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(54) **Szűrés netrin-1 aktivitású, rák elleni vegyületekre**

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(54) SCREENING FOR ANTI-CANCER COMPOUNDS USING NETRIN-1 ACTIVITY

SUCHE NACH VERBINDUNGEN GEGEN KREBS UNTER VERWENDUNG VON NETRIN-1-AKTIVITÄT

CRIBLAGE DE COMPOSÉS ANTICANCÉREUX EN UTILISANT L'ACTIVITÉ DE LA NÉTRINE-1

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Description

[0001] The subject matter of the present invention relates to an *in vitro* method for the screening of anti-cancer compounds based on the capacity for these compound to interact with netrin-1 and/or to inhibit the dimerization of the intracellular domain of the netrin-1 receptor expressed in tumor cells. The invention also relates to a method for predicting the presence of metastatic cancer, or for determining the efficiency of an anti-cancer treatment based on the measuring of the expression level of netrin-1. The invention further comprises kits and compounds as a medicament for the treatment of metastatic cancer such as breast cancer, related to the overexpression of netrin-1 by the tumor cells.

[0002] Netrin-1, a diffusible laminin-related protein, has been shown to play a major role in the control of neuronal navigation during the development of the nervous system [31], by interacting with its main receptors, DCC (Deleted in Colorectal Cancer) [1,2,3] and UNC5H [4, 5]. However, more recently, netrin-1 has emerged as a completely different molecule that regulates cell survival. Indeed, the netrin-1 receptors DCC and UNC5H, -i.e., UNC5H1, UNC5H2, and UNC5H3- belong to the so-called dependence receptor family [6, 7]. Dependence receptors form a group of receptors that share the ability to induce cell death when expressed in settings in which their ligand is not available [44]. Such receptors, which also include RET [8], β-integrins [9], Patched [10], neogenin [11], $p75^{NTR}$ [12] and the androgen receptor [40], share the functional property of inducing cell death when disengaged from their ligands, while the presence of their ligand blocks this pro-apoptotic activity. Such receptors thus create cellular states of dependence on their respective ligands [13, 14].

[0003] This dependence effect has been suggested to act as a mechanism for eliminating tumor cells that would develop in settings of ligand unavailability: proliferation of tumor cells in a cell environment with constant and limited ligand presence or migration of metastatic tumor cells towards tissues where the ligand is not expressed. A selective advantage for a tumor cell would then be to lose the pro-apoptotic activity of its dependence receptors. It was predicted from genetic screens that the C. elegans netrin-1 -UNC6-interacted with UNC40 and with UNC5 [42]. Four orthologues of UNC5 were identified in mammals: UNC5H1, H2, H3, H4 and UNC40 was found to be the orthologue of the vertebrate DCC (Deleted in Colorectal Cancer) [39]. Along this line, DCC was proposed in the early 1990s to be a tumor suppressor gene, whose expression is lost in the vast majority of human cancers [15, 16]. This hypothesis also fits with the recent observation that UNC5H genes are down-regulated in the vast majority of colorectal tumors, hence suggesting that the loss of UNC5H genes represents a selective advantage for tumor development [17]. Interestingly, in mice, both inactivation of UNC5H3 and overexpression of netrin-1 in the gastro-intestinal tract are associated with intestinal tumor progression [18, 19], hence demonstrating *per se* that the loss of netrin-1 dependence receptors in the human pathology is a causal factor for tumor progression. However, although an initial series of reports supported the fact that DCC acted as a tumor suppressor (for a review see [29]), doubts have arisen, mainly because of the rarity of point mutations in the DCC coding sequence and because of the lack of tumor predisposition in DCC hemizygous mice [41].

[0004] However, the model described above predicts that both loss of the netrin-1 receptors and gain of ligand expression -i.e., autocrine expression- should be observed in human cancers, as they should represent similar selective advantages. This question is important not only for basic knowledge, but is crucial for therapy: indeed, inhibiting the extracellular interaction between netrin-1 dependence receptors and netrin-1 could represent an appealing strategy to trigger tumor regression.

[0005] It is particular desirable to provide simple and consistent means for identifying and characterizing new compounds which can be used for the treatment of cancer.

[0006] Surprisingly, the inventors have first demonstrated that, rather than losing netrin-1 dependence receptors, the majority of metastatic breast tumors show increased netrin-1 expression, a trait that may be used in therapy to trigger death of metastasic tumor.

[0007] If the pro-apoptotic signaling of DCC and/or UNC5H is beginning to be documented, an important question in this death/life signature dictated by DCC, UNC5H and more generally by the other known dependence receptors, is how does the presence of the ligand inhibit their pro-apoptotic activity [50].

