserted along the alkylene group one or more oxygen, sulfur or optionally substituted nitrogen atoms, wherein the nitrogen substituent is alkyl as described previously.

"Alkenylene" refers to an alkylene group containing at least one carbon-carbon double bond. Exemplary alkenylene groups include ethenylene (-CH=CH-) and propenylene (CH=CHCH2--).

"Cycloalkyl" refers to any stable monocyclic or bicyclic ring having from about 3 to about 10 carbons, and all combinations and subcombinations of rings therein. The cycloalkyl group may be optionally substituted with one or more cycloalkyl-group substituents. Exemplary cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl groups.

"Cycloalkyl-substituted alkyl" refers to a linear alkyl group, preferably a lower alkyl group, substituted at a terminal carbon with a cycloalkyl group, preferably a C3-C8 cycloalkyl group. Exemplary cycloalkyl-substituted alkyl

groups include cyclohexylmethyl, cyclohexylethyl, cyclopentylethyl, cyclopentylpropyl, cyclopropylmethyl and the like.

"Cycloalkenyl" refers to an olefinically unsaturated cycloaliphatic group having from about 4 to about 10 carbons, and all combinations and subcombinations of rings therein.

"Alkoxy" refers to an alkyl-O-group where alkyl is as previously described. Exemplary alkoxy groups include, for example, methoxy, ethoxy, propoxy, butoxy and heptoxy.

"Alkoxy-alkyl" refers to an alkyl-O-alkyl group where alkyl is as previously described.

"Acyl" means an alkyl-CO group wherein alkyl is as previously described. Exemplary acyl groups include acetyl, propanoyl, 2-methylpropanoyl, butanoyl and palmitoyl.

"Aryl" refers to an aromatic carbocyclic radical containing from about 6 to about 10 carbons, and all combinations and subcombinations of rings therein. The aryl group may be optionally substituted with one or two or more aryl group substituents. Exemplary aryl groups include phenyl and naphthyl.

"Aryl-substituted alkyl" refers to a linear alkyl group, preferably a lower alkyl group, substituted at a terminal carbon with an optionally substituted aryl group, preferably an optionally substituted phenyl ring. Exemplary aryl-substituted alkyl groups include, for example, phenylmethyl, phenylethyl and 3-(4-methylphenyl)propyl.

"Heterocyclic" refers to a monocyclic or multicyclic ring system carbocyclic radical containing from about 4 to about 10 members, and all combinations and subcombinations of rings therein, wherein one or more of the members of the ring is an element other than carbon, for example, nitrogen, oxygen or sulfur. The heterocyclic group may be aromatic or nonaromatic. Exemplary heterocyclic groups include, for example, pyrrole and piperidine groups.

"Halo" refers to fluoro, chloro, bromo or iodo.

"Peripheral," in reference to opioid antagonists, designates opioid antagonists that act primarily on physiological systems and components external to the central nervous system, e.g., they do not readily cross the blood-brain barrier in an amount effective to inhibit the central effects of opioids. In other words, peripheral opioid antagonists do not effectively inhibit the analgesic effects of opioids when administered peripherally, e.g., they do not reduce the analgesic effect of the opioids. For example, the peripheral opioid antagonist compounds employed in the methods of the present invention exhibit high levels of activity with respect to gastrointestinal tissue, while exhibiting reduced or substantially no central nervous system (CNS) activity. The peripheral opioid antagonist compounds employed in the present methods suitably exhibit less than about 5-15% of their pharmacological activity in the CNS, with about 0% (e.g., no CNS activity) being most suitable. The non-central acting characteristic of a peripheral opioid antagonist is often related to charge, polarity and/or size of the molecule. For example, peripherally-acting quaternary amine opioid antagonists are positively charged while the central-acting tertiary amine opioid antagonists are neutral molecules. The peripheral opioid antagonists useful in the present invention are typically mu and/or kappa opioid antagonists.

As used herein, "antiangiogenesis" or "antiangiogenic" is meant to refer to the capability of a molecule/compound to attenuate, e.g., inhibit, reduce or modulate, proliferation of new blood vessels, in general, and for example, to reduce or inhibit migration and proliferation of human microvascular endothelial cells in culture in the presence of certain growth factors. As described above, the formation of new blood vessels by endothelial cells involves migration, proliferation and differentiation of the cells.

In the following description of the methods of the invention, process steps are carried out at room temperature and atmospheric pressure unless otherwise specified. It also is specifically understood that any numerical range recited herein includes all values from the lower value to the upper value, e.g., all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this

application. For example, if a concentration range or beneficial effect range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended.

In one aspect, the present invention relates to methods of attenuating abnormal or undesirable cellular, particularly endothelial cell migration and/or proliferation, and angiogenesis in tissue or an organ of a subject. The methods comprise providing or administering one or more opioid antagonists in an effective amount to endothelial cells of the tissue or organ of a patient to inhibit endothelial cell migration and proliferation, and angiogenesis. The angiogenesis may, in part, be the result of receiving opioid treatment, particularly for pain management in cancer patients, or having high levels of endogenous opioids.

It was observed that morphine and the mu agonist enkephalin DAMGO ([D-Ala^2, N-McPhe^4, Gly^5-ol] enkephalin), each cause a dose-dependent increase in migration of endothelial cells similar to that of vascular endothelial growth factor (VEGF) as measured by, e.g., a chemotaxis assay (as detailed in the examples below) or other similar assays used to identify factors in tumor angiogenesis and the drugs that affect it. At clinically relevant concentrations of morphine, the magnitude of the effect is approximately 70% of that which is achieved by VEGF. This morphine-based endothelial cell migration is attenuated by the mu opioid antagonist methylnaltrexone (MNTX) in a dose-dependent fashion. For example, endothelial cell migration induced by morphine, in concentrations as low as 10^{-7}M, is significantly blocked by 10^{-7}M MNTX (FIG 2). This attenuation strongly suggesting that endothelial cell migration is mediated by morphine action on the mu opioid receptor (MOR). As described in the examples below, the effect via the MOR rather than other opioid receptors is confirmed by experiments that show the highly selective synthetic enkephalin mu agonist DAMGO also induces migration. The migratory effect induced by DAMGO is also blocked by MNTX (FIG. 3).

In one comprehensive review (Neumann et al. Pain 1982;13:247-52), analgesia in cancer patients was associated with a range of steady state

concentrations of morphine and plasma ranging from 6 to 364 ng/mL. It was observed an effect of morphine that causes endothelial cell migration at 100 ng/mL well within the clinical dose range. It therefore is believed by the inventors herein that a dose of MNTX which will maintain plasma levels of MNTX at minimum levels of plasma MNTX between about 25 and 150 ng/mL would be suitable. Such doses are attainable and are well tolerated (Yuan et al., J Clin Pharmacol 2005;45:538-46)

Alvimopan, another selective peripheral opioid antagonist given orally, is in late stage development for prophylaxis of postoperative ileus and the management of opioid induced constipation (Moss et al., Pain relief without side effects: peripheral opioid antagonists. In Schwartz, A.J., editor. 33rd ASA Refresher Course in Anesthesiology. Philadelphia: Lippincott Williams & Wilkins (in press).) There is some transpassage of alvimopan across the membrane (J. Foss, et al., Clin. Pharm. & Ther. 2005, PII-90, p. 74) and it may, therefore, possess the ability to reverse some of the systemic effects of opioids without affecting analgesia even when given orally.

Without being bound by any particular theory, it may be that the mechanism of mu opioid effect on endothelial cell migration occurs at the membrane level as MNTX, unlike naloxone, is a charged molecule at physiological pH. Morphine acts via G-protein coupled receptors, while VEGF acts by receptor tyrosine kinases. While the actions of mu agonists and VEGF may be independent, there is growing evidence of receptor transactivation as a mechanism. A prior investigation demonstrated that pertussis toxin dependent GPCRs transactivate VEGF receptor- 2/F1 K1 (Zeng, H. et al., J. Biol. Chem. 2003;278:20738-45). By this manner morphine could transactivate F11c-1 and promote an environment where endothelial cell proliferation and tumor growth could occur. A recent study of MOR knockout mice infected with T241 fibrosarcoma cells demonstrated significant differences in the incidences of tumor growth and a 10-fold increase in F11 c-1 expression in morphine treated mice versus controls, versus no increase in morphine treated KO mice (K. Gupta, personal communication). This provides further evidence that morphine

stimulates endothelial cell proliferation and promotes tumor growth probably by transactivating FLK1 phosphorylation. As such, the present invention provides potential clinical strategies using MNTX as well as other peripheral opioid antagonist in conjunction with current therapies targeting VEGF. Although a direct effect by receptor transactivation is possible, a potential additional factor involved in the proliferation of tumors may well be the role of chemokines as integrators of pain and inflammation. A recent review on this subject (White et al., Nature Rev. Drug Discovery 2005;4:834-44) also suggests a possible role for leukocytes in activating opioid receptors.

Furthermore, it was observed that morphine, DAMGO and VEGF stimulate RhoA activation which is inhibited by opioid antagonists, such as MNTX. RhoA is an important signaling molecule involved in angiogenesis (Aepfelbacher et al., 1997; Cascone et al., 2003; Hoang et al., 2004; Liu and Sanger, 2004.) VEGF receptor transactivation is important for opiate-induced RhoA activation. Silencing RhoA expression blocked opioid and VEGF induced EC proliferation and migration, demonstrating a role for RhoA activation in agonist-induced EC angiogenic activity. The MNTX mediated attenuation of RhoA activation may be important for the inhibitory role of MNTX on opioid and VEGF induced angiogenesis.

Because morphine and other opioids at clinical doses enhance endothelial cell migration, the present invention may be of therapeutic value in opioid antagonist treatment for patients on significant and sustained doses of opioids that have tumors relying on the angiogenic process. Further, while the inventor's clinical observations have focused on morphine, which is exogenously administered, endogenous opioids, which are released by stress or pain, may also play a role in endothelial cell migration. Based on endothelial cell migration experiments detailed below in the examples, MNTX and opioid antagonists generally are of therapeutic value as an antiangiogenic therapy even absent exogenous opioid administration (as detailed herein). It is envisioned that the methods of the present invention will inhibit or reduce the growth of blood vessels within and to a tumor. Inhibiting the growth of blood vessels within tumors

prevents nutrients and oxygen from being supplied to the tumor to support growth beyond a certain size. Minimizing the number of blood vessels or other tumors also lessens the probability that the tumor will metastasize.

The present invention may be of therapeutic value in opioid antagonist treatment for patients who have tumors relying on the angiogenic process. Tumors that rely on angiogenic processes are solid tumors, leukemias and myelomas. Solid tumors include, but are not limited to adrenal cortical carcinoma, tumors of the bladder: squamous cell carcinoma, urothelial carcinomas; tumors of the bone: adamantinoma, aneurysmal bone cysts, chondroblastoma, chondroma, chondromyxoid fibroma, chondrosarcoma, fibrous dysplasia of the bone, giant cell tumour, osteochondroma, osteosarcoma; breast tumors: secretory ductal carcinoma, chordoma; colon tumors: colorectal adenocarcinoma; eye tumors: posterior uveal melanoma, fibrogenesis imperfecta ossium, head and neck squamous cell carcinoma; kidney tumors: chromophobe renal cell carcinoma, clear cell renal cell carcinoma, nephroblastoma (Wilms tumor), kidney: papillary renal cell carcinoma, primary renal ASPSCR1-TFE3 tumor, renal cell carcinoma; liver tumors: hepatoblastoma, hepatocellular carcinoma; lung tumors: non-small cell carcinoma, small cell cancer; malignant melanoma of soft parts; nervous system tumors: medulloblastoma, meningioma, neuroblastoma, astrocytic tumors, ependymomas, peripheral nerve sheath tumors, phaeochromocytoma; ovarian tumors: epithelial tumors, germ cell tumors, sex cord-stromal tumors, pericytoma; pituitary adenomas; rhabdoid tumor; skin tumors: cutaneous benign fibrous histiocytomas; smooth muscle tumors: intravenous leiomyomatosis; soft tissue tumors: liposarcoma, myxoid liposarcoma, low grade fibromyxoid sarcoma, leiomyosarcoma, alveolar soft part sarcoma, angiomatoid fibrous histiocytoma (AFH), clear cell sarcoma, desmoplastic small round cell tumor, elastofibroma, Ewing's tumors, extraskeletal myxoid chondrosarcoma, inflammatory myofibroblastic tumor, lipoblastoma, lipoma / benign lipomatous tumors, liposarcoma / malignant lipomatous tumors, malignant myoepithelioma, rhabdomyosarcoma, synovial sarcoma, squamous cell cancer; tumors of the testis: germ cell tumors, spermatocytic seminoma; thyroid

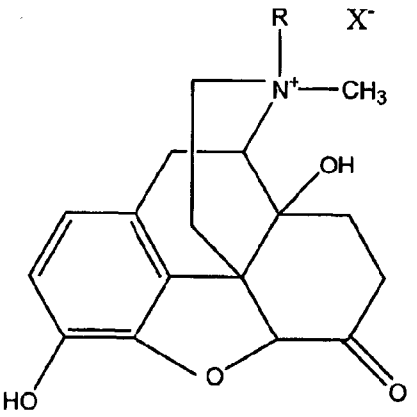
tumors: anaplastic (undifferentiated) carcinoma, oncocytic tumors, papillary carcinoma; uterus tumors: carcinoma of the cervix, endometrial carcinoma, leiomyoma etc.

In one embodiment of the invention the tumors are prostate cancer, gastrointestinal tumors such as colon or pancreatic cancer and the compounds of the invention are co-administered with other anticancer agents as described herein.

The opioid antagonists in accordance with the present invention include both centrally and peripherally acting opioid antagonists. It is contemplated that those antagonists of particular value are suitably the peripheral opioid antagonists. Especially suitable may be a mu opioid antagonist, especially a mu peripheral opioid antagonist. Opioid antagonists form a class of compounds that can vary in structure while maintaining the peripheral restrictive property. These compounds include tertiary and quaternary morphinans, in particular noroxymorphone derivatives, N-substituted piperidines, and in particular, piperidine-N-alkylcarboxylates, and tertiary and quaternary benzomorphans. Peripherally restricted antagonists, while varied in structure, are typically charged, polar and/or of high molecular weight, each of which impedes their crossing the blood-brain barrier.

Examples of opioid antagonists, which cross the blood-brain barrier and are centrally (and peripherally) active, include, e.g., naloxone, naltrexone (each of which is commercially available from Baxter Pharmaceutical Products, Inc.) and nalmefene (available, e.g., from DuPont Pharma). These may be of value in attenuating angiogenesis in the central nervous system or in patients not being treated for pain management or other opioid treatment.

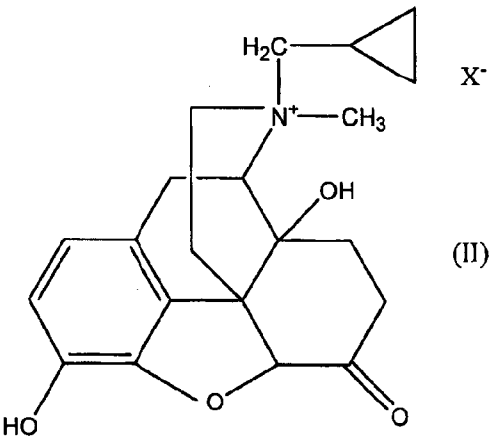
A peripheral opioid antagonist useful for the present invention may be a compound which is a quaternary morphinan derivative, and in particular, a quaternary noroxymorphone of formula (I):



(I)

wherein R is alkyl, alkenyl, alkynyl, aryl, cycloalkyl-substituted alkyl or aryl-substituted alkyl, and X' is the anion, especially a chloride, bromide, iodide or methylsulfate anion. The noroxymorphone derivatives of formula (I) can be prepared, for example, according to the procedure in U.S. Patent No. 4,176,186, ; see also, U.S. Patent Nos. 4,719,215; 4,861,781; 5,102,887; 5,972,954 and 6,274,591, U.S. Patent Application Nos. 2002/0028825 and 2003/0022909; and PCT publication Nos. WO 99/22737 and WO 98/25613

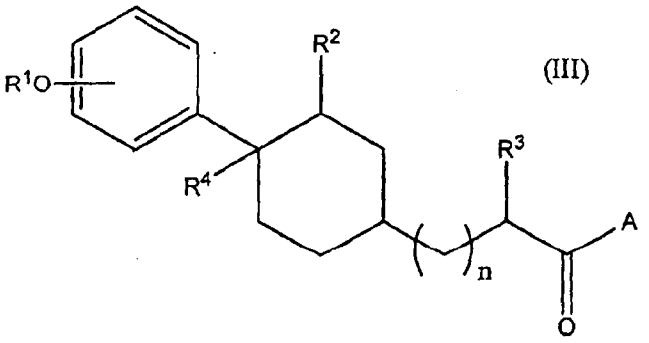
A compound of formula (I) of particular value is N-methylnaltrexone (or simply methylnaltrexone), wherein R is cyclopropylmethyl as represented in formula (II):



(II)

wherein X^- is as described above. Methylnaltrexone is a quaternary derivative of the opioid antagonist naltrexone. Methylnaltrexone exists as a salt, and "methylnaltrexone" or "MNTX", as used herein, therefore embraces salts. "Methylnaltrexone" or "MNTX" specifically includes, but is not limited to, bromide salts, chloride salts, iodide salts, carbonate salts, and sulfate salts of methylnaltrexone. Names used for the bromide salt of MNTX in the literature include: methylnaltrexone bromide; *N*-methylnaltrexone bromide; naltrexone methobromide; naltrexone methyl bromide; SC-37359, MRZ-2663-BR, and N-cyclopropylmethylnoroxy-morphine-methobromide.Methylnaltrexone is commercially available from, e.g., Mallinckrodt Pharmaceuticals, St. Louis, Mo. Methylnaltrexone is provided as a white crystalline powder, freely soluble in water, typically as the bromide salt. The compound as provided is 99.4% pure by reverse phase HPLC, and contains less than 0.011% unquaternized naltrexone by the same method. Methylnaltrexone can be prepared as a sterile solution at a concentration of, e.g., about 5 mg/mL..

Other suitable peripheral opioid antagonists may include N-substituted piperidines, and in particular, piperidine-N-alkylcarboxylates as represented by formula (III):



(III)

wherein

R^1 is hydrogen or alkyl;

R^2 is hydrogen, alkyl, or alkenyl;

R^3 is hydrogen, alkyl, alkenyl, aryl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkyl or aryl-substituted alkyl;

R^4 is hydrogen, alkyl, or alkenyl;

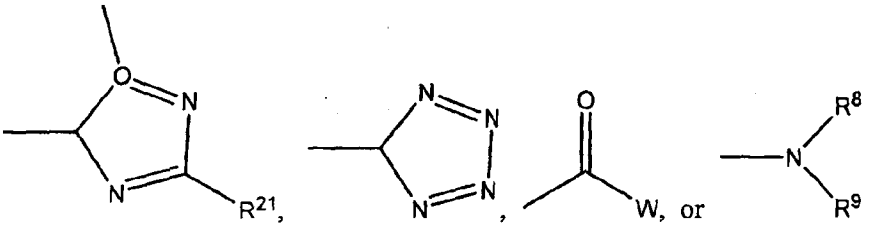
A is OR^5 or NR^6R^7; wherein

R^5 is hydrogen, alkyl, alkenyl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkyl or aryl-substituted alkyl;

R^6 is hydrogen or alkyl;

R^7 is hydrogen, alkyl, alkenyl, aryl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkyl or aryl-substituted alkyl, or alkylene-substituted B or together with the nitrogen atom to which they are attached, R^6 and R^7 form a heterocyclic ring selected from pyrrole and piperidine;

B is



wherein R^8 is hydrogen or alkyl;

R^9 is hydrogen, alkyl, alkenyl, aryl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkyl or aryl-substituted alkyl or together with the nitrogen atom to which they are attached, R^8 and R^9 form a heterocyclic ring selected from pyrrole and piperidine;

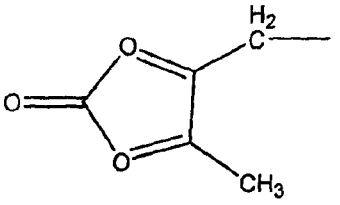
W is OR^{10}, $NR^{11}R^{12}$, or OE; wherein

R^{10} is hydrogen, alkyl, alkenyl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkenyl, or aryl-substituted alkyl;

R^{11} is hydrogen or alkyl;

R^{12} is hydrogen, alkyl, alkenyl, aryl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkyl, aryl-substituted alkyl or alkylene-substituted C(=O)Y or, together with the nitrogen atom to which they are attached, R^{11} and R^{12} form a heterocyclic ring selected from pyrrole and piperidine;

E is



alkylene-substituted (C=0)D, or -R^{13}0C(=O)R^{14}; wherein

R^{13} is alkyl-substituted alkylene;

R^{14} is alkyl;

D is OR^{15} or $NR^{16}R^{17}$; wherein

R^{15} is hydrogen, alkyl, alkenyl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl substituted alkyl, or aryl-substituted alkyl;

R^{16} is hydrogen, alkyl, alkenyl, aryl, aryl-substituted alkyl, cycloalkyl, cycloalkenyl, cycloalkyl substituted alkyl or cycloalkenyl-substituted alkyl;

R^{17} is hydrogen or alkyl or, together with the nitrogen atom to which they are attached, R^{16} and R^{17} form a heterocyclic ring selected from the group consisting of pyrrole or piperidine;

Y is OR^{18} or $NR^{19}R^{20}$; wherein

R^{18} is hydrogen, alkyl, alkenyl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkyl, or aryl-substituted alkyl;

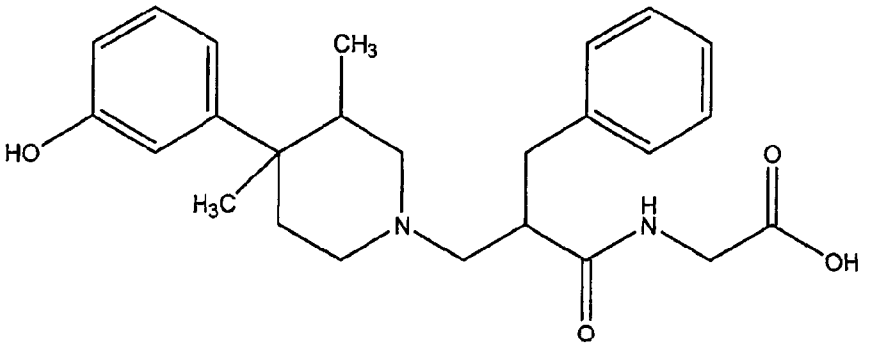
R^{19} is hydrogen or alkyl;

R^{20} is hydrogen, alkyl, alkenyl, aryl, cycloalkyl, cycloalkenyl, cycloalkyl-substituted alkyl, cycloalkenyl-substituted alkyl, or aryl-substituted alkyl or, together with the nitrogen atom to which they are attached, R19 and R20 form a heterocyclic ring selected from pyrrole and piperidine;

R^{21} is hydrogen or alkyl;

and n is 0 to 4.