[0008] In a second time, the inventors have analyzed whether netrin-1 induced DCC and/or UNC5H multimerization could be the critical step that inhibits DCC and/or UNC5H pro-apoptotic activity. Surprisingly, they have demonstrated that netrin-1 receptor, such as DCC and/or UNC5H multimerizes in response to netrin-1, a process sufficient to inhibit apoptosis.

[0009] In a first aspect, the present invention is directed to an *in vitro* method for selecting a compound for the prevention or the treatment of cancer, wherein said method comprises the following steps of:

a) having a medium containing netrin-1, or a fragment thereof, and a netrin-1 receptor, or a fragment thereof, wherein:

- said netrin-1, or a fragment thereof, and said netrin-1 receptor, or a fragment thereof, is able to specifically interact together to form a binding pair, and/or
- said netrin-1, or a fragment thereof, is able to induce the dimerization or multimerization of said netrin-1 receptor, or a fragment thereof, particularly the intracellular domain of said netrin-1 receptor;

b) contacting said medium with the compound to be tested;
c)

- measuring the inhibition of the interaction between netrin-1, or a fragment thereof, and said netrin-1 receptor, or a fragment thereof, and/or

- determine whether said compound inhibit the dimerization or multimerization of said netrin-1 receptor, or a fragment thereof, particularly the dimerization of the intracellular domain of said netrin-1 receptor; and

d) selecting said compound if:

- the measuring in step c) demonstrates a significantly inhibition of the interaction between netrin-1, or a fragment thereof, and netrin-1 receptor, or a fragment thereof, in presence of said compound, and/or
- the determination in step c) demonstrates a significantly inhibition of the dimerization or multimerization of said netrin-1 receptor, or a fragment thereof, in presence of said compound, particularly the dimerization of the intracellular domain of said netrin-1 receptor.

[0010] By the terms interaction between netrin-1 and its netrin-1 receptor, it is intended to designate in the present application the interaction which result to the selective advantage for tumor cells to escape netrin-1 dependence receptors induced apoptosis, preferably due to elevated netrin-1 level.

[0011] So, the inhibition of this interaction can be obtained for example by the complete or partial inhibition of the binding of netrin-1 to its receptor, notably in presence of a competitive ligand (such as an antibody which is directed to this extracellular membrane domain of said netrin-1 receptor), or in presence of a compound able to form a specific complex with the netrin-1 (such as a soluble extracellular membrane domain of its netrin-1 receptor, or part thereof).

[0012] In a preferred embodiment, the method according to the present invention is characterized in that said cancer to be prevent or treated is a cancer wherein tumoral cells express or overexpress netrin-1.

[0013] In another preferred embodiment, the method according to the present invention is characterized in that said cancer to be prevent or treated is selected from the group consisting of breast cancer, colorectal cancer, lung cancer, neuroblastoma, glioma, acute myeloid leukemia, sarcoma, melanoma, ovarian adenocarcinoma, renal adenocarcinoma pancreatic adenocarcinoma, uterus adenocarcinoma, stomac adenocarcinoma, kidney adenocarcinoma and rectal adenocarcinoma.

[0014] In another preferred embodiment, the method according to the present invention is characterized in that said cancer to be prevent or treated is a metastatic or an aggressive cancer.

[0015] In the method according to the invention, said netrin-1 receptor is preferably selected from the group of DCC, UNC5H (particularly UNC5H1, UNC5H2 and UNC5H3), neogenin and the adenosine A2b, more preferably selected from the group of DCC, UNC5H1, UNC5H2 and UNC5H3.

[0016] In another preferred embodiment, the method according to the present invention is characterized in that at step a):

- said netrin-1 receptor fragment comprises or is the extracellular domain of the netrin-1 receptor, or part thereof able to interact with netrin-1; and/or
- said netrin-1 receptor fragment comprises or is the intracellular domain of the netrin-1 receptor, or part thereof able to dimerize or multimerize in presence of netrin-1.

[0017] In another preferred embodiment, the method according to the present invention is characterized in that said netrin-1 or/and said netrin-1 receptor are from mammal, particularly from mouse, rat or human.

[0018] In a particular aspect of the method of the present invention, at step a) said netrin-1 is from chicken.

[0019] In another preferred embodiment, the method according to the present invention is characterized in that said netrin-1 or/and said netrin-1 receptor and/or the compound to be tested is labelled by a marker able to be directly or indirectly measured.