Particular piperidine-N-alkylcarbonylates which may be of value are N-alkylamino-3, 4, 4 substituted piperidines, such as alvimopan represented below as formula (IV):



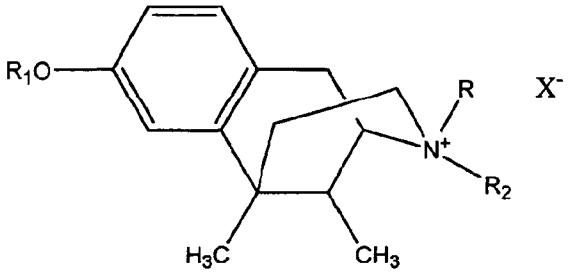
(IV)

Suitable N-substituted piperidines may be prepared as disclosed in U.S. Patent Nos. 5,270,328; 6,451,806; 6,469,030.

Alvimopan is available from Adolor Corp., Exton, PA. Such compounds have moderately high molecular weights, a zwitterion form and a polarity which prevent penetration of the blood-brain barrier.

Still other suitable peripheral opioid antagonist compounds may include quaternary benzomorphan compounds. The quaternary benzomorphan compounds employed in the methods of the present invention exhibit high levels of morphine antagonism, while exhibiting reduced, and preferably substantially no, agonist activity.

The quaternary benzomorphan compounds which may be employed in the methods of the present invention have the following formula (V):



(V)

wherein;

R^1 is hydrogen, acyl or acetoxy; and

R^2 is alkyl or alkenyl;

R is alkyl, alkenyl or alkynyl

and

X^- is an anion, especially a chloride, bromide, iodide or methylsulfate anion.

Specific quaternary derivatives of benzomorphan compounds that may be employed in the methods of the present invention include the following compounds of formula (V): 2'-hydroxy 5,9-dimethyl-2,2-diallyl-6,7-benzomorphanium-bromide; 2'-hydroxy-5,9-dimethyl-2-n-propyl-2 allyl-6,7-benzomorphanium-bromide; 2'-hydroxy-5,9-dimethyl-2-n-propyl-2-propargyl-6,7 benzomorphanium-bromide; and 2'-acetoxy-5,9-dimethyl-2-n-propyl-2-ally)-6,7 benzomorphanium-bromide.

Other quaternary benzomorphan compounds that may be employed in the methods of the present invention are described, for example, in U.S. Pat. No. 3,723,440.

The compounds employed in the methods of the present invention may exist in prodrug form. As used herein, "prodrug" is intended to include any covalently bonded carriers which release the active parent drug according to formulas (I) to (V) or other formulas or compounds employed in the methods of the present invention in vivo when such prodrug is administered to a mammalian subject. Since prodrugs are known to enhance numerous desirable qualities of pharmaceuticals (e.g., solubility, bioavailability, manufacturing, etc.), the compounds employed in the present methods may, if desired, be delivered in prodrug form. Thus, the present invention contemplates methods of delivering prodrugs. Prodrugs of the compounds employed in the present invention may be prepared by modifying functional groups present in the compound in such a way that the modifications are cleaved, either in routine manipulation or in vivo, to the parent compound.

Accordingly, prodrugs include, for example, compounds described herein in which a hydroxy, amino, or carboxy group is bonded to any group that, when the prodrug is administered to a mammalian subject, cleaves to form a free hydroxyl, free amino, or carboxylic acid, respectively.

Examples include, but are not limited to, acetate, formate and benzoate derivatives of alcohol and amine functional groups; and alkyl, carbocyclic, aryl, and alkylaryl esters such as methyl, ethyl, propyl, iso-propyl, butyl, isobutyl, sec-butyl, tert-butyl, cyclopropyl, phenyl, benzyl, and phenethyl esters, and the like.

As noted, the compounds employed in the methods of the present invention may be prepared in a number of ways well known to those skilled in the art. All preparations disclosed in association with the present invention are contemplated to be practiced on any scale, including milligram, gram, multigram, kilogram, multikilogram or commercial pharmaceutical scale.

Compounds employed in the present methods may contain one or more asymmetrically substituted carbon atom, and may be isolated in optically active or racemic form. Thus, all chiral, diastereomeric, racemic form, epimeric form and all geometric isomeric form of a structure are intended, unless the specific stereochemistry or isomeric form is specifically indicated. It is well known in the art how to prepare and isolate such optically active form. For example, mixtures of stereoisomers may be separated by standard techniques including, but not limited to, resolution of racemic form, normal, reverse-phase, and chiral chromatography, preferential salt formation, recrystallization, and the like, or by chiral synthesis either from chiral starting materials or by deliberate synthesis of target chiral centers.

In some embodiments of the invention, the opioid antagonist may be a mu opioid antagonist. In other embodiments, the opioid antagonist may be a kappa opioid antagonist. The invention also encompasses administration of more than one opioid antagonist, including combinations of mu antagonists, combinations of kappa antagonists and combinations of mu and kappa antagonists, for example, a combination of methylnaltrexone and alvimopan.

The methods of the present invention encompass providing a therapeutic or prophylactic role in other endothelial-based diseases, e.g., in a variety of angiogenesis and/or proliferation-related neoplastic and non-neoplastic diseases, e.g., sickle cell disease, neovascular disease of the eye (such as diabetic retinopathy, neovascular glaucoma, retinopathy of prematurity, age-related

macular degeneration), endothelial proliferation in the kidneys or lung and psoriasis. Non-neoplastic conditions that are amenable to treatment include rheumatoid arthritis, psoriasis, atherosclerosis, diabetic and other proliferative retinopathies including retinopathy of prematurity, retrolental fibroplasia, neovascular glaucoma, age-related macular degeneration, thyroid hyperplasias (including Grave's disease), corneal and other tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial effusion (such as that associated with pericarditis), and pleural effusion. For example, it has been shown that morphine induced proliferative retinopathy in sickle cell disease (Gupta et al., personal communication). It is anticipated that treatment with an opioid antagonist may significantly inhibit the retinopathy, particularly with opioid-induced retinopathy in sickle cell patients that are in active opioid therapy, and receive opioids for long periods of time, including chronic therapy for weeks, months or even years.

The methods of the present invention are also envisioned to be of value in reducing the risk of recurrence of a malignancy or neoplasm after treatment with other therapeutic modalities, e.g., after surgical intervention. For example, the present invention provides a method for reducing the risk of recurrence of postoperative cancer. The cancers may include, for example, breast cancer or prostate cancer, and reduced risk may be achieved by providing to the patient suffering from such cancer an effective amount of an opioid antagonist, particularly a peripheral opioid antagonist. For example, as described above, patients undergoing breast cancer surgery had a significant difference (fourfold) in the incidence of recurrence at 2-4 years depending on whether the patients received regional or general anesthesia (with morphine during their initial surgery. Co-administration of the opioid antagonists, especially peripheral antagonist, in accordance with the present invention with surgical treatment may be of value to reduce the incidence of recurrence of the cancer.

It is also contemplated that the invention provides a method of inhibiting the activity of VEGF by providing to the affected cells or subject an effective amount of an opioid antagonist under conditions sufficient to inhibit VEGF-

induced angiogenesis. In other words, the compounds of the present invention have VEGF-inhibitory or antagonist activity.

As also shown in the examples below, it has further been found that a peripheral opioid antagonist, MNTX, attenuates not only VEGF-induced endothelial cell migration, but also induction of endothelial migration and/or proliferation by other pro-migration/pro-proliferative factors such as platelet derived growth factor (PDGF), or sphingosine 1-phosphate (S1P). Such attenuation ranges from about 10% to 60%, and provides further evidence that the methods of the present invention have value in inhibiting pro-migration, pro-angiogenic factors.

The methods of the invention also encompass treating patients, e.g., cancer patients, who are undergoing treatment with opioid agonists. Opioid agonists include, but are not limited to, morphine, methadone, codeine, meperidine, fentidine, fentanil, sufentanil, alfentanil and the like. As described above, opioid agonists are classified by their ability to agonize one type of receptor an order of magnitude more effectively than another. For example, the relative affinity of morphine for the mu receptor is 200 times greater than for the kappa receptor, and is therefore classified as a mu opioid agonist. Some opioid agonists may act as agonists towards one receptor and antagonists toward another receptor and are classified as agonist/antagonists, (also known as mixed or partial agonists). "Agonist/antagonist," "partial agonist," and "mixed agonist" are used interchangeably herein. These opioids include, but are not limited to, pentazocine, butorphanol, nalorphine, nalbufine, buprenorphine, bremazocine, and bezocine. Many of the agonist/antagonist group of opioids are agonists at the kappa receptors and antagonists at the mu receptors. Further, it is envisioned the active metabolites of opioid agonists may also be active as angiogenesis inducers. For example, the metabolites of morphine, morphine 3-glucuronide (M3G) and morphine 6-glucuronide (M6G) may be active proangiogenic factors.

Generally, the peripheral opioid antagonists in accordance with the present invention may be administered in an effective amount such that the patient's plasma level of the peripheral opioid antagonist is in the range from 10^{-6} M to 10^{-}

^{9}M. Patient drug plasma levels may be measured using routine HPLC methods known to those of skill in the art.

As described in the examples below, the enkephalin analog DAMGO induces endothelial migration. Thus, the methods of the present invention may be of value to patient suffering from angiogenic-related or hyperproliferative diseases, e.g., cancer, quite apart from treatment with opioid agonists.

The particular mode of administration of the opioid antagonist selected will depend, of course, upon the particular combination of drugs selected, the severity of the tumor progression being treated, in the cancer patient, the general health condition of the patient, and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, e.g., any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical (as by powder, ointment, drops, transdermal patch or iontophoretic devise), transdermal, sublingual, intramuscular, infusion, intravenous, pulmonary, intramuscular, intracavity, as an aerosol, aural (e.g., via eardrops), intranasal, inhalation, intraocular or subcutaneous. Direct injection could also be used for local delivery. Oral or subcutaneous administration may be suitable for prophylactic or long term treatment because of the convenience of the patient as well as the dosing schedule. For ocular diseases, ophthalmic formulations may be injected or instilled directly.

Additionally, the opioid antagonists may be administered as an enterically coated tablet or capsule. In some embodiments, the opioid antagonist is administered by a slow infusion method or by a time-release or controlled-release method or as a lyophilized powder.

When administered, the compounds of the invention are given in pharmaceutically acceptable amounts and in pharmaceutically acceptable compositions or preparations. Such preparations may routinely contain salts, buffering agents, preservatives, and optionally other therapeutic ingredients. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare

pharmaceutically acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluenesulfonic, tartaric, citric, methanesulfonic, formic, succinic, naphthalene-2-sulfonic, pamoic, 3-hydroxy-2-naphthalenecarboxylic, and benzene sulfonic. Suitable buffering agents include, but are not limited to, acetic acid and salts thereof (1-2% WN); citric acid and salts thereof (1-3% WN); boric acid and salts thereof (0.5-2.5% WN); and phosphoric acid and salts thereof (0.8-2% WN).

Suitable preservatives include, but are not limited to, benzalkonium chloride (0.003-0.03% WN); chlorobutanol (0.3-0.9% W/N); parabens (0.01-0.25% WN) and thimerosal (0.004-0.02% WN).

For ease of administration, a pharmaceutical composition of the peripheral opioid antagonist may also contain one or more pharmaceutically acceptable excipients, such as lubricants, diluents, binders, carriers, and disintegrants. Other auxiliary agents may include, e.g., stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, coloring, flavoring and/or aromatic active compounds.

A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. For example, suitable pharmaceutically acceptable carriers, diluents, solvents or vehicles include, but are not limited to, water, salt (buffer) solutions, alcohols, gum arabic, mineral and vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, vegetable oils, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxy methylcellulose, polyvinyl pyrrolidone, etc. Proper fluidity may be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. Prevention of the action of microorganisms may be ensured by the

inclusion of various antibacterial and antifungal agents such as paraben, chlorobutanol, phenol, sorbic acid and the like.

If a pharmaceutically acceptable solid carrier is used, the dosage form of the analogs may be tablets, capsules, powders, suppositories, or lozenges. If a liquid carrier is used, soft gelatin capsules, transdermal patches, aerosol sprays, topical cream, syrups or liquid suspensions, emulsions or solutions may be the dosage form.

For parental application, particularly suitable are injectable, sterile solutions, preferably nonaqueous or aqueous solutions, as well as dispersions, suspensions, emulsions, or implants, including suppositories. Ampoules are often convenient unit dosages. Injectable depot form may also be suitable and may be made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide, poly(orthoesters) and poly(anhydrides). Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled.

Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues. The injectable formulations may be sterilized, for example, by filtration through a bacterial-retaining filter or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable media just prior to use.

For enteral application, particularly suitable are tablets, dragees, liquids, drops, suppositories, or capsules such as soft gelatin capsules. A syrup, elixir, or the like can be used wherein a sweetened vehicle is employed.

As noted, other delivery system may include time-release, delayed-release or sustained-release delivery system. Such system can avoid repeated administrations of the compounds of the invention, increasing convenience to the patient and the physician and maintain sustained plasma levels of compounds. Many types of controlled-release delivery system are available and known to those of ordinary skill in the art. Sustained- or controlled-release compositions can be formulated, e.g., as liposomes or those wherein the active compound is

protected with differentially degradable coatings, such as by microencapsulation, multiple coatings, etc.

For example, compounds of this invention may be combined with pharmaceutically acceptable sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. A sustained-release matrix, as used herein, is a matrix made of materials, usually polymers, which are degradable by enzymatic or acid-base hydrolysis or by dissolution. Once inserted into the body, the matrix is acted upon by enzymes and body fluids. A sustained-release matrix may be desirably chosen from biocompatible materials such as liposomes, polymer-based system such as polylactides (polylactic acid), polyglycolide (polymer of glycolic acid), polylactide co-glycolide (copolymers of lactic acid and glycolic acid), polyanhydrides, poly(ortho)esters, polysaccharides, polyamino acids, hyaluronic acid, collagen, chondroitin sulfate, polynucleotides, polyvinyl propylene, polyvinyl pyrrolidone, and silicone; nonpolymer system such as carboxylic acids, fatty acids, phospholipids, amino acids, lipids such as sterols, hydrogel release system; silastic system; peptide-based system; implants and the like. Specific examples include, but are not limited to: (a) erosional system in which the polysaccharide is contained in a form within a matrix, found in U.S. Pat. Nos. 4,452,775, 4,675,189, and 5,736,152

and (b) diffusional system in which an active component permeates at a controlled rate from a polymer such as described in U.S. Pat. Nos. 3,854,480, 5,133,974 and 5,407,686.

In addition, pump-based hard-wired delivery system can be used, some of which are adapted for implantation. Suitable enteric coatings are described in PCT publication No. WO 98/25613 and U.S. Pat. No. 6,274,591.

Use of a long-term sustained-release implant may be particularly suitable for treatment of chronic conditions. "Long-term" release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 7 days, and suitably 30 to 60 days. Long-term

sustained-release implants are well-known to those of ordinary skill in the art and include some of the release system described above.

For topical application, there are employed as nonsprayable form, viscous to semi-solid or solid form comprising a carrier compatible with topical application and having a dynamic viscosity preferably greater than water. Suitable formulations include, but are not limited to, solutions, suspensions, emulsions, cream, ointments, powders, liniments, salves, aerosols, etc., which are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, etc.

Transdermal or iontophoretic delivery of pharmaceutical compositions of the peripheral opioid antagonists is also possible.

Respecting MNTX specifically, aqueous formulations may include a chelating agent, a buffering agent, an anti-oxidant and, optionally, an isotonicity agent, preferably pH adjusted to between 3.0 and 3.5. Preferred such formulations that are stable to autoclaving and long term storage are described in application serial no. 10/821811, now published as 20040266806, entitled "Pharmaceutical Formulation.".

In one embodiment, compounds of the invention are administered in a dosing regimen which provides a continuous dosing regimen of the compound to a subject, e.g., a regimen that maintains minimum plasma levels of the opioid antagonist, and preferably eliminates the spikes and troughs of a drug level with conventional regimens. Suitably, a continuous dose may be achieved by administering the compound to a subject on a daily basis using any of the delivery methods disclosed herein. In one embodiment, the continuous dose may be achieved using continuous infusion to the subject, or via a mechanism that facilitates the release of the compound over time, for example, a transdermal patch, or a sustained release formulation. Suitably, compounds of the invention are continuously released to the subject in amounts sufficient to maintain a concentration of the compound in the plasma of the subject effective to inhibit or reduce opioid induced angiogenesis; or in cancer patients, to attenuate growth of a tumor. Compounds in accordance with the present invention, whether provided alone or in combination with other therapeutic agents, are provided in an

antiangiogenic effective amount. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration; the route of administration; the rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed and like factors well known in the medical arts. For example, it is well within the level of ordinary skill in the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

If desired, the effective daily dose may be divided into multiple doses for purposes of administration. Consequently, single dose compositions may contain such amounts or submultiples thereof to make up the daily dose. As noted, those of ordinary skill in the art will readily optimize effective doses and co-administration regimens (as described herein) as determined by good medical practice and the clinical condition of the individual patient.

Generally, oral doses of the opioid antagonists, particularly peripheral antagonists, will range from about 0.01 to about 80 mg/kg body weight per day. It is expected that oral doses in the range from 1 to 20 mg/kg body weight will yield the desired results. Generally, parenteral administration, including intravenous and subcutaneous administration, will range from about 0.001 to 5 mg/kg body weight. It is expected that doses ranging from 0.05 to 0.5 mg/kg body weight will yield the desired results. Dosage may be adjusted appropriately to achieve desired drug levels, local or systemic, depending on the mode of administration. For example, it is expected that the dosage for oral administration of the opioid antagonists in an enterically coated formulation would be from 10 to 30% of the non-coated oral dose. In the event that the response in a patient is insufficient of

such doses, even higher doses (or effectively higher 30 dosage by a different, more localized delivery route) may be employed to the extent that the patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds. Appropriate system levels can be determined by, for example, measurement of the patient's plasma level of the drug using routine HPLC methods known to these of skill in the art.

In some embodiments of the invention, the opioid antagonists are co-administered with the opioid. The term "co-administration" is meant to refer to a combination therapy by any administration route in which two or more agents are administered to a patient or subject. Co-administration of agents may also be referred to as combination therapy or combination treatment. The agents may be in the same dosage formulations or separate formulations. For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times. The agents may be administered simultaneously or sequentially (e.g., one agent may directly follow administration of the other or the agents may be give episodically, e.g., one can be given at one time followed by the other at a later time, e.g., within a week), as long as they are given in a manner sufficient to allow both agents to achieve effective concentrations in the body. The agents may also be administered by different routes, e.g., one agent may be administered intravenously while a second agent is administered intramuscularly, intravenously or orally. In other words, the co-administration of the opioid antagonist compound in accordance with the present invention with an opioid is suitably considered a combined pharmaceutical preparation which contains an opioid antagonist and a opioid agent, the preparation being adapted for the administration of the peripheral opioid antagonist on a daily or intermittent basis, and the administration of opioid agent on a daily or intermittent basis. Thus, the opioid antagonists may be administered prior to, concomitant with, or after administration of the opioids. Co-administrable agents also may be formulated as an admixture, as, for example, in a single formulation or single tablet. These formulations may be parenteral or oral,

such as the formulations described, e.g., in U.S. Pat. Nos. 6,277,384; 6,261,599; 5,958,452 and PCT publication No. WO 98/25613.

It is further contemplated that the present method can be used alone or in conjunction with other treatments to control the growth or migration of endothelial cells in connection with the various conditions described above. The peripheral opioid antagonist may be co-administered with another therapeutic agent that is not an opioid or opioid antagonist. Suitable such therapeutic agents include anticancer agents, e.g., chemotherapeutic agents, radiotherapy, or other antiangiogenic agents such as suramin, or anti-VEGF mab, an endostatin or radiotherapy. It is envisioned that the opioid antagonists in accordance with the present invention are of particular value when co-administered with those agents that inhibit VEGF activity, e.g., anti-VEGF mab. The anti-VEGF antibodies are useful in the treatment of various neoplastic and non-neoplastic diseases and disorders, including endometrial hyperplasia, endometriosis, abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors and Meigs' syndrome. One example of a anti-VEGF mab is bevacizumab (Avastin, Genentech) described in US Patent No 6,884,879 and WO94/10202 In a certain embodiments of the invention, MNTX is co-administered with Avastin.™

In other words, the compounds of the present invention may also be useful for the treatment of cancer in patients, as described above, either when used alone or in combination with one or more other anticancer agents, e.g., radiotherapy and/or other chemotherapeutic, including antiangiogenic, treatments conventionally administered to patients for treating cancer. The main categories and examples of such drugs are listed herein and include, but are not limited to metalloproatease inhibitors, inhibitors of endothelial cell proliferation/migration, antagonists of angiogenic growth factors, inhibitors of Integrin/Survival signaling, and chelators of copper.