[0020] In another preferred embodiment, the method according to the present invention is characterized in that at step c):

- the measure of the inhibition of the interaction between netrin-1, or a fragment thereof, and said netrin-1 receptor, or a fragment thereof, is carried out by immunoassay (particularly by ELISA or by Immunoradiometric Assay (IRMA)), by Scintillation Proximity Assay (SPA) or by Fluorescence Resonance Energy Transfer (FRET); and/or
- the dimerization or multimerization, or its inhibition, of said netrin-1 receptor, or fragment thereof, particularly the intracellular domain, is carried out by immunoprecipitation or FRET.

[0021] In another particular preferred embodiment, the method according to the present invention is characterized in that at step a) said medium contains cells which express at their surface membrane an endogenous or a recombinant netrin-1 receptor, particularly a recombinant extracellular domain of said netrin-1 receptor.

[0022] In a preferred embodiment, said recombinant netrin-1 receptor also comprises the intracellular domain of said netrin-1 receptor.

[0023] In another particular preferred embodiment, the method according to the present invention is characterized in that at step a) said medium contains tumoral cells, preferably metastatic tumoral cells, which express endogenously said netrin-1 receptor at their membrane surface and which express or overexpress netrin-1, and wherein at step c) the inhibition of the interaction between netrin-1 and its netrin-1 receptor in presence of the compound to be tested, is measured by the apoptosis or cells death induced by the presence of the compound to be tested, preferably analysed using the trypan blue staining method as indicated in the examples below.

[0024] In a preferred embodiment said tumoral cells are selected from the group consisting of 4T1 cells, CAL51 cells, T47D cells, SKBR7 cells, IMR32 cells, GL26 cells and H358 cells, notably CAL51 cell lines, such as CAL51-36 cell line, which are much more susceptible to cell death in response to the presence of DCC-EC-Fc.

[0025] The present invention is also directed to an *in vitro* method for selecting a compound for the prevention or the treatment of cancer, wherein said method comprises the following steps of:

a) having a medium containing a mammal cell expressing an endogenous or a recombinant netrin-1 receptor, or a fragment thereof comprising at least its intracellular domain, preferably a tumor cell, more preferably a cell presenting dimerization or multimerization of its netrin-1 receptor intracellular domain or a cell wherein its netrin-1 receptor intracellular domain is able to dimerize or multimere in presence of netrin-1;
b) contacting said medium with the compound to be tested, optionally the medium further containing netrin-1, or a fragment thereof able to interact with the extracellular domain of the netrin-1 receptor;
c) determine whether the dimerization or multimerization of said netrin-1 receptor intracellular domain is inhibited in presence of said compound to be tested;
d) optionally, determine (for example by the blue trypan method) whether the presence of the compound to be tested induces the cell death of said mammal cell; and
e) selecting said compound if the determination in step c) demonstrates a significantly inhibition of the dimerization or multimerization of the intracellular domain of said netrin-1 receptor and/or if the determination in step d) demonstrates the cell death of said mammal cell.

[0026] In a second aspect, the present invention is directed to an *in vitro* method for predicting the presence of a metastatic cancer or an aggressive cancer (such as neuroblastome) in a patient having a primary tumor from a biopsy of said patient containing primary tumors cells, said method comprising the following step of:

(a) measuring of the netrin-1 expression level in said biopsy.

[0027] In a preferred embodiment, the method for predicting according to the present invention is characterized in that at step a) wherein an increase of the netrin-1 expression level in said biopsy, compared with expression of netrin-1 in non-metastatic primary tumor biopsies or in non-aggressive cancer biopsies is significant of the presence of a metastatic cancer or an aggressive cancer.

[0028] In a more preferred embodiment, the method for predicting according to the present invention is characterized in that a ratio superior to 2, preferably to 2.5, to 3, to 3.5, to 4, to 4.5 and to 5, between netrin-1 expression in the biopsy to be tested and in the non-metastatic or non-aggressive reference biopsy is significant of the presence of a metastatic or an aggressive cancer.

[0029] In a third aspect, the present invention is directed to an method for determining *in vitro* the efficiency of an anti-cancer treatment for a patient or for selecting patients who responds to a specific anti-cancer treatment, said method comprising the following step of:

(a) obtaining a primary tumor biopsy of said treated patient; and
(b) measuring of the netrin-1 expression level in said biopsy,

wherein the efficiency of said anti-cancer treatment is correlated with the decrease of the amount of the netrin-1 expression level measured in said biopsy, or wherein the selected patients who respond to a specific anti-cancer treatment are patients where the amount of the netrin-1 expression level measured in their biopsy has been decreased after said specific treatment.

[0030] In a preferred embodiment, the method for determining *in vitro* the efficiency of an anti-cancer treatment for a patient or for selecting patients who responds to a specific anti-cancer treatment, is characterized in that said cancer

induced an overexpression of netrin-1 and/or is a metastatic or an aggressive cancer.