In certain embodiments the compounds of the invention can be combined with known combinations of anticancer agents. The compounds of the invention

can be combined with an antiangiogenic agent and a chemotherapeutic agent and administered to a cancer patient. For example, MNTX can be administered to cancer patients in combination with Avastin and 5-fluorouracil.

It is anticipated that the opioid antagonists co-administered with various anticancer drugs, radiotherapy or other antiangiogenic drugs can give rise to a significantly enhanced antiproliferative effect on cancerous cells, and thus providing an increased therapeutic effect, e.g., employing peripheral opioid antagonists to certain tumors can potentiate their response to other therapeutic regimens. Specifically, a significantly increased antiangiogenic or antihyperproliferative effect is obtained with the above disclosed co-administered combinations, even if utilizing lower concentrations of the anticancer, a lower dosing of radiation, or other antiangiogenic drugs compared to the treatment regimes in which the drugs or radiation are used alone. Therefore there is the potential to provide therapy wherein adverse side effects associated with the anticancer or other antiangiogenic drugs or radiotherapy are considerably reduced than normally observed with the anticancer or other antiangiogenic drugs or radiotherapy used alone in larger doses. For example, co-administration of an opioid antagonist in accordance with the present invention with an anti-VEGF agent, e.g., anti-VEGF mab, may reduce the dose of the anti-VEGF agent or increase potency or efficacy or both. Further, as detailed herein, the co-administration of an opioid antagonist in accordance with the present invention with other anticancer modalities may have prophylactic value.

When used in the treatment of hyperproliferative diseases, compounds of the present invention may be co-administered with metalloprotease inhibitors such as for example: Marimastat, synthetic matrix metalloprotease inhibitor (MMPI), British Biotech; Bay 12-9566, synthetic MMPI and inhibitor tumor growth, Bayer; AG3340, synthetic MMPI, Agouron/Warner-Lambert; CGS 27023A, synthetic MMPI, Novartis; CGS 27023A, Synthetic MMPI; COL-3, synthetic MMPI, tetracycline derivative, Collagenex; AE-941 (Neovastat), naturally occurring MMPI, AEterna, BMS-275291, synthetic MMPI, Bristol-Myers Squibb; Penicillamine, urokinase inhibitor, NCI-NABTT.

When used in the treatment of hyperproliferative diseases, compounds of the present invention may be co-administered with direct inhibitors of endothelial cell proliferation/migration such as: TNP-470 (fumagillin derivative), inhibits endothelial cell growth, TAP Pharmaceuticals; Squalamine, inhibits sodium-hydrogen exchanger, NIHE3, Magainin; Combretastatin, induction of apoptosis in proliferating endothelial cells, Oxigene; Endostatin, inhibition of endothelial cells, EntreMed; Penicillamine, blocks endothelial cell migration and proliferation, NCI – NABTT; Farnesyl Transferase Inhibitor (FTI), blocks endothelial cell migration and proliferation, NCI – NABTT, -L-778,123 Merck, -SCH66336 Schering-Plough, -R115777 Janssen.

When used in the treatment of hyperproliferative diseases, compounds of the present invention may be co-administered with antagonists of angiogenic growth factors such as: anti-VEGF antibody, monoclonal antibody that inactivates VEGF, Genentech; thalidomide, blocks activity of angiogenic growth factors (bFGF, VEGF, TNF-alpha), Celgene; SU5416, blocks VEGF receptor (Flk-1/KDR) signaling (tyrosine kinase), Sugen-NCI; ribozyme (Angiozyme), attenuates mRNA of VEGF receptors, Ribozyme Pharmaceuticals, Inc; SU6668, blocks VEGF, bFGF, and PDGF receptor signaling, Sugen; PTK787/ZK22584, blocks VEGF receptor signaling, Novartis; Interferon-alpha, inhibition of bFGF and VEGF production; Suramin, blocks binding of growth factor to its receptor, NCI – NABTT.

When used in the treatment of hyperproliferative diseases, compounds of the present invention may be co-administered with drugs that inhibit endothelial-specific Integrin/Survival signaling: Vitaxin, antibody to alpha-v-beta3 integrin present on endothelial cell surface, Ixsys; EMD121974, small molecule blocker of integrin present on endothelial cell surface, Merck KGaA.

When used in the treatment of hyperproliferative diseases, compounds of the present invention may be co-administered with chelators of copper, such as: penicillamine, sulfhydryl group binds copper; clears copper through urinary excretion, NCI-NABTT; tetrathiomolybdate, thiol groups tightly bind copper, inactivate copper available to tumor, University of Michigan Cancer Center;

captopril, chelates copper and zinc; also, inhibitor of MMP and angiotensin converting enzyme, Northwestern University.

When used in the treatment of hyperproliferative diseases, compounds of the present invention may be co-administered with angiogenesis antagonists with distinct mechanisms: CAI, inhibitor of calcium influx, NCI; ABT-627, endothelin receptor antagonist, Abbott/NCI; CM101/ZDO101, group B Strep toxin that selectively disrupts proliferating endothelium by interaction with the (CM201) receptor, CarboMed/Zeneca; Interleukin-12, induction of interferon-gamma, down-regulation of IL-10, induction of IP-10, M.D. Anderson Cancer Center/Temple University, Temple University, Genetics Institute, Hoffman LaRoche; IM862, blocks production of VEGF and bFGF; increases production of the inhibitor IL-12, Cytran; PNU-145156E, blocks angiogenesis induced by Tat protein, Pharmacia and Upjohn.

When used in the treatment of hyperproliferative diseases, compounds of the present invention may be co-administered with chemotherapeutic agents such as, for example, alpha interferon, COMP (cyclophosphamide, vincristine, methotrexate and prednisone), etoposide, mBACOD (methortrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine and dexamethasone), PRO-MACE/MOPP (prednisone, methotrexate (w/leucovin rescue), doxorubicin; cyclophosphamide, paclitaxol, docetaxol, etoposide/mechlorethamine, vincristine, prednisone and procarbazine), vincristine, vinblastine, angioinhibins, TNP-470, pentosan polysulfate, platelet factor 4, angiostatin, LM-609, SU-101, CM-101, Techgalan, thalidomide, SP-PG and the like.

Anticancer agents which may be co-administered with compounds of the present invention also suitably include antimetabolites (e.g., 5-fluoro-uracil, methotrexate, fludarabine), antimicrotubule agents (e.g., vincristine, vinblastine, taxanes such as paclitaxel, docetaxel), an alkylating agent (e.g., cyclophosphamide, melphalan, biochoroethylnitrosurea, hydroxyurea), nitrogen mustards, (e.g., mechloethamine, melphan, chlorambucil, cyclophosphamide and Ifosfamide); nitrosoureas (e.g., carmustine, lomustine, semustine and streptozocin;), platinum agents (e.g., cisplatin, carboplatin, oxaliplatin, JM-216,

C1-973), anthracyclines (e.g., doxrubicin, daunorubicin), antibiotics (e.g., mitomycin, idarubicin, adriamycin, daunomycin), topoisomerase inhibitors (e.g., etoposide, camptothecins), alkyl sulfonates including busulfan; triazines (e.g., dacarbazine); ethyenimines (e.g., thiotepa and hexamethylmelamine); folic acid analogs (e.g., methotrexate); pyrimidine analogues (e.g., 5 fluorouracil, cytosine arabinoside); purine analogs (e.g., 6-mercaptopurine, 6-thioguanine); antitumor antibiotics (e.g., actinomycin D; bleomycin, mitomycin C and methramycin); hormones and hormone antagonists (e.g., tamoxifen, cortiosteroids) and any other cytotoxic agents, (e.g., estramustine phosphate, prednimustine).

It will be understood that agents which can be combined with the compounds of the present invention for the inhibition, treatment or prophylaxis of angiogenesis and/or cancers are not limited to those listed above, but include, in principle, any agents useful for the treatment opioid induced angiogenic diseases and tumor growth.

The present invention is further explained by the following examples, which should not be construed by way of limiting the scope of the present invention.

EXAMPLES

Example 1: Endothelial Cell Migration Assay

The antiangiogenic activity of the peripheral opioid antagonists in accordance with the present invention was evaluated in experiments testing the ability of the antagonist to inhibit or modulation capillary endothelial cell migration using a modified Boyden chamber.

The endothelial cell migration assay was performed as described by Lingen, M.W., Methods in Molecular Medicine, 78: 337-347 (2003). Briefly, Human Microvascular Endothelial Cells (HMVEC) (Cell Systems, Kirkland, WA.) were starved overnight in Endothelial Growth Medium (EGM) containing 0.1% bovine serum albumin (BSA). Cells were then trypsinized and resuspended in Dulbecco's Modified Eagle Medium (DME) with 0.1 % BSA at a concentration of 1×10^6

cells/mL. Cells were added to the bottom of a 48-well modified Boyden chamber (NeuroPore Corporation, Pleasanton, CA.). The chamber was assembled and inverted, and cells were allowed to attach for 2 hours at 37°C to polycarbonate chemotaxis membranes (5 μm pore size) (NeuroProbe) that had been soaked in 0.1% gelatin overnight and dried. The chamber was then reinverted and the compound to be tested at varying concentrations in quadruple, vascular endothelial growth factor (VEGF) (as a positive control) or vehicle were added to the wells of the upper chamber (to a total volume of 50 mL); the apparatus was then incubated for 4 hours at 37°C. Membranes were recovered, fixed and stained (DiffQuick, Fisher Scientific, Pittsburgh, Pa.) and the number of cells that had migrated to the upper chamber per 10 high power fields were counted. Background migration to DME+0.1 % BSA was subtracted and the data reported as the number of cells migrated per 10 high power fields (400 times). Each substance was tested in quadruplicate in each experiment, and all experiments were repeated to least twice. VEGF (R&D System, Minneapolis, MN) was used as a positive control at a concentration of 200 pg/mL. The optimal concentration for VEGF was determined previously by dose-response experiments (data not shown). The compounds tested as described above were morphine, naloxone, methylnaltrexone, and the combination of methylnaltrexone and morphine. The concentrations of each tested substance ranged for 0.001 to 10.0 μM. The concentration of the morphine was constant at 0.1 μM. The results are shown in FIG. 1.

FIG. 1 shows that morphine increased migration in a concentration-dependent manner. The co-addition of methylnaltrexone and morphine, however, decreased migration in a concentration-dependent manner. Neither methylnaltrexone or naloxone alone affected migration.

Example 2: Endothelial Cell Migration Assay

Another set of experiments was conducted in accordance with the procedure described in Example 1. In this set of experiments, methylnaltrexone and the combination of methylnaltrexone and morphine was again tested for

ability to inhibit endothelial cell migration. The methylnaltrexone concentrations when tested alone varied from 0.001 to 10.0 μM. In combination, the concentrations of methylnaltrexone varied from 0.001 to 10.0 μM, while the morphine concentration remained constant at 0.1 μM as described in Example 1. The results are shown in FIG. 2.

FIG. 2 shows the combination of methylnaltrexone and morphine decreased migration in a concentration-dependent manner, while methylnaltrexone alone did not affect migration.

Example 3: Endothelial Cell Migration induced by DAMGO

The drugs used in this study were [D-Ala 2, N-McPhe4, Gly5-ol] enkephalin or DAMGO (Sigma, St. Louis, MO); naloxone (Sigma, St. Louis, MO); N-methylnaltrexone bromide or methylnaltrexone (Mallinckrodt Specialty Chemicals, Phillipsburg, NJ). The endothelial cell migration assay was performed as previously described (9). Human dermal microvascular endothelial cells (Cell Systems, Kirkland, WA) were starved overnight in media containing 0.1% bovine serum albumin (BSA), harvested, resuspended into Dulbecco's Modified Eagle's media (DME) with 0.1% BSA, and plated on a semi-porous gelatinized membrane in a modified Boyden chamber (Nucleopore Corporation, Pleasanton, CA). Test substances were then added to the wells of the upper chamber and cells were allowed to migrate for four hours at 37° C.

Membranes were recovered, fixed, and stained and the number of cells that had migrated to the upper chamber per ten high power fields counted by a blinded observer. Background migration to DME + 0.1 % BSA was subtracted and the data reported as the number of cells migrated per 10 high power fields (400x). Each substance was tested in quadruplicate in each experiment and all experiments were repeated at least twice. The concentration of DAMGO was 1 μM, VEGF (R&D Systems, Minneapolis, MN) was used as a positive control at a concentration of 200 pg/mL. The optimal concentration for VEGF was determined previously by dose-response experiments (data not shown).

The results are shown in FIG. 3 which shows that methylnaltrexone and DAMGO decreased migration in a concentration-dependent manner. FIG. 4 illustrates similar results with naloxone and DAMGO. The inactive morphine metabolite M3G exerts no angiogenic activity while M6G known to act at the mu receptor exhibited a concentration dependent effect on angiogenesis (FIG. 5).

Example 4: Treatment of Human and Mammalian Subjects with Methylnaltrexone

In a first set of experiments, mice are induced to develop tumors by transformation, inbreeding or transplantation of tumor cells. Thirty-six mice, each bearing tumors having a volume of at least 60 mm^3, are randomly divided into three groups. The first group receives a control substance comprising neither an opioid nor an opioid antagonist. The second group receives an opioid, e.g. morphine administered orally at a dose of 0.5 mg/kg/day. The third group receives an opioid, e.g. morphine administered orally at a dose of 0.5 mg/kg/day, and the peripheral opioid antagonist methylnaltrexone, administered orally at a dose of 5 mg/kg/day.

The compounds are administered daily for a period of eight weeks. Differences in the rate of tumor growth, tumor size, a reduction in angiogenesis in the tumor and mortality of the mice between each of the groups are recorded. The results demonstrate a reduction in tumor growth and angiogenesis compared to controls or morphine alone.

In a second set of experiments, human cancer patients are enrolled in a study. Enrollees in the study are controlled for age, stage of disease, treatment types and genetic and familial factors. Participants are divided into two groups according to whether they are receiving opioids, e.g. morphine. The group receiving opioids is further randomly divided into two subgroups. One of the two subgroups receiving opioids receives a peripheral opioid antagonist, e.g., methylnaltrexone administered orally at a dose of 5 mg/kg/day for a period of eight weeks. The other of the two subgroups receives placebo for the same period. Differences in the rate of tumor growth, tumor size, a reduction in

angiogenesis in the tumor and mortality of the participants in each of the groups are recorded.

Example 5: Treatment of Human and Mammalian Subjects with Alvimopan

Mice that have been induced to develop tumors are subjected to the protocol as described in Example 3, except that the peripheral opioid antagonist is alvimopan. The results demonstrate a reduction in tumor growth and angiogenesis compared to controls or opioid alone.

Human cancer patients are enrolled in a study conducted as described in Example 4, except that the peripheral opioid antagonist is alvimopan.

Example 6: Therapies Comprising Co-administration of the Peripheral Opioid Antagonist Methylnaltrexone and Second Therapeutic Agent

In a first set of experiments, mice are induced to develop tumors by transformation, inbreeding or transplantation of tumor cells. Forty-eight mice, each bearing tumors having a volume of at least 60 mm^3, are randomly divided into six groups. The first group receives a control substance which does not comprise an opioid, an opioid antagonist, or an anticancer agent. The second group receives an opioid, e.g. morphine administered orally at a dose of 0.5 mg/kg/day. The third group receives an opioid, e.g. morphine administered orally at a dose of 0.5 mg/kg/day, and the peripheral opioid antagonist methylnaltrexone, administered orally at a dose of 5 mg/kg/day. The fourth group receives an opioid, e.g. morphine administered orally at a dose of 0.5 mg/kg/day, and the peripheral opioid antagonist methylnaltrexone administered orally at a dose of 5 mg/kg/day with an anticancer therapeutic agent, e.g. bevacizumab (Avastin) at a dose of 5 mg/kg every 14 days. The sixth group receives an opioid, e.g. morphine, at a dose of 0.5 mg/kg/day and an anticancer therapeutic agent, e.g. bevacizumab (Avastin) at a dose of 5 mg/kg every 14 days.

The compounds are administered daily for a period of eight weeks. Differences in the rate of tumor growth, tumor size, a reduction in angiogenesis in

the tumor and mortality of the mice in each of the groups are recorded. The results demonstrate an enhanced result (e.g., reduction in angiogenesis and tumor growth) for the groups administered the combination of opioid, opioid antagonist, and anticancer agent compared to the other groups.

In a second set of experiments, human cancer patients receiving an opioid, e.g. morphine, an anticancer therapeutic agent, e.g. bevacizumab (Avastin™) or both are enrolled in a study. Enrollees in the study are controlled for age, stage and type of disease, treatment types and genetic and familial factors. Participants receiving an opioid are randomly divided into first and second groups; participants receiving an anticancer therapeutic agent, e.g. bevacizumab (Avastin™) are randomly divided into third and fourth groups; participants receiving an opioid plus an anticancer agent, e.g. bevacizumab (Avastin™) are randomly divided into fifth and sixth groups. The first, third and fifth groups each receive a peripheral opioid antagonist, e.g., methylnaltrexone administered orally at a dose of 5 mg/kg/day for a period of eight weeks. The second, fourth and sixth groups receive placebo for the same period. Differences in the rate of tumor growth, tumor size, a reduction in angiogenesis in the tumor and mortality of the participants in each of the groups are recorded. The results demonstrate an enhanced result (e.g., reduction in angiogenesis and tumor growth) for the groups administered the combination of opioid, opioid antagonist, and anticancer agent compared to the other groups.

Example 7: Therapies Comprising Co-administration of the Peripheral Opioid Antagonist Alvimopan and Second Therapeutic Agent

Mice that have been induced to develop tumors are subjected to the protocol as described in Example 5, except that the peripheral opioid antagonist is alvimopan. The results demonstrate an enhanced result (e.g., reduction in angiogenesis and tumor growth) for the groups administered the combination of opioid, opioid antagonist, and anticancer agent compared to the other groups.

Human cancer patients are enrolled in a study conducted as described in Example 6, except that the peripheral opioid antagonist is alvimopan. The results

demonstrate an enhanced result(e.g., reduction in angiogenesis and tumor growth) for the groups administered the combination of opioid, opioid antagonist, and anticancer agent compared to the other groups.

Example 8: Effect of Opioid Antagonists on Endothelial Cell Migration/Proliferation

Cell culture and reagents- Human dermal microvascular endothelial cells (Cell Systems, Kirkland, WA) and human pulmonary microvascular endothelial cells (Clonetics, Walkersville, MD) were cultured as previously described in EBM-2 complete medium (Clonetics) at 37°C in a humidified atmosphere of 5% CO_2, 95% air, with passages 6-10 used for experimentation (Garcia, J. G., Liu, F., Verin, A. D., Birukova, A., Dechert, M. A., Gerthoffer, W. T., Bamberg, J. R., and English, D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. J Clin Invest, 108: 689-701, 2001). Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). Reagents for SDS-PAGE electrophoresis were purchased from Bio-Rad (Richmond, CA), Immobilon-P transfer membrane from Millipore (Millipore Corp., Bedford, MA). The drugs used in this study were [D-Ala^2, N-MePhe^4, Gly^5-ol] enkephalin or DAMGO (Sigma, St. Louis, MO); naloxone, morphine-3- glucuronide (M3G) and morphine-6-glucuronide (M6G) (Sigma, St. Louis, MO); N-methylnaltrexone bromide or methylnaltrexone (Mallinckrodt Specialty Chemicals, Phillipsburg, NJ), morphine (Baxter, Deerfield, Illinois). VEGF Receptor Tyrosine Kinase Inhibitor was purchased from Calbiochem (San Diego, CA). Mouse anti-RhoA antibody, mouse anti-phosphotyrosine antibody and rho binding domain (RBD)-conjugated beads were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-VEGF receptor 1 (Flt-I) and anti- VEGF receptor 2 (Flk-I) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-β-actin antibody was purchased from Sigma (St. Louis, MO). Secondary horseradish peroxidase (HRP)-labeled antibodies were purchased from Amersham Biosciences (Piscataway, NJ).

Immunoprecipitation and immunoblotting- Cellular materials were incubated with IP buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgC12, 1% Triton X-100, 0.1% SDS, 0.4 mM Na3VO4, 40 mM NaF, 50 µM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1 :250 dilution of

Calbiochem protease inhibitor mixture 3). The samples were then immunoprecipitated with anti-VEGF receptor 1 or anti-VEGF receptor 2 IgG followed by SDS-PAGE in 4-15% polyacrylamide gels, transfer onto ImmobilonTM membranes, and developed with specific primary and secondary antibodies. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

Determination of tyrosine phosphorylation of VEGF Receptors 1 and 2 – Solubilized proteins in IP buffer (see above) were immunoprecipitated with either rabbit anti-VEGF receptor 1 or rabbit anti-VEGF receptor 2 antibody followed by SDS-PAGE in 4-15% polyacrylamide gels and transfer onto ImmobilonTM membranes (Millipore Corp., Bedford, MA). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rabbit anti-VEGF receptor 1 antibody, rabbit anti-VEGF receptor 2 antibody or mouse anti-phosphotyrosine antibody followed by incubation with horseradish peroxidase (HRP)-labeled goat anti-rabbit or goat anti-mouse IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

Construction and transfection of siRNA against RhoA – The siRNA sequence targeting human against RhoA was generated using mRNA sequences from Genbank™ (gi:33876092). For each mRNA (or scramble), two targets were identified. Specifically, RhoA target sequence 1 (5'-AAGAAACTGGTGATTGTTGGT-3') (SEQ ID NO:1), RhoA target sequence 2 (5'-AAAGACATGCTTGCTCATAGT-3') (SEQ ID NO:2), scrambled sequence 1 (5'-AAGAGAAATCGAAACCGAAAA-3') (SEQ ID NO:3), and scramble sequence 2 (5'-AAGAACCCAATTAAGCGCAAG-3') (SEQ ID NO:4), were utilized. Sense and antisense oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). For construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). Human lung microvascular EC were then transfected with siRNA using siPORTamineTM as the transfection reagent (Ambion, TX) according to the protocol provided by Ambion. Cells (~ 40% confluent) were serum-starved for 1

hour followed by incubated with 3 μM (1.5 μM of each siRNA) of target siRNA (or scramble siRNA or no siRNA) for 6 hours in serum-free media. The serum-containing media was then added (1% serum final concentration) for 42 h before biochemical experiments and/or functional assays were conducted.

RhoA activation assay - After agonist and/or inhibitor treatment, EC are solubilized in solubilization buffer and incubated with rho bonding domain (RBD)-conjugated beads for 30 minutes at 4^0C. The supernatant is removed and the RBD-beads with the GTP-bound form of RhoA bound are washed extensively. The RBD beads are boiled in SDS-PAGE sample buffer and the bound RhoA material is run on SDS-PAGE, transferred to ImmobilonTM and immunpblotted with anti-RhoA antibody (Garcia et al 2001).

Human dermal microvascular EC migration assay - The endothelial cell migration assay was performed as previously described (Lingen MW. Endothelial cell migration assay: A quantitative assay for prediction of in vivo biology. In: DiPietro LA and Burns-Harring AL, editors. Wound Healing: Methods and Protocols. Totowa, NJ: Humana Press, Inc; 2002. p. 337-47). Human dermal microvascular endothelial cells (Cell Systems, Kirkland, WA) were starved overnight in media containing 0.1% bovine serum albumin (BSA), harvested, resuspended into Dulbecco's Modified Eagle's media (DME) with 0.1% BSA, and plated on a semi-porous gelatinized membrane in a modified Boyden chamber (Nucleopore Corporation, Pleasanton, CA). Test substances were then added to the wells of the upper chamber, and cells were allowed to migrate for 4 hr at 37^0C. Membranes were recovered, fixed, and stained and the number of cells that had migrated to the upper chamber per 10 high-power fields was counted by a blinded observer. Background migration to DME + 0.1% BSA was subtracted, and the data were reported as the number of cells migrated per 10 high-power fields (400x). Each substance was tested in quadruplicate in each experiment and all experiments were repeated at least twice. Vascular endothelial growth factor (VEGF, R&D Systems, Minneapolis, MN) was used as a positive control at a concentration of 200 pg/mL. The optimal concentration for VEGF was determined previously by dose-response experiments (data not shown).

Human pulmonary microvascular EC migration assay - Twenty-four Transwell™ units with 8 M pore size were used for monitoring in vitro cell migration. HPMVEC (~1 x 10^4 cells/well) were plated with various treatments

(100 nM MNTX, 10 μM VEGF Receptor Tyrosine Kinase Inhibitor or siRNA) to the upper chamber and various agonists were added to the lower chamber (100 nM MS, DAMGO or VEGF). Cells were allowed to migrate for 18 hours. Cells from the upper and lower chamber were quantitated using the CellTiter96™ MTS assay (Promega, San Luis Obispo, CA) and read at 492 nm. % migration was defined as the # of cells in the lower chamber % the number of cells in both the upper and lower chamber. Each assay was set up in triplicate, repeated at least five times and analyzed statistically by Student's t test (with statistical significance set at $P < 0.05$).

Human pulmonary microvascular EC proliferation assay – For measuring cell growth, HPMVEC [5×10^3 cells/well pretreated with various agents (100 nM MNTX, 10 μM VEGF Receptor Tyrosine Kinase Inhibitor or siRNA) were incubated with 0.2 mL of serum-free media containing various agonists (100 nM MS, DAMGO or VEGF) for 24 h at 37°C in 5%CO2/95% air in 96-well culture plates. The in vitro cell proliferation assay was analyzed by measuring increases in cell number using the CellTiter96™ MTS assay (Promega, San Luis Obispo, CA) and read at 492 nm. Each assay was set up in triplicate, repeated at least five times and analyzed statistically by Student's t test (with statistical significance set at $P < 0.05$).

Using the endothelial cell migration assay, it was found that MS caused a concentration-dependent increase in endothelial migration. Naloxone and MNTX alone had no effect on endothelial cell migration over a wide range of concentrations. This is demonstrated in representative photomicrographs and quantitatively (FIGS. 6 and 1, respectively). At clinically relevant concentrations of morphine, the magnitude of the effect was approximately 70% of that achieved by VEGF. Endothelial cell migration induced by morphine in concentrations as low as 10^{-7}M (FIG. 2). Morphine-based endothelial cell migration was attenuated by the mu opioid antagonists naloxone and MNTX (in doses as low as 10^{-8} μM) in a concentration-dependent fashion, strongly suggesting that endothelial cell migration is mediated by morphine's action on the mu opioid receptor (MOR). That the effect is via the MOR rather than other opioid receptors was confirmed

by our observations that the highly selective synthetic enkephalin mu agonist DAMGO also induced migration in a concentration dependent fashion. The effect of DAMGO was also blocked by MNTX (FIG. 3). That the inactive morphine metabolite M3G exerts no angiogenic activity, while M6G, known to act at the mu receptor, exhibits a concentration-dependent effect on angiogenesis, confirms our hypothesis that morphine's effect on the endothelium is mediated by mu receptors (McQuay et al. 1997) (FIG. 5).

In order to assess the mechanisms of opioid and MNTX-induced effects on angiogenesis, a well-characterized EC line was used, human pulmonary microvascular endothelial cells (HPMVEC). In agreement with the effects on human dermal microvascular EC, it was observed that MS, DAMGO and VEGF induce HPMVEC migration which is inhibited by MNTX (FIG. 7B). It was shown that MS, DAMGO and VEGF also stimulate HPMVEC proliferation which is attenuated by MNTX (FIG. 7A).

Considering the inhibitory effects of MNTX, a mu opioid receptor antagonist, on VEGF-induced EC proliferation and migration, the role of opioids on VEGF receptor transactivation was examined. FIG. 8A shows that MS and DAMGO induce tyrosine phosphorylation of both VEGF receptor 1 (Flt-1) and 2 (Flk-1) which is blocked by MNTX. Further, MNTX attenuates the tyrosine phosphorylation of VEGF receptors 1 and 2 induced by VEGF. These results indicate that opioids induce VEGF receptor transactivation.

In order to address if VEGF receptor tyrosine kinase activity is required for opioid-induced angiogenesis, EC were pre-treated with a VEGF receptor 1 and 2 tyrosine kinase inhibitor and measured opioid-induced EC proliferation and migration (FIG. 8B). The results indicate that the tyrosine kinase activity of VEGF receptors is important in opioid-induced EC angiogenic functions.

One important signaling molecule involved in angiogenesis is the small G-protein, RhoA (Aepfelbacher et al. 1997; Cascone et al. 2003; Hoang et al. 2004; Liu and Senger 2004). It was observed that MS, DAMGO and VEGF stimulate RhoA activation which is inhibited by MNTX (Figure 9A). Further, VEGF receptor transactivation is important for opioid-induced RhoA activation (Figure

9B). Silencing RhoA expression blocks opioid and VEGF-induced EC proliferation and migration (FIG. 10). These results indicate the pivotal role of RhoA activation on agonist-induced EC angiogenic activity.

Taken as a whole these findings suggest a model in which the peripheral mu opioid receptor antagonist, MNTX, attenuates opioid and VEGF-induced VEGF receptor and RhoA activation. This attenuation is important for the inhibitory role of MNTX on opioid and VEGF-mediated angiogenesis (FIG. 11).

Example 9: Methylnaltrexone inhibits SIP, VEGF and PDGF-induced angiogenesis: Role of receptor transactivation

Assays were conducted according to the procedure similar to that described in Examples 1-3. It was observed that SIP, VEGF, PDGF, morphine and DAMGO induced proliferation (FIG. 12) (as measured by the colorimetric CellTiter™ (Promega) MTS assay) and migration (Figure 13) (as measured by the Transwell™ (Costar) permeable membrane filter assay (8 μm pore diameter)) of EC which were inhibited by pretreatment with MNTX (0.1 μM, 1 hour). Silencing mu opioid receptor expression (siRNA) blocks morphine and DAMGO-induced EC proliferation (FIG. 14) and migration (FIG. 15) while also significantly inhibiting SIP, VEGF and PDGF-induced EC proliferation (FIG. 14) and migration (FIG. 15). Immunoprecipitation followed by immunoblot analyses indicate that SIP, VEGF and PDGF treatment of EC induced serine/threonine phosphorylation of the mu opioid receptor (FIG. 16) (indicating receptor transactivation) and activation of the cytoskeletal regulatory small G-protein, RhoA (FIG. 17). Further, morphine and DAMGO treatment of EC induced tyrosine phosphorylation of the VEGF receptor (Figure 18), PDGF receptor (FIG. 18) and S1 P3 receptor (FIG. 19) along with RhoA activation. MNTX pretreatment of EC attenuated morphine, DAMGO, SIP, VEGF and PDGF induced receptor phosphorylation events and RhoA activation. Finally, silencing RhoA expression (siRNA) blocked agonist-induced EC proliferation (FIG. 20) and migration (FIG. 21). Taken together, these results indicate that MNTX inhibits agonist induced EC proliferation and migration via inhibition of receptor

phosphorylation/transactivation and subsequent inhibition of RhoA activation (FIG. 22). These results suggest that MNTX inhibition of angiogenesis can be a useful therapeutic intervention for cancer treatment.

Example 10: Methylnaltrexone and antiproliferative compounds synergistically inhibit VEGF-induced proliferation and migration

Assays were conducted according to the procedure similar to that described in Examples 1-3. It was observed that methylnaltrexone and 5-FU synergistically inhibit VEGF induced proliferation of endothelial cells.(Figure 23). It was likewise observed that methylnaltrexone and Bevacizumab synergistically inhibit VEGF induced migration of endothelial cells.(Figure 24).

Example 11: Effects of MNTX on various cancer cell lines

The antiproliferative effects of methylnaltrexone alone and in combination with another anti-cancer drug were evaluated. In general, human cancer cells were allowed to grow under suitable conditions known in the art. The cells were then treated with MNTX and/or 5-fluorouracil (5-FU) or vehicle, for 2-3 days, and the cells were counted. Vehicle-treated cells were taken as controls, and as such, the cell numbers were taken as 100% proliferation. Cell numbers of treated groups were taken as a percentage of control.

The effects of MNTX on SW 480 human colorectal cancer cell line were evaluated. As shown in Figure 25, it was observed that MNTX itself possesses antiproliferation activity in SW 480 cells (**, $P < 0.01$ compared to control). In addition, MNTX enhanced 5-FU's tumoricidal effect (*, $P< 0.05$ compared to 5-FU 10 uM only, approx. IC50 for this cell line). As shown in Figures 26, 27, and 28, respectively, similar results were obtained in HCT116 human colorectal cancer cell line, MCF-7 human breast cancer cell line, and non-small cell lung cancer cell (NSLCC) line.

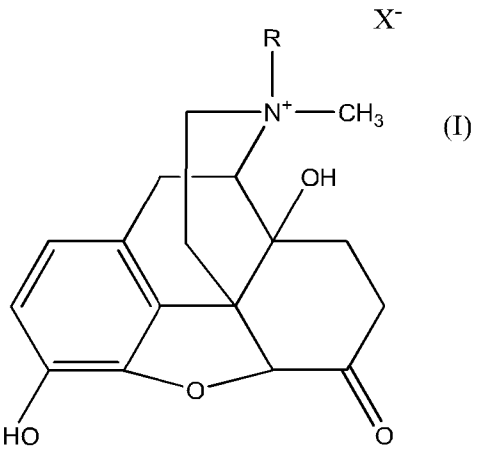
In summary, the present invention provides methods of attenuating endothelial cell migration and/or proliferation associated with angiogenesis and/or enhancing endothelial cell barrier function in tissue or an organ of a subject in

need therefor by administering one or more opioid antagonists, especially peripheral opioid antagonists, in an effective amount to the patient to inhibit the migration and/or proliferation and angiogenesis, and/or improve barrier function. The methods of the present invention may also involve administering a peripheral opioid antagonist to a patent receiving opioid treatment. Especially suitable may be a mu peripheral opioid antagonist. The present invention also provides methods of co-administering an opioid and a peripheral opioid antagonist to a subject in need therefore. The peripheral opioid antagonist may also be co-administered with an anticancer agent, as may the combination of the opioid and peripheral opioid antagonist be co-administered with an anticancer agent.

While the present invention has now been described and exemplified with some specificity, those skilled in the art will appreciate the various modifications, including variations, additions, and omissions that may be made in what has been described. Accordingly, it is intended that these modifications also be encompassed by the present invention and that the scope of the present invention be limited solely by the broadest interpretation that lawfully can be accorded the appended claims.

CLAIMS

1. Use of a combination of a peripheral opioid antagonist and 5-fluorouracil (5-FU) for treating a disorder characterized by hyperproliferation of endothelial cells in a subject, wherein the peripheral opioid antagonist is a compound of formula (I):



(I)

wherein R is alkyl, alkenyl, alkynyl, aryl, cycloalkyl-substituted alkyl or aryl-substituted alkyl, and X^- is a chloride, bromide, iodide or methylsulfate anion, wherein the disorder is cancer, sickle cell disease, vascular wounds, proliferative retinopathy, unwanted endothelial cell proliferation in the kidneys or the lung, neovascular disease of the eye, psoriasis, rheumatoid arthritis, diabetes, atherosclerosis, retrolental fibroplasia, neovascular glaucoma, thyroid hyperplasia, Grave's disease, tissue transplantation, chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial with pericarditis, or pleural effusion.

2. The use of claim 1, wherein the compound of formula (I) is methylnaltrexone.

3. The use of claim 1 or 2, wherein the peripheral opioid antagonist is for use simultaneously or sequentially with the 5-fluorouracil (5-FU).

4. The use of any one of claims 1 to 3, wherein the cells are vascular endothelial cells.

5. The use of any one of claims 1 to 4, wherein the disorder is cancer.

6. The use of claim 5, wherein the cancer is colon cancer, breast cancer or non-small cell lung cancer.

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7. The use of any one of claims 1 to 4, wherein the neovascular disease of the eye is diabetic retinopathy, neovascular glaucoma, retinopathy of prematurity, or age-related macular degeneration.

8. The use of any one of claims 1 to 7, wherein the subject is taking concurrent opioid therapy.

9. The use of any one of claims 1 to 7, wherein the subject is not taking concurrent opioid therapy.

10. The use of any one of claims 1 to 7, wherein the subject is taking concurrent chronic opioid therapy.

11. The use of any one of claims 1 to 7, wherein the subject is not taking concurrent chronic opioid therapy.

12. The use of any one of claims 1 to 11, wherein the peripheral opioid antagonist is for use such that the subject has effective circulating blood plasma levels of peripheral opioid antagonist continuously for at least 1 week.

13. The use of any one of claims 1 to 11, wherein the hyperproliferation is exogenously opioid-induced hyperproliferation.

14. The use of any one of claims 1 to 11, wherein the hyperproliferation is agonist-induced hyperproliferation.

15. The use of claim 14, wherein the agonist is VEGF, platelet-derived growth factor (PDGF), or sphingosine 1-phosphate (SIP).

16. The use of claim 1, wherein the tissue transplantation is corneal transplantation.

17. Use of a combination of a peripheral opioid antagonist and 5-fluorouracil (5-FU) for treating a disorder characterized by hyperproliferation of cells overexpressing mu-opioid receptors, wherein the opioid antagonist is methylnaltrexone, and wherein the disorder is cancer, sickle cell disease, vascular wounds, proliferative retinopathy, unwanted endothelial cell proliferation in the kidneys or the lung, neovascular disease of the eye, psoriasis, rheumatoid arthritis, diabetes, atherosclerosis, retrolental fibroplasia, neovascular glaucoma, thyroid hyperplasia, Grave's disease, tissue transplantation,

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chronic inflammation, lung inflammation, nephrotic syndrome, preeclampsia, ascites, pericardial with pericarditis, or pleural effusion.

18. The use of claim 17, wherein the tissue transplantation is corneal transplantation.

19. The use of claim 17, wherein the neovascular disease of the eye is diabetic retinopathy, neovascular glaucoma, retinopathy of prematurity, or age-related macular degeneration.

20. Use of a combination of a peripheral opioid antagonist and 5-fluorouracil (5-FU) for inhibiting proliferation of cancer cells, wherein the peripheral opioid antagonist is methylnaltrexone.

21. Use of a combination of a peripheral opioid antagonist and 5-fluorouracil (5-FU) for treating a disorder which is cancer, sickle cell anemia, ocular neovascular diseases, diabetes, ocular retinopathy, or hyperproliferations in the kidneys, eyes, or lung.

22. The use of claim 21, where in the peripheral opioid antagonist is a quaternary morphinan or a N-substituted piperidine.

23. The use of claim 21, wherein the quaternary morphinan is a quaternary noroxymorphone.

24. The use of claim 21, wherein the N-substituted piperidine is a piperidine-N-alkylcarboxylate.

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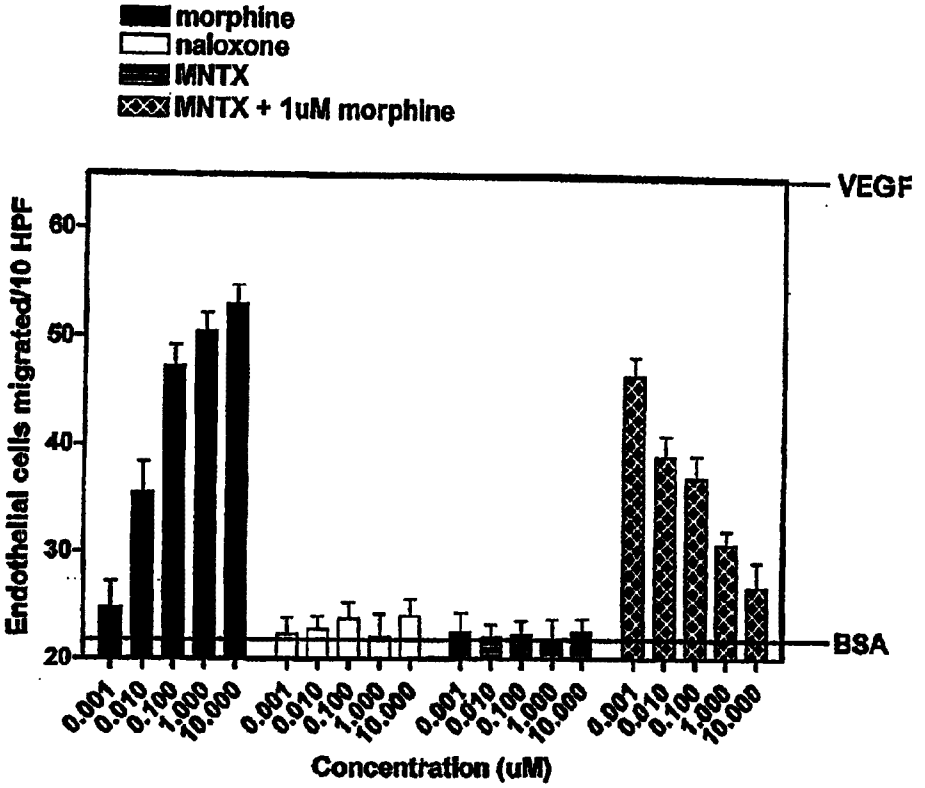

morphine
naloxone
MNTX
MNTX + 1uM morphine
VEGF
BSA
Endothelial cells migrated/10 HPF
60
50
40
30
20
0.001
0.010
0.100
1.000
10.000
Concentration (uM)

FIG. 1

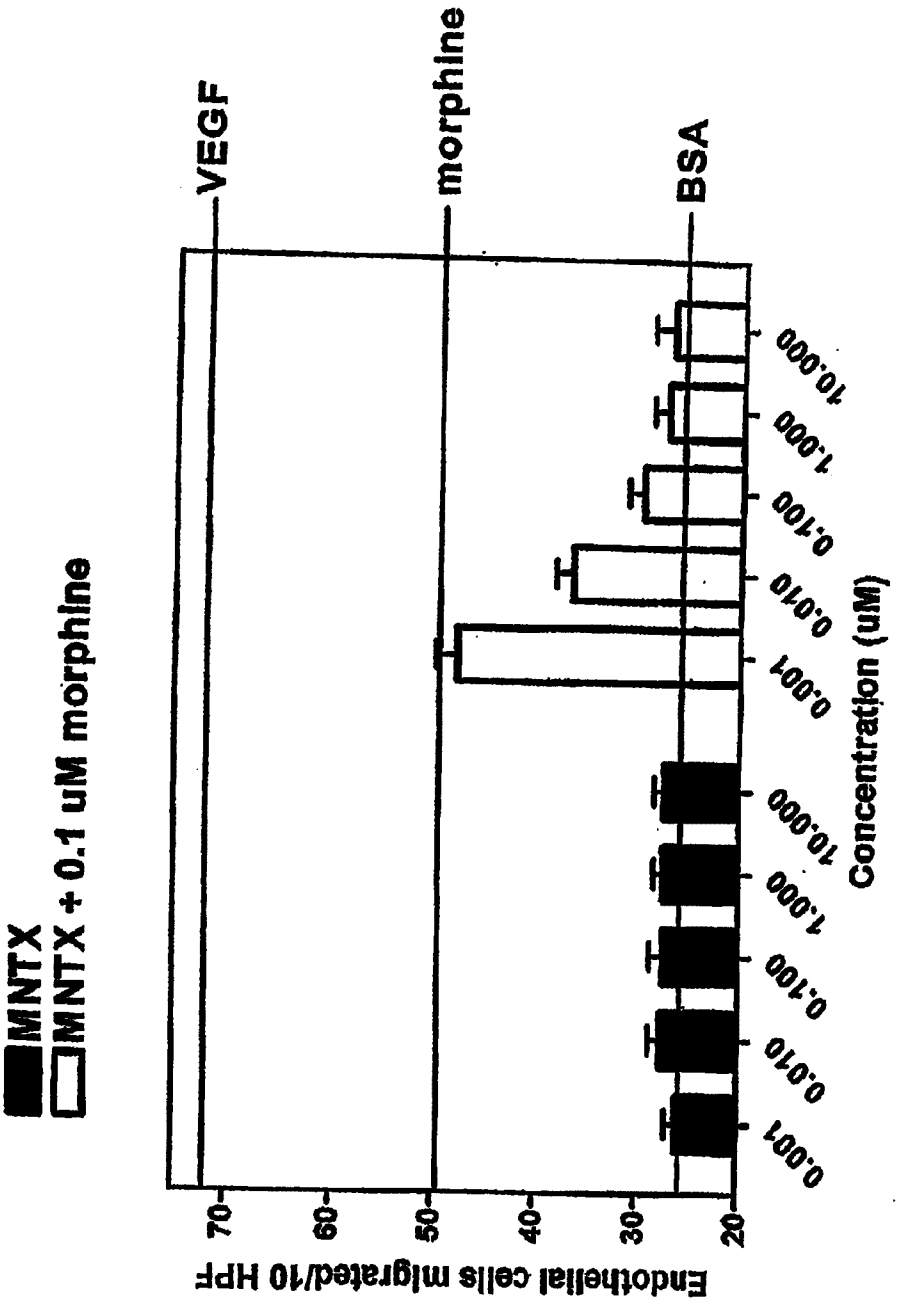

VEGF
morphine
BSA
MNTX
MNTX + 0.1 uM morphine
Concentration (uM)
10,000
1,000
0,100
0,010
0,001
10,000
1,000
0,100
0,010
0,001
Endothelial cells migrated/10 HPF
70
60
50
40
30
20

FIG. 2

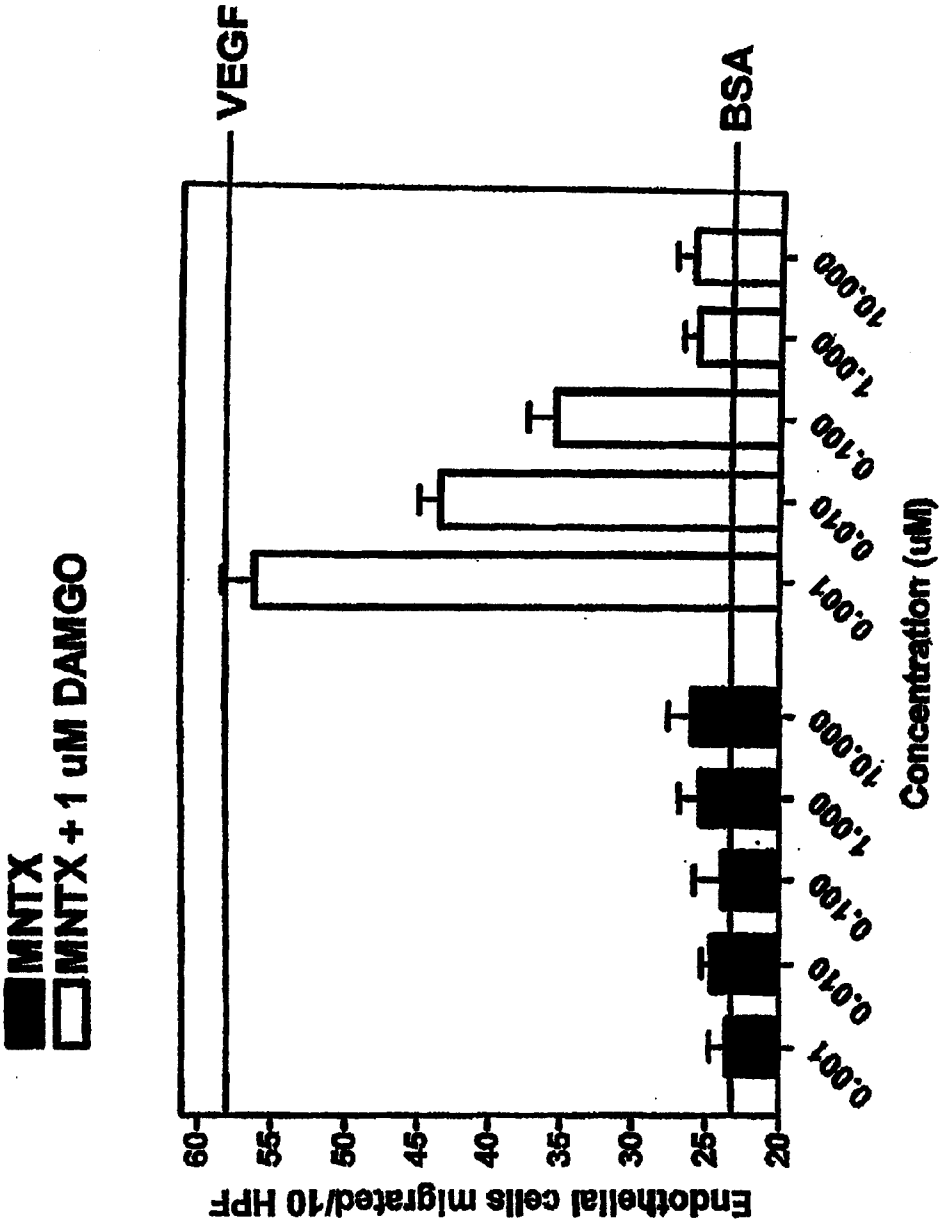

VEGF
BSA
MNTX
MNTX + 1 uM DAMGO
Endothelial cells migrated/10 HPF
60
55
50
45
40
35
30
25
20
Concentration (uM)
10.000
1.000
0.100
0.010
0.001
10.000
1.000
0.100
0.010
0.001

FIG. 3

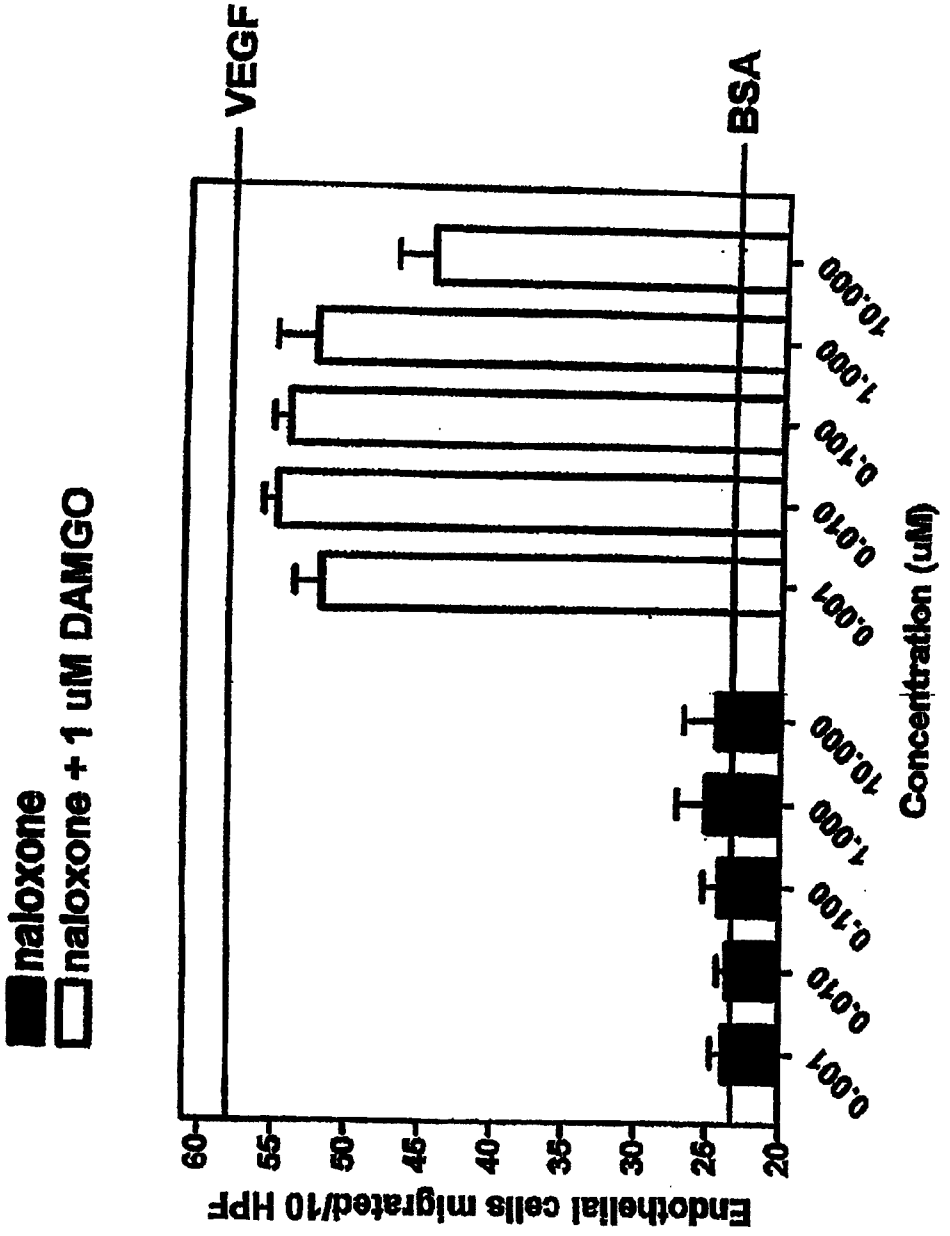

VEGF
BSA
naloxone
naloxone + 1 uM DAMGO
Endothelial cells migrated/10 HPF
60
55
50
45
40
35
30
25
20
10,000
1,000
0,100
0,010
0,001
Concentration (uM)

FIG. 4

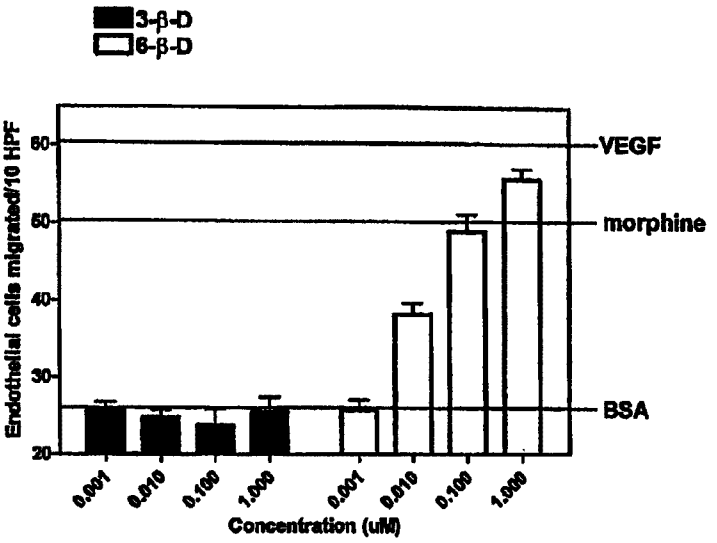

3-β-D
6-β-D
Endothelial cells migrated/10 HPF
60
50
40
30
20
VEGF
morphine
BSA
0.001
0.010
0.100
1.000
0.001
0.010
0.100
1.000
Concentration (uM)

FIG. 5

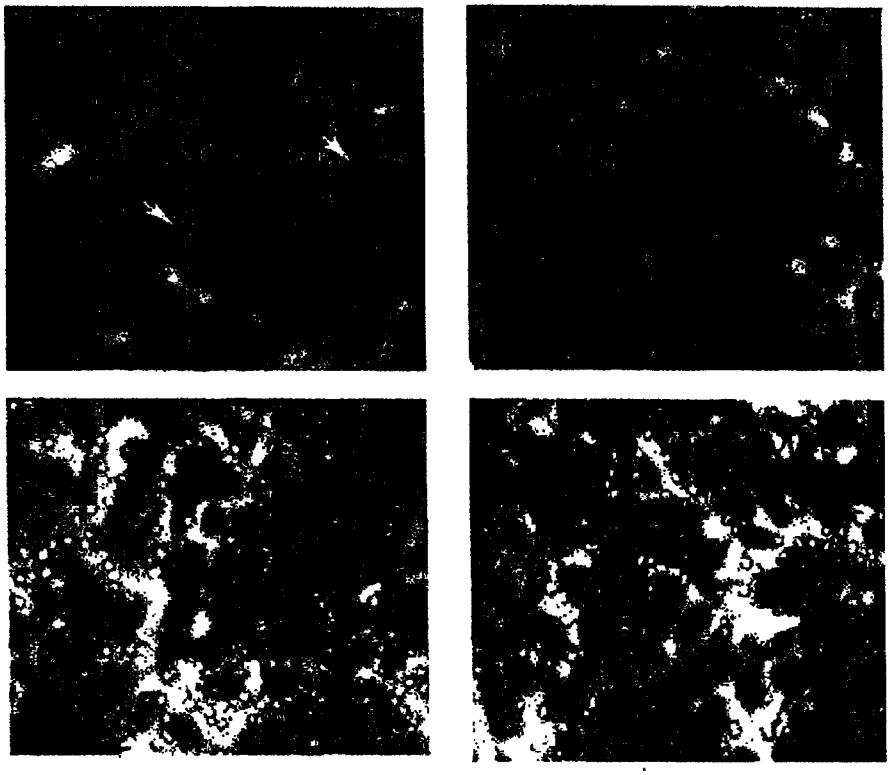


FIG. 6

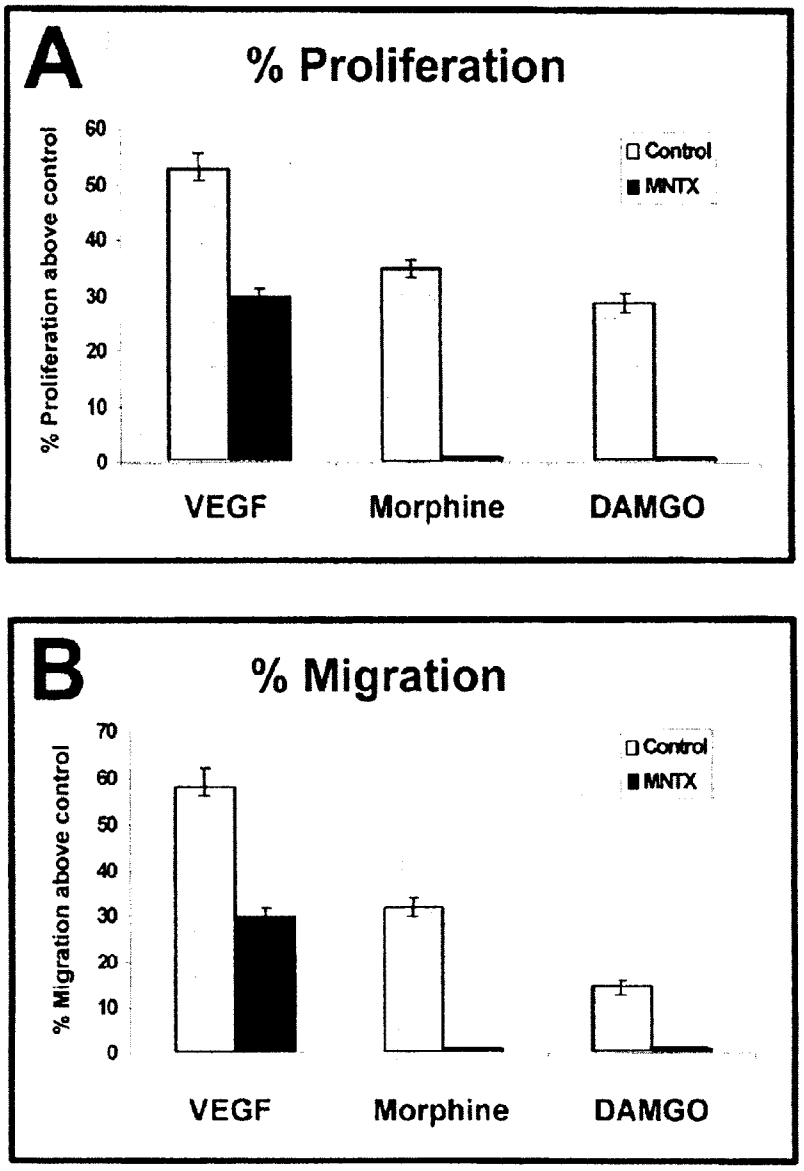
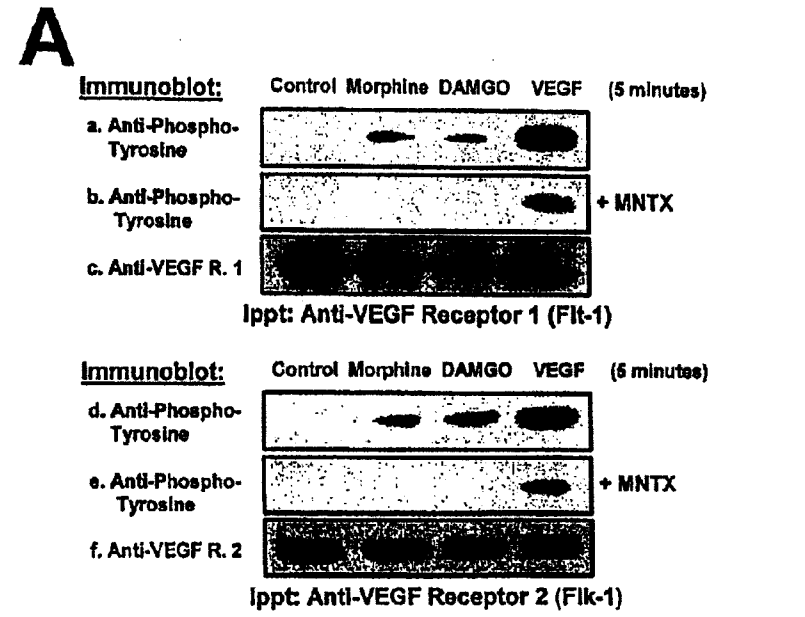

A
% Proliferation
% Proliferation above control
60
50
40
30
20
10
0
Control
MNTX
VEGF
Morphine
DAMGO
B
% Migration
% Migration above control
70
60
50
40
30
20
10
0
Control
MNTX
VEGF
Morphine
DAMGO

FIG. 7


A
Immunoblot:
Control Morphine DAMGO VEGF (5 minutes)
a. Anti-Phospho-Tyrosine
b. Anti-Phospho-Tyrosine
+ MNTX
c. Anti-VEGF R. 1
Ippt: Anti-VEGF Receptor 1 (Flt-1)
Immunoblot:
Control Morphine DAMGO VEGF (5 minutes)
d. Anti-Phospho-Tyrosine
e. Anti-Phospho-Tyrosine
+ MNTX
f. Anti-VEGF R. 2
Ippt: Anti-VEGF Receptor 2 (Flk-1)

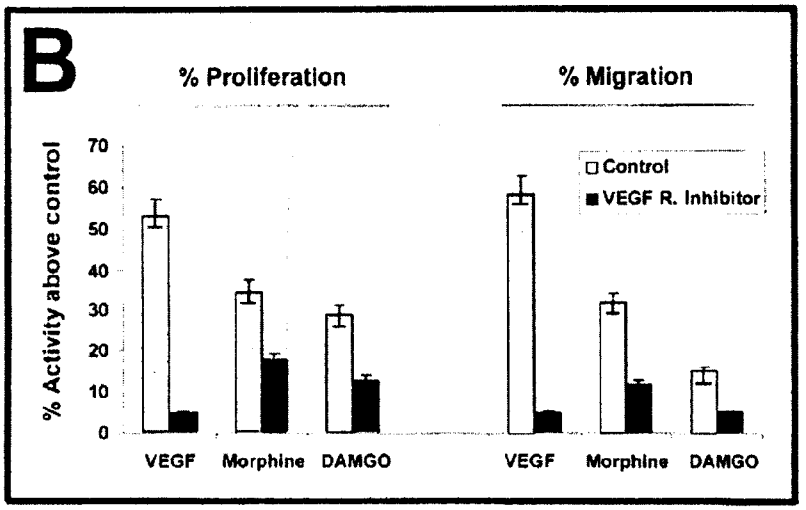

B
% Proliferation
% Migration
% Activity above control
70
60
50
40
30
20
10
0
Control
VEGF R. Inhibitor
VEGF
Morphine
DAMGO
VEGF
Morphine
DAMGO

FIG. 8

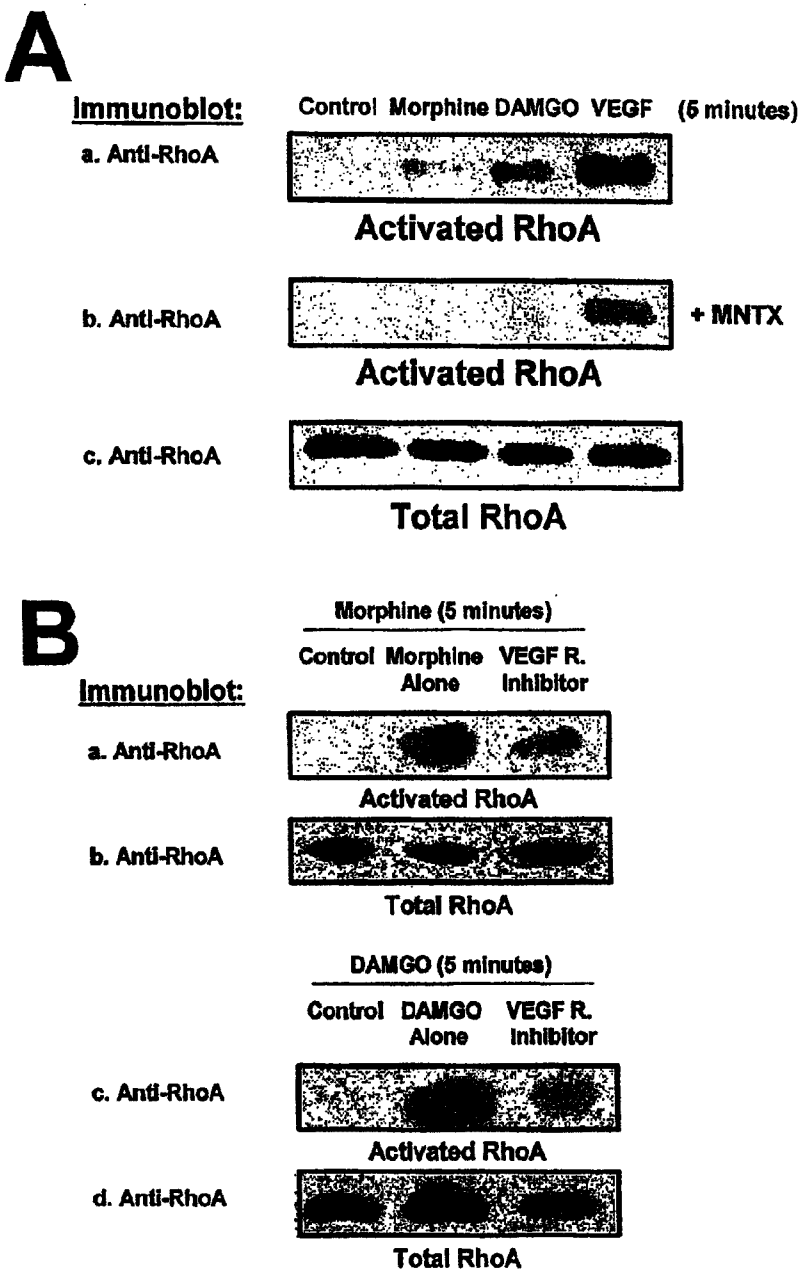

A
Immunoblot:
Control Morphine DAMGO VEGF (5 minutes)
a. Anti-RhoA
Activated RhoA
b. Anti-RhoA
+ MNTX
Activated RhoA
c. Anti-RhoA
Total RhoA
B
Immunoblot:
Morphine (5 minutes)
Control Morphine Alone
VEGF R. Inhibitor
a. Anti-RhoA
Activated RhoA
b. Anti-RhoA
Total RhoA
DAMGO (5 minutes)
Control DAMGO Alone
VEGF R. Inhibitor
c. Anti-RhoA
Activated RhoA
d. Anti-RhoA
Total RhoA

FIG. 9

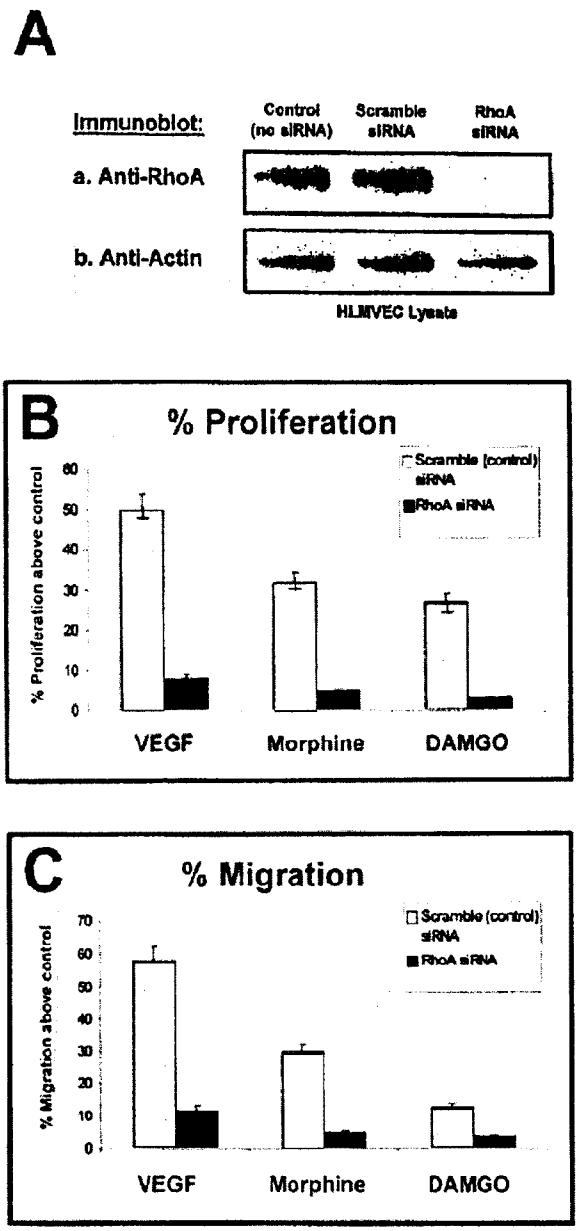

A
Immunoblot:
Control (no siRNA)
Scramble siRNA
RhoA siRNA
a. Anti-RhoA
b. Anti-Actin
HLMVEC Lysate
B
% Proliferation
Scramble (control) siRNA
RhoA siRNA
% Proliferation above control
0
10
20
30
40
50
60
VEGF
Morphine
DAMGO
C
% Migration
Scramble (control) siRNA
RhoA siRNA
% Migration above control
0
10
20
30
40
50
60
70
VEGF
Morphine
DAMGO

FIG. 10

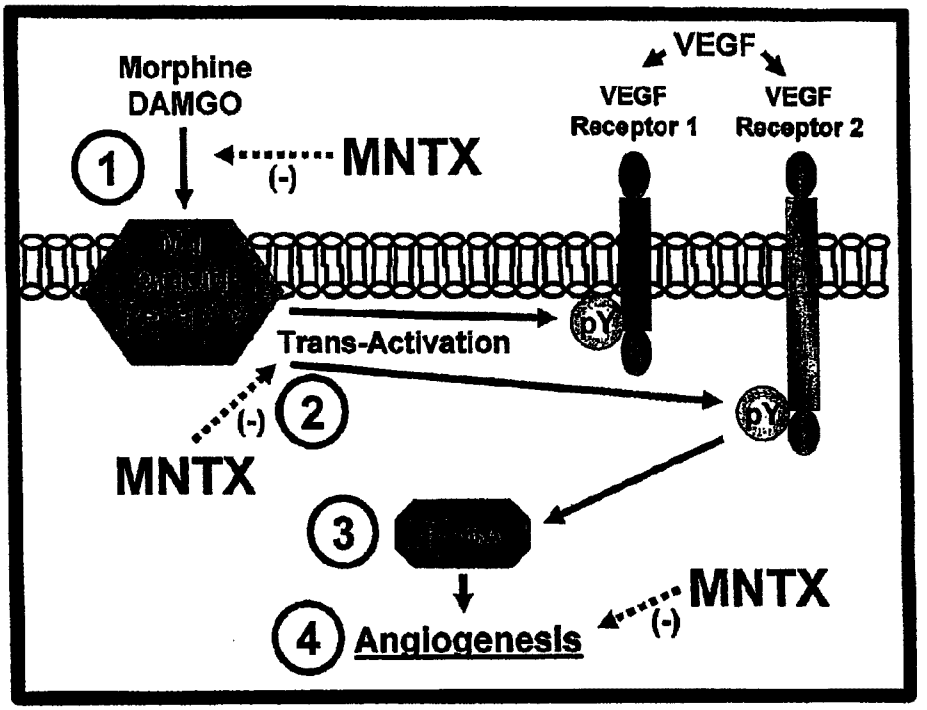

Morphine
DAMGO
VEGF
VEGF Receptor 1
VEGF Receptor 2
1
(-)
MNTX
Trans-Activation
pY
pY
(-)
2
MNTX
3
4
Angiogenesis
(-)
MNTX

FIG. 11

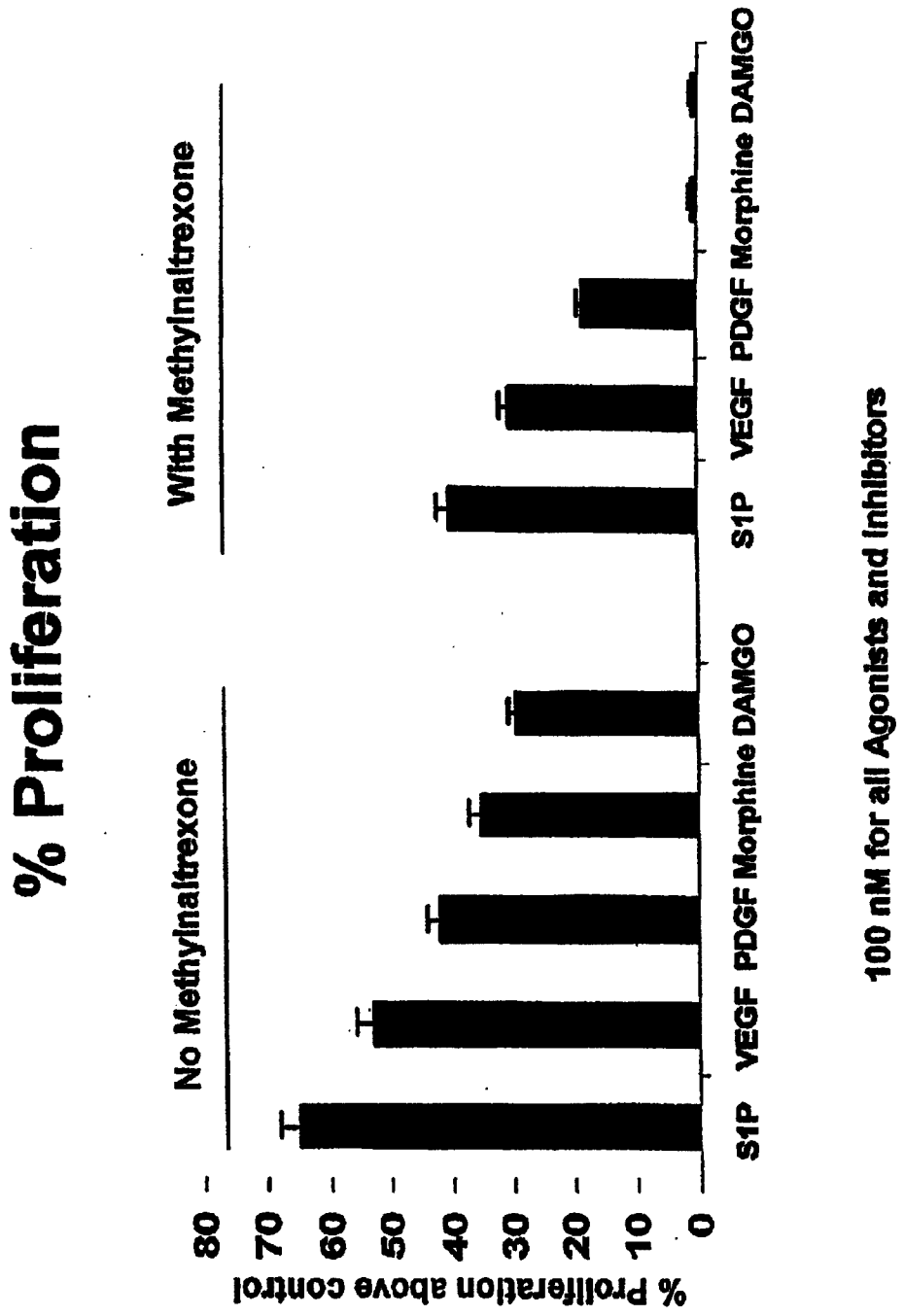

% Proliferation
No Methylnaltrexone
With Methylnaltrexone
% Proliferation above control
80
70
60
50
40
30
20
10
0
S1P
VEGF
PDGF
Morphine
DAMGO
S1P
VEGF
PDGF
Morphine
DAMGO
100 nM for all Agonists and Inhibitors

FIG. 12

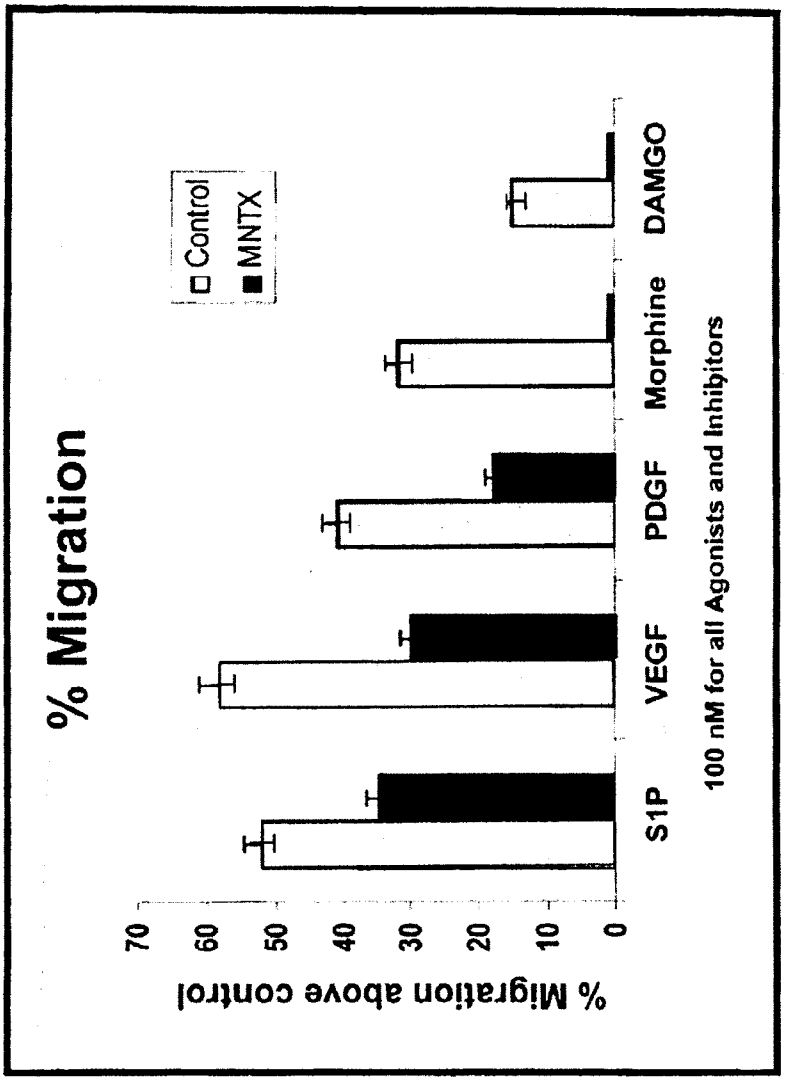

% Migration
Control
MNTX
% Migration above control
0
10
20
30
40
50
60
70
S1P
VEGF
PDGF
Morphine
DAMGO
100 nM for all Agonists and Inhibitors

FIG. 13

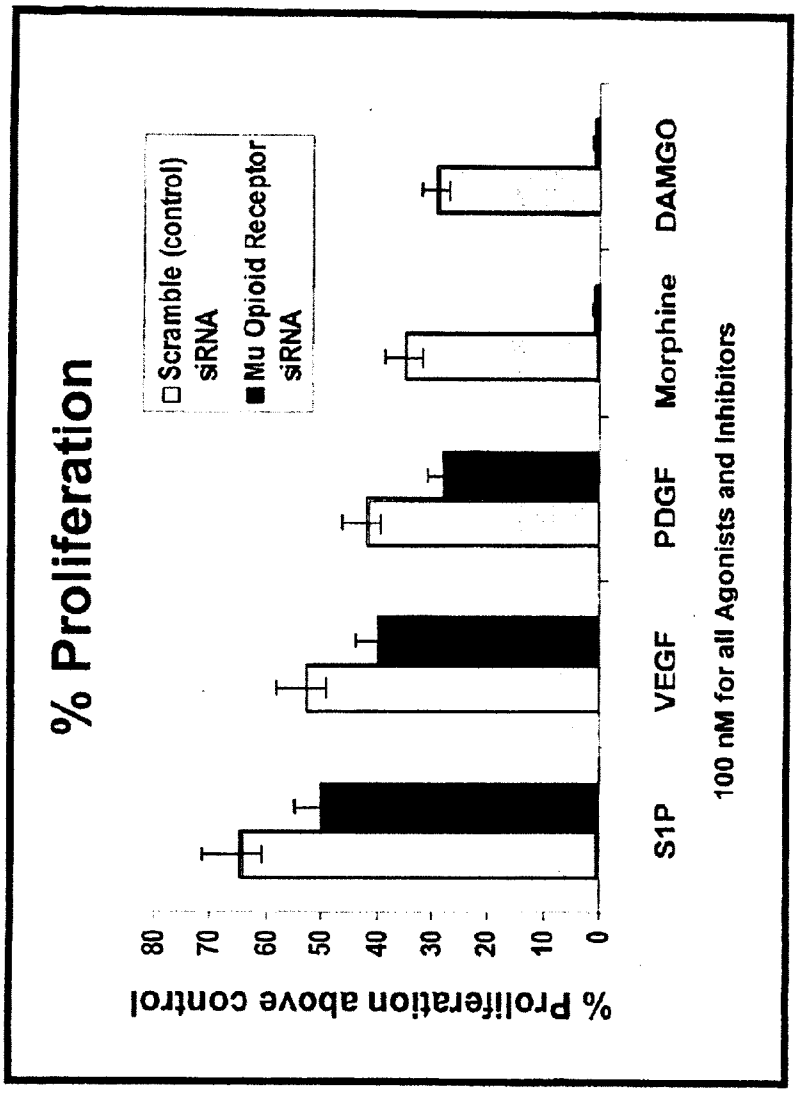

% Proliferation
Scramble (control) siRNA
Mu Opioid Receptor siRNA
% Proliferation above control
80
70
60
50
40
30
20
10
0
S1P
VEGF
PDGF
Morphine
DAMGO
100 nM for all Agonists and Inhibitors

FIG. 14

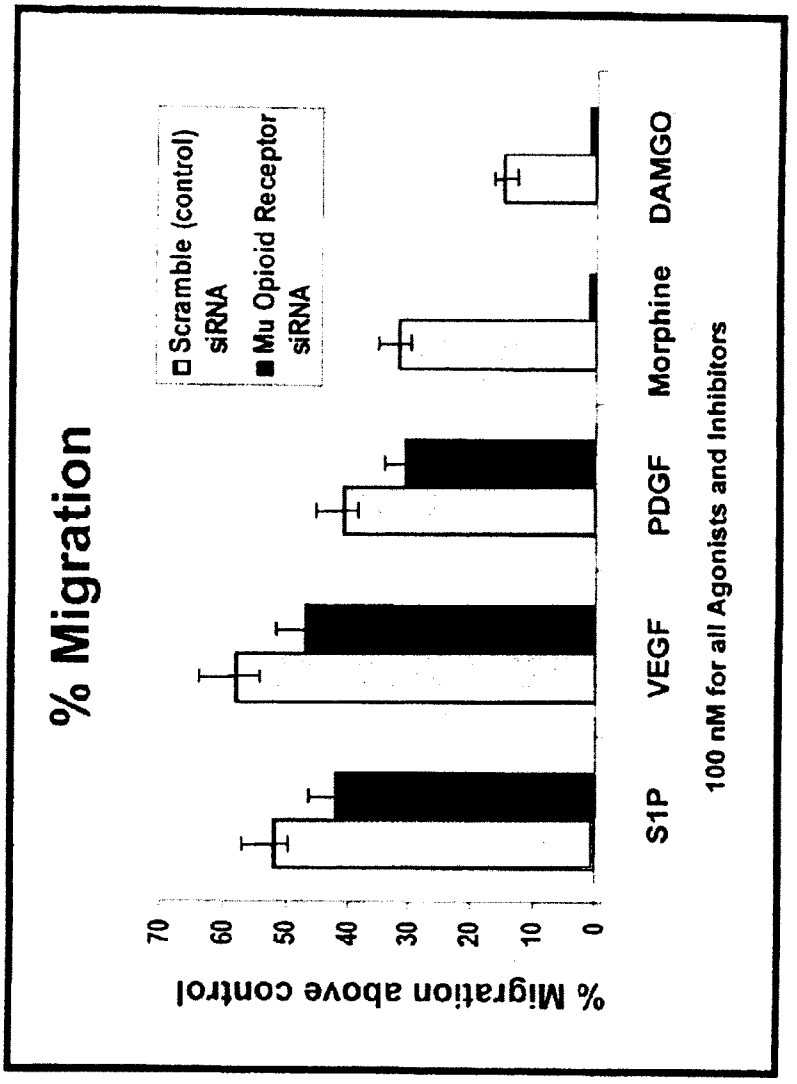

% Migration
Scramble (control) siRNA
Mu Opioid Receptor siRNA
% Migration above control
70
60
50
40
30
20
10
0
S1P
VEGF
PDGF
Morphine
DAMGO
100 nM for all Agonists and Inhibitors

FIG. 15

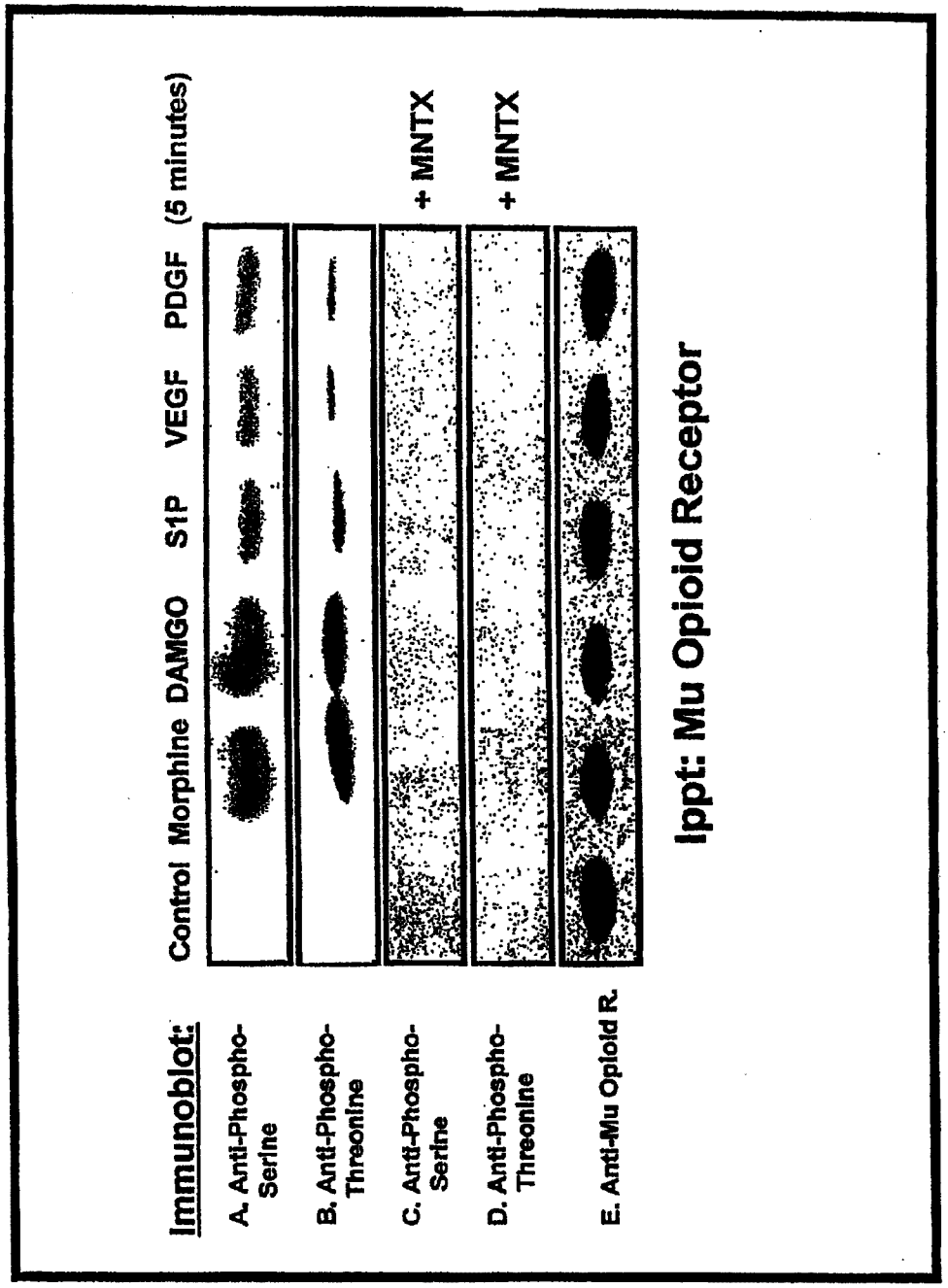

Immunoblot:
Control
Morphine
DAMGO
S1P
VEGF
PDGF
(5 minutes)
A. Anti-Phospho-Serine
B. Anti-Phospho-Threonine
C. Anti-Phospho-Serine
+ MNTX
D. Anti-Phospho-Threonine
+ MNTX
E. Anti-Mu Opioid R.
Ippt: Mu Opioid Receptor

FIG. 16

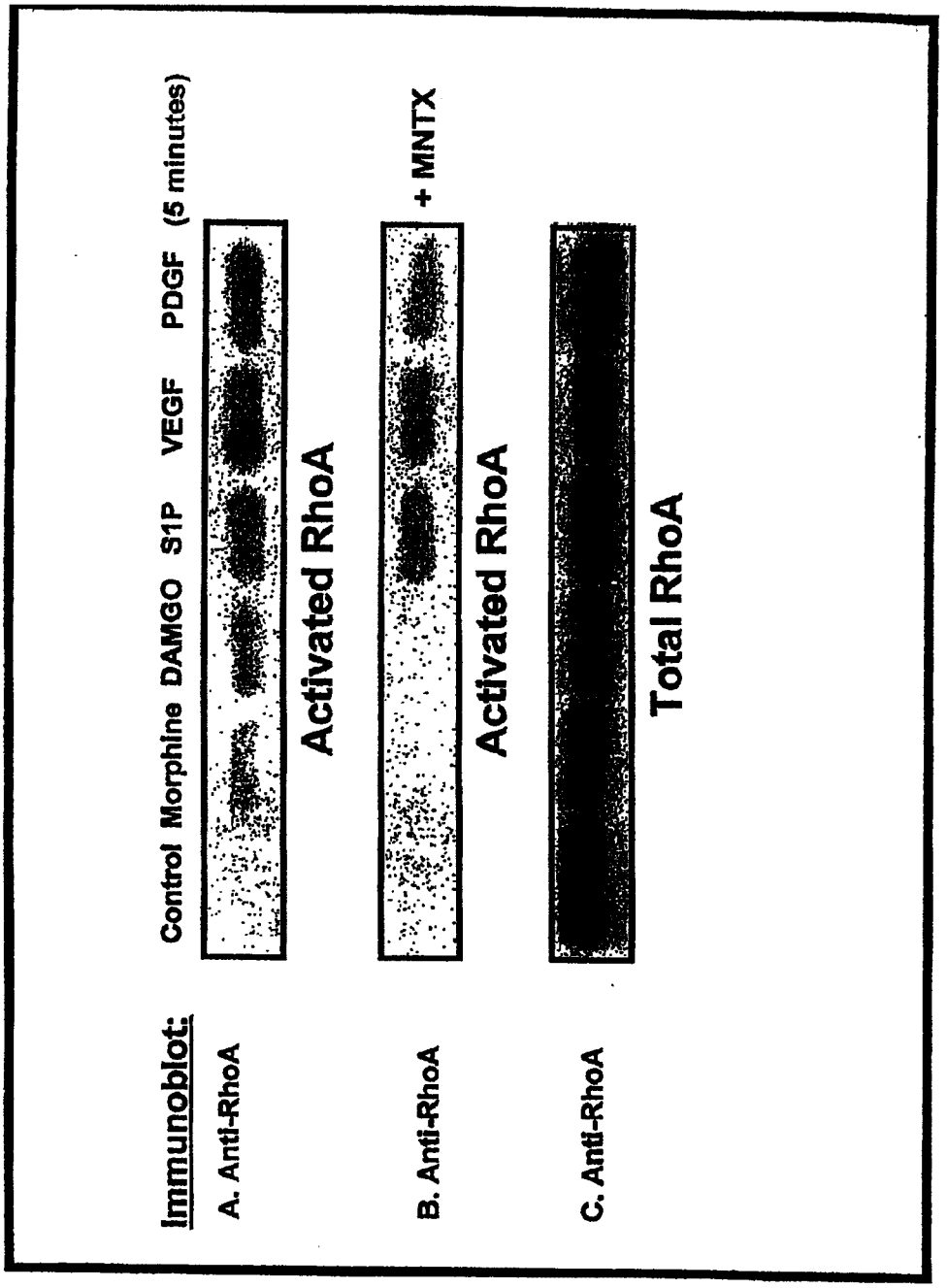

Immunoblot:
A. Anti-RhoA
B. Anti-RhoA
C. Anti-RhoA
Control Morphine DAMGO S1P VEGF PDGF (5 minutes)
+ MNTX
Activated RhoA
Activated RhoA
Total RhoA

FIG. 17

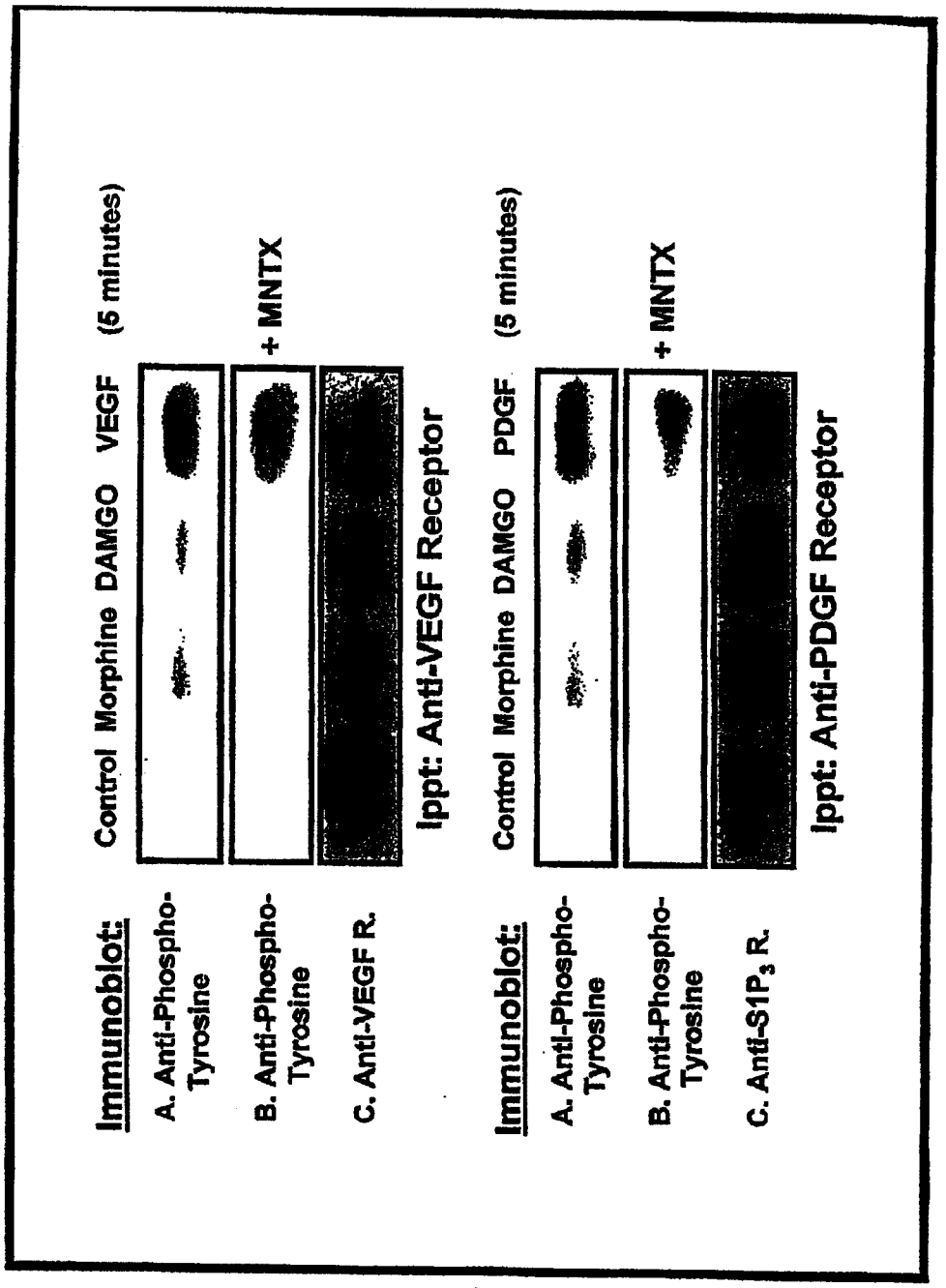

Immunoblot:
A. Anti-Phospho-Tyrosine
B. Anti-Phospho-Tyrosine
C. Anti-VEGF R.
Control
Morphine
DAMGO
VEGF
(5 minutes)
+ MNTX
Ippt: Anti-VEGF Receptor
Immunoblot:
A. Anti-Phospho-Tyrosine
B. Anti-Phospho-Tyrosine
C. Anti-S1P3 R.
Control
Morphine
DAMGO
PDGF
(5 minutes)
+ MNTX
Ippt: Anti-PDGF Receptor

FIG. 18

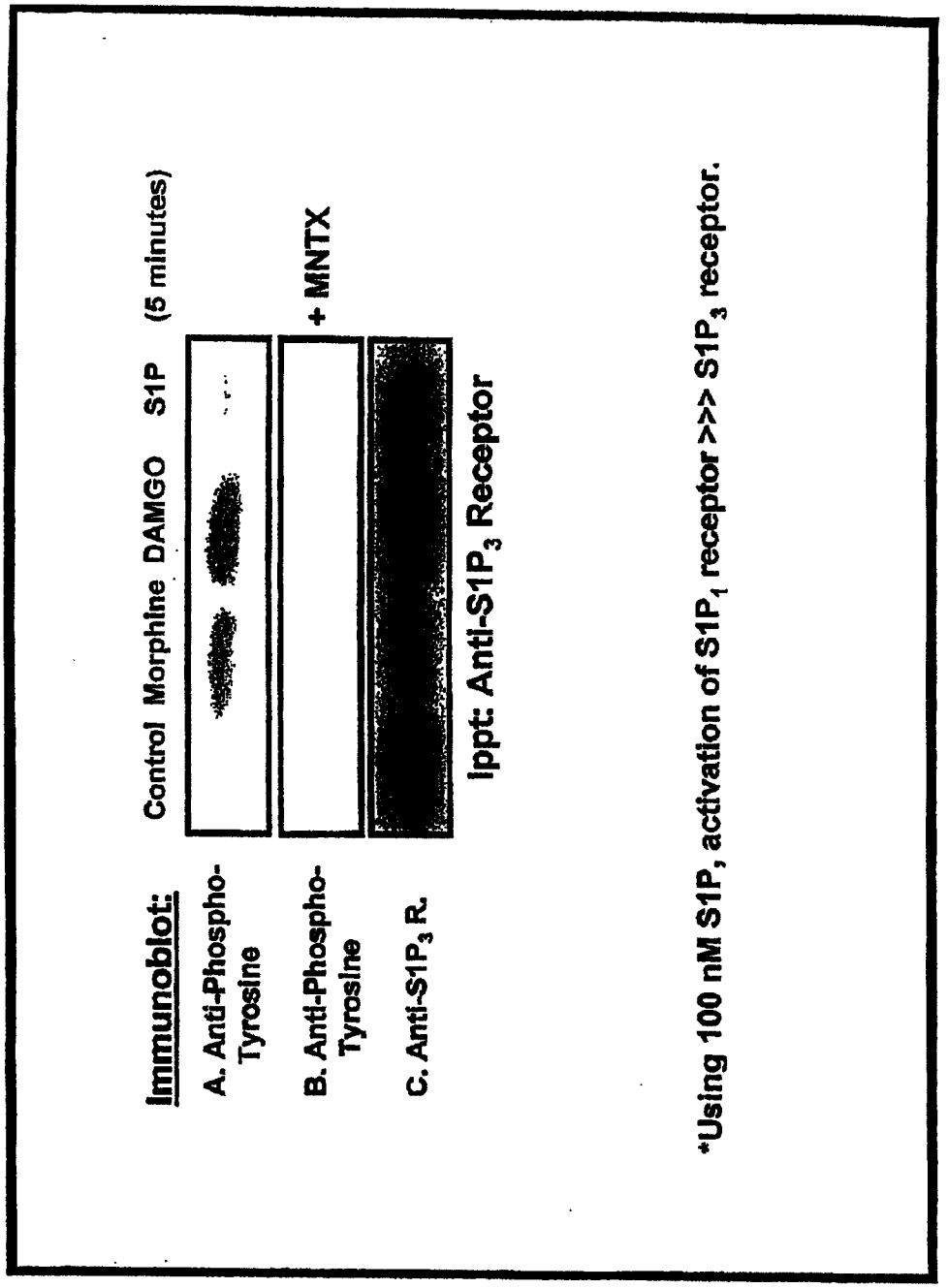

Immunoblot:
A. Anti-Phospho-Tyrosine
B. Anti-Phospho-Tyrosine
C. Anti-S1P3 R.
Control Morphine DAMGO S1P (5 minutes)
+ MNTX
Ippt: Anti-S1P3 Receptor
*Using 100 nM S1P, activation of S1P1 receptor >>> S1P3 receptor.

FIG. 19

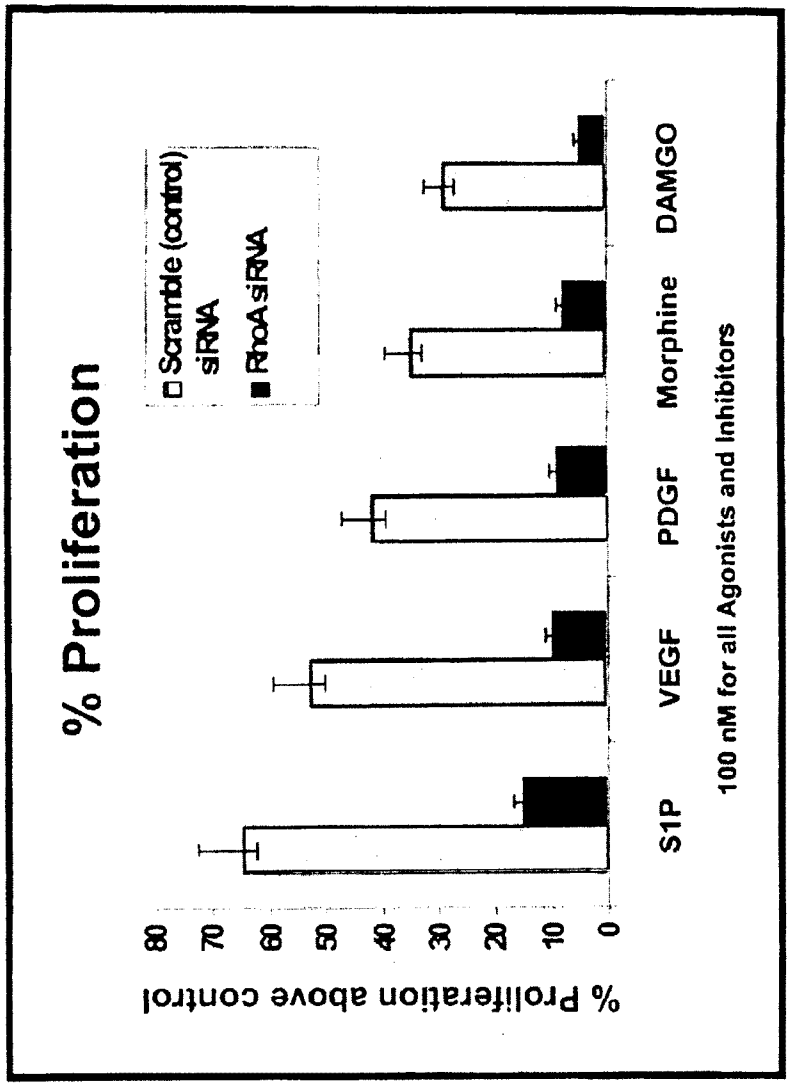

% Proliferation
Scramble (control) siRNA
RhoA siRNA
% Proliferation above control
80
70
60
50
40
30
20
10
0
S1P
VEGF
PDGF
Morphine
DAMGO
100 nM for all Agonists and Inhibitors

FIG. 20

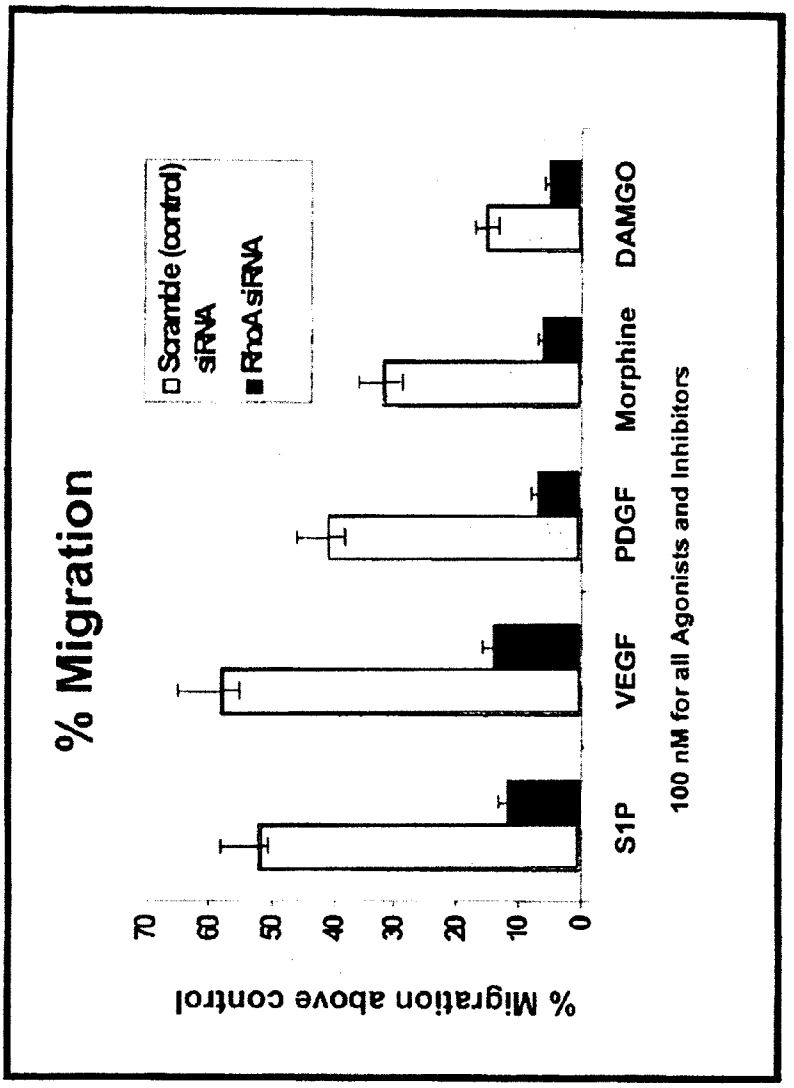

% Migration
Scramble (control) siRNA
RhoA siRNA
% Migration above control
70
60
50
40
30
20
10
0
S1P
VEGF
PDGF
Morphine
DAMGO
100 nM for all Agonists and Inhibitors

FIG. 21

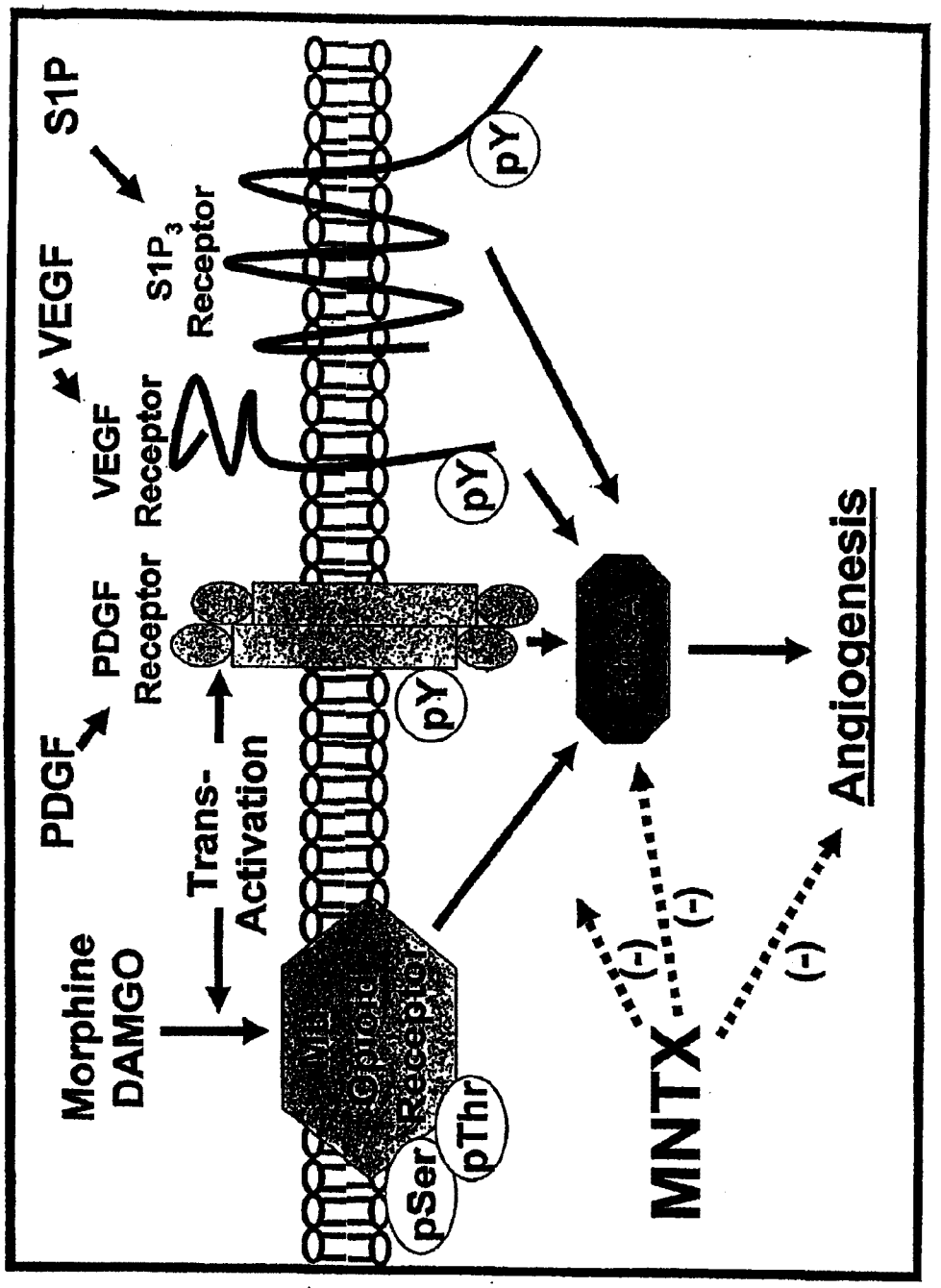

S1P
VEGF
PDGF
Morphine
DAMGO
S1P3 Receptor
VEGF Receptor
PDGF Receptor
Trans-Activation
Opioid Receptor
pY
pY
pY
pSer
pThr
MNTX
(-)
(-)
(-)
Angiogenesis

FIG. 22

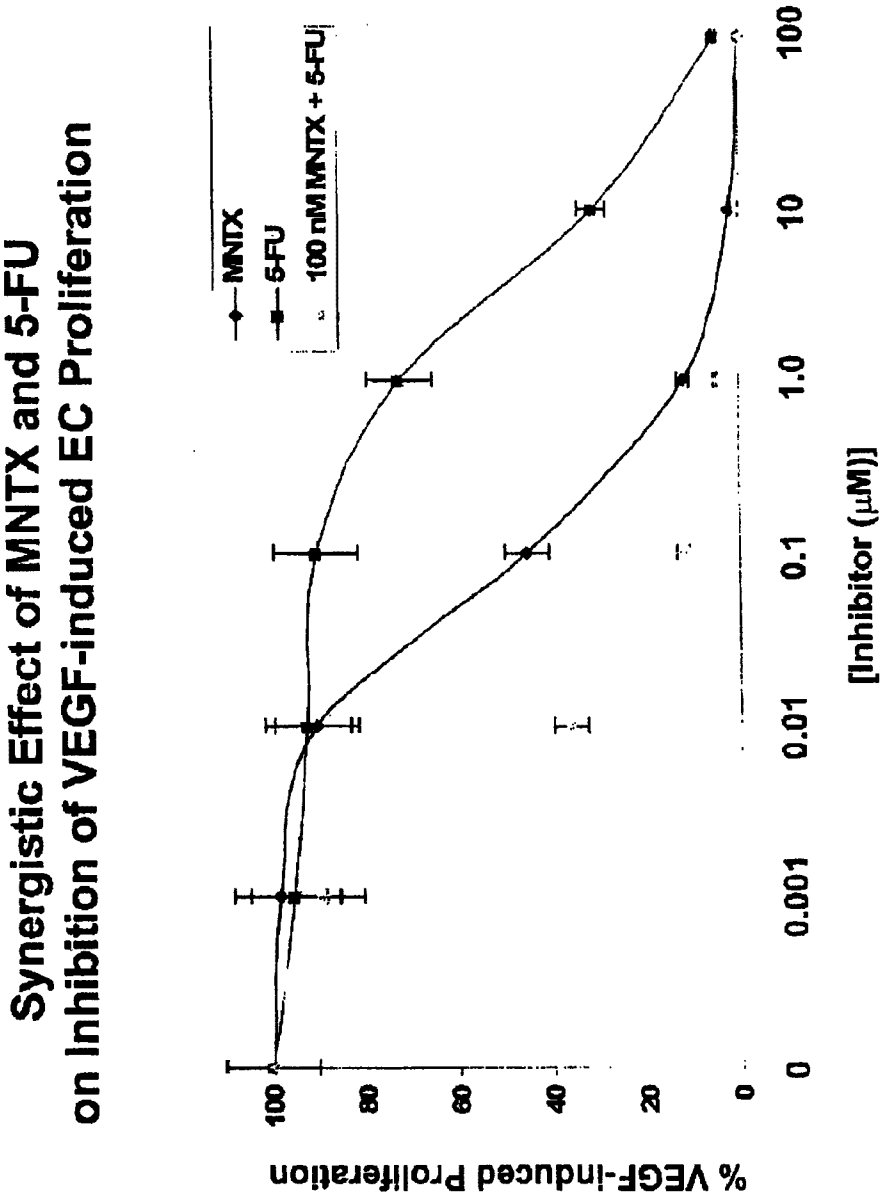

Synergistic Effect of MNTX and 5-FU
on Inhibition of VEGF-induced EC Proliferation
MNTX
5-FU
100 nM MNTX + 5-FU
% VEGF-Induced Proliferation
100
80
60
40
20
0
0
0.001
0.01
0.1
1.0
10
100
[Inhibitor (μM)]

FIG. 23

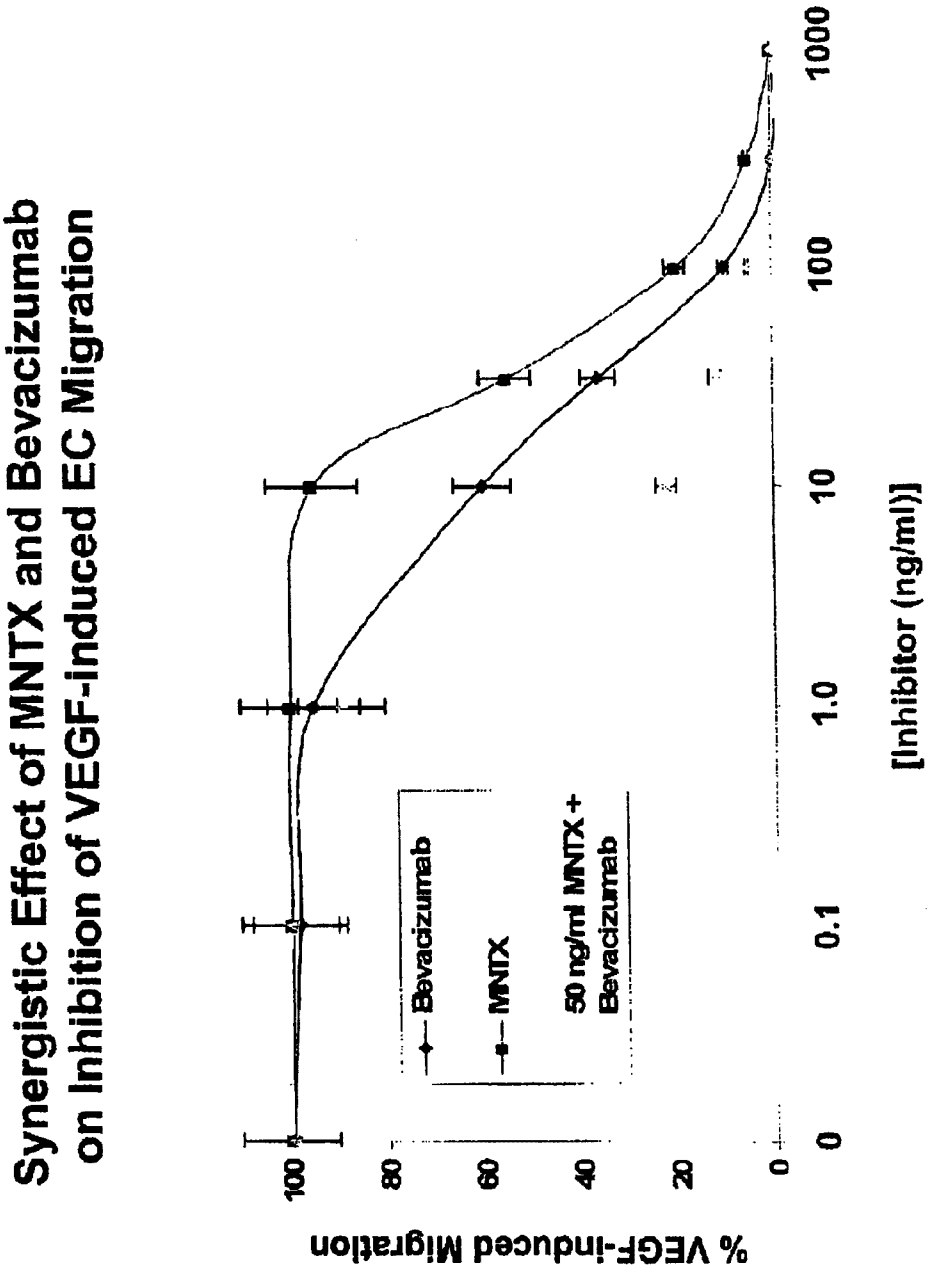

Synergistic Effect of MNTX and Bevacizumab on Inhibition of VEGF-induced EC Migration
% VEGF-induced Migration
100
80
60
40
20
0
0
0.1
1.0
10
100
1000
[Inhibitor (ng/ml)]
Bevacizumab
MNTX
50 ng/ml MNTX + Bevacizumab

FIG. 24

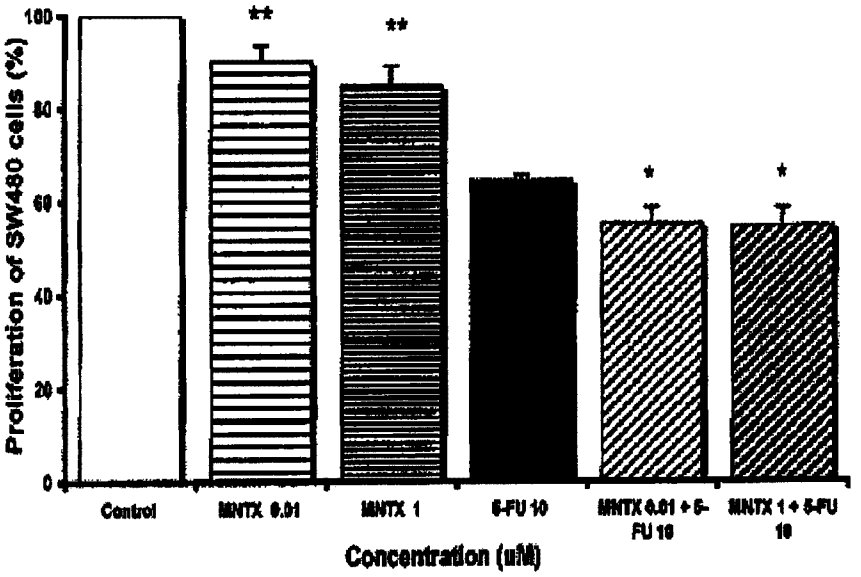

Proliferation of SW480 cells (%)
100
80
60
40
20
0
**
**
*
*
Control
MNTX 0.01
MNTX 1
5-FU 10
MNTX 0.01 + 5-FU 10
MNTX 1 + 5-FU 10
Concentration (uM)

FIG. 25

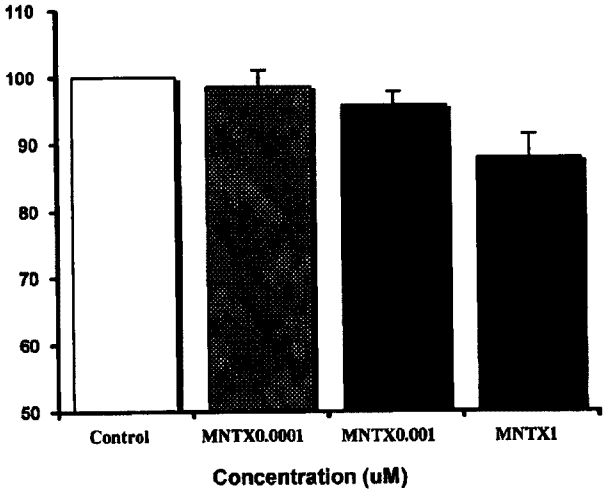

110
100
90
80
70
60
50
Control
MNTX0.0001
MNTX0.001
MNTX1
Concentration (uM)

FIG. 26

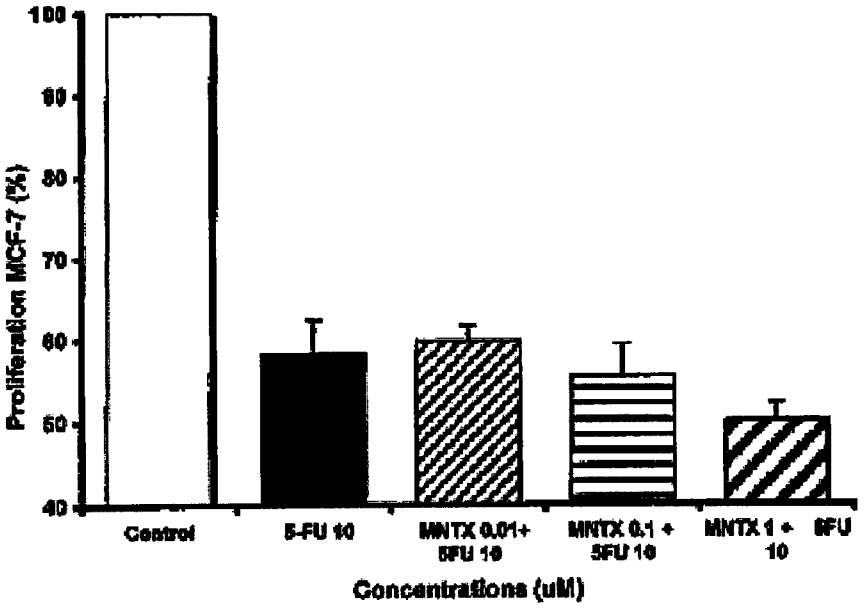

100
90
80
70
60
50
40
Proliferation MCF-7 (%)
Control
5-FU 10
MNTX 0.01+ 5FU 10
MNTX 0.1 + 5FU 10
MNTX 1 + 10
5FU
Concentrations (uM)

FIG. 27

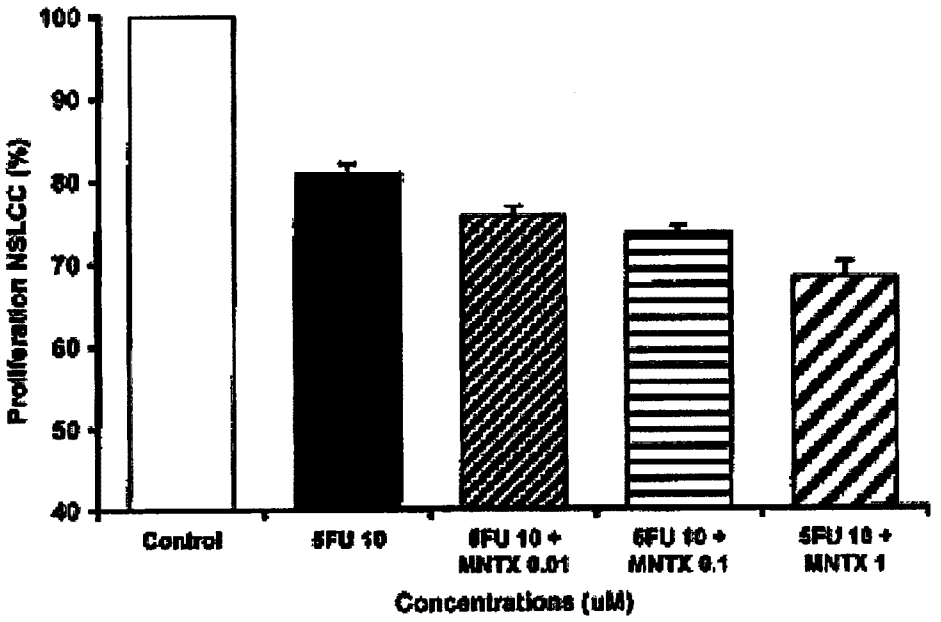
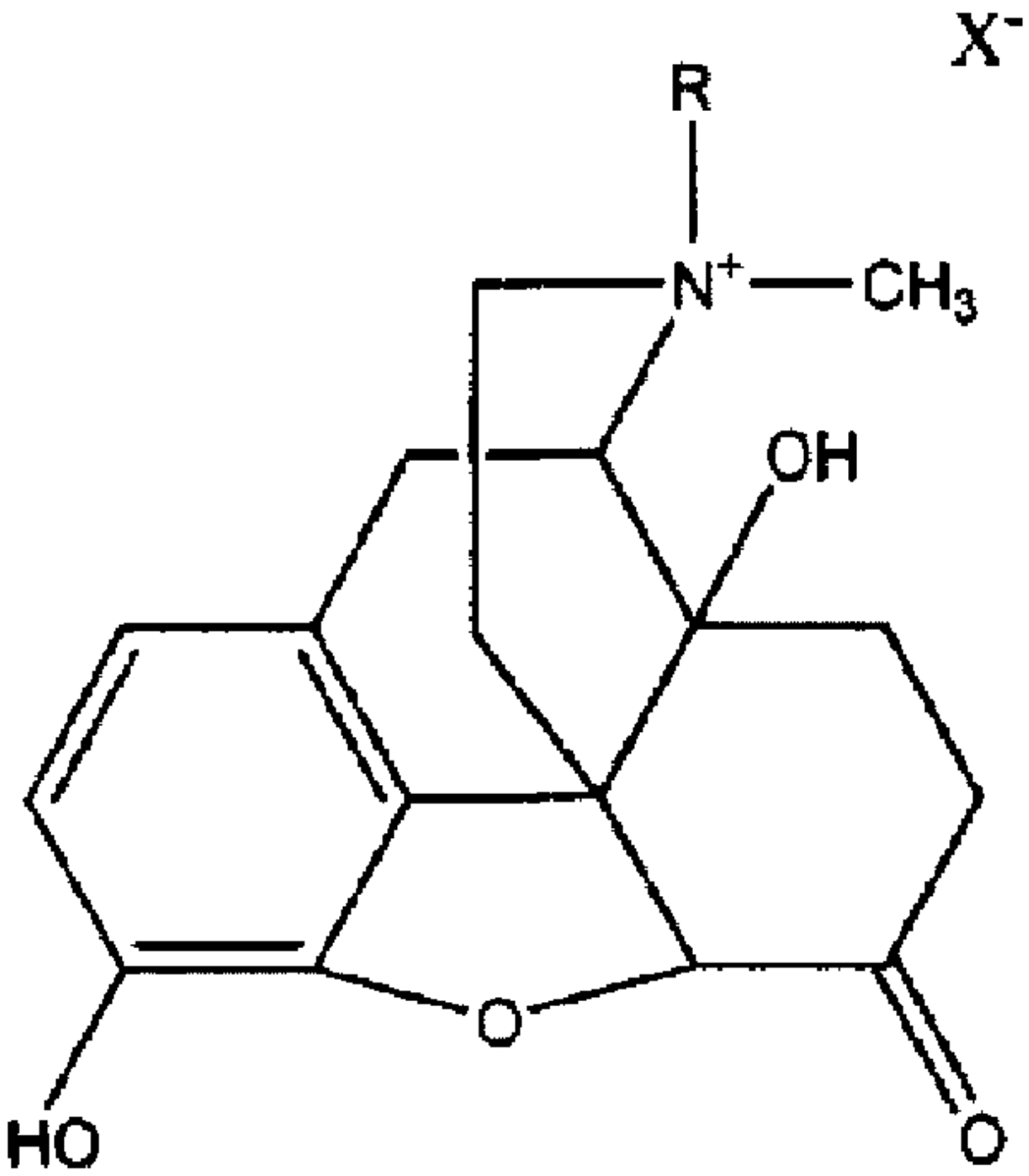

100
90
80
70
60
50
40
Proliferation NSLCC (%)
Control
5FU 10
5FU 10 + MNTX 0.01
5FU 10 + MNTX 0.1
5FU 10 + MNTX 1
Concentrations (uM)

FIG. 28



(I)