[0031] In a preferred embodiment, the method for prediction or for determining *in vitro* the efficiency of an anti-cancer treatment for a patient is characterized in that the measured netrin-1 expression product is the RNA encoding netrin-1, particularly measured by a quantitative real time reverse PCR method, or in that the expression level of netrin-1 which is measured is the measure of the netrin-1 protein level, particularly by a method using specific antibodies able to specifically recognize said netrin-1 protein.

[0032] In a preferred embodiment, the method for prediction or for determining *in vitro* the efficiency of an anti-cancer treatment for a patient is characterized in that the primary tumor is a primary tumor of a cancer selected from the group consisting of breast cancer, colorectal cancer, lung cancer, neuroblastoma, glioma, acute myeloid leukemia, sarcoma, melanoma, ovarian adenocarcinoma, renal adenocarcinoma pancreatic adenocarcinoma, uterus adenocarcinoma, stomac adenocarcinoma, kidney adenocarcinoma and rectal adenocarcinoma.

[0033] In another aspect, the present invention is directed to a kit for the selection of a compound for the prevention or the treatment of cancer, wherein said kit comprises:

- a netrin-1 receptor protein, or a fragment thereof able to specifically interact with the netrin-1 protein to form a binding pair, preferably recombinant protein; and
- netrin-1 protein, or a fragment thereof able to specifically interact with said netrin-1 receptor protein to form a binding pair, preferably recombinant protein.

[0034] Said netrin-1 receptor being also preferably selected from the group of DCC, UNC5H (particularly UNC5H1, UNC5H2 and UNC5H3), neogenin and the adenosine A2b, more preferably selected from the group of DCC, UNC5H1, UNC5H2 and UNC5H3, more preferably from mammal such as from mouse, rat or human.

[0035] In a preferred embodiment, said kit comprises:

- tumoral cells which express netrin-1 receptor and which express or overexpress netrin-1, particularly cells from metastatic tumoral cell line, preferably selected from the group consisting of of 4T1 cells, CAL51 cells, T47D cells, SKBR7 cells, IMR32 cells, GL26 cells and H358 cells, notably CAL51 cell lines, such as CAL51-36 cell line, which are much more susceptible to cell death in response to the presence of DCC-EC-Fc.

[0036] In another aspect, the present invention comprises a compound selected from the group consisting of:

- a compound comprising an extracellular domain of netrin-1 receptor or fragment thereof able to specifically inhibit the interaction between the netrin-1 and said netrin-1 receptor, and/or able to inhibit the dimerization or multimerization of said netrin-1 receptor, or a fragment thereof, particularly to inhibit the intracellular domain of said netrin-1 receptor; and
- a monoclonal or polyclonal antibody directed specifically against netrin-1 or netrin-1 receptor, particularly directed to the extracellular domain of said netrin-1 receptor or to the netrin-1 fragment able to interact with the extracellular domain of said netrin-1 receptor,

as a medicament.

[0037] The amino acid sequence of human netrin-1 or human netrin receptor such as UNC5H1, UNC5H2 and UNC5H3 (Unc-5 homolog 1, 2 and 3 equivalent to Unc-5 homolog A, B and C) are well known by the skilled man. Example of these amino acid sequences with the localization of their particular domain can be found in Genbank under the accession number AAD09221 or NP_004813 for human netrin-1, NP_588610 for human netrin receptor Unc-5 homolog 1, Q8IZJ1 for netrin receptor Unc-5 homolog 2 and 095185 for Unc-5 homolog 3.

[0038] Preferably, in the compounds of the present invention, said extracellular domain of netrin-1 receptor or fragment thereof is selected from the group of DCC, UNC5H (particularly UNC5H1, UNC5H2 and UNC5H3), neogenin and the adenosine A2b, more preferably selected from the group of DCC, UNC5H1, UNC5H2 and UNC5H3, more preferably from mammal such as from mouse, rat or human.

[0039] In a more preferred embodiment, said compound according to the present invention comprises an extracellular domain of netrin-1 receptor from DCC, preferably said compound is DCC-EC-Fc or DCC-5Fbn.

[0040] In another aspect, the present invention pertains to the use of the level of netrin-1 expression as a marker for the identification of metastatic cancer in a patient, preferably of metastatic breast or colorectal cancer, the most preferred being the metastatic breast cancer.

[0041] In another aspect, the present invention pertains to a method of treatment for inducing the apoptosis or the cell death of tumor cells which have acquired the selective advantage to escape netrin-1 dependence receptors induced apoptosis, preferably by elevated netrin-1 level, in a patient comprising administering a compound able to inhibit the interaction between netrin-1 and its netrin-1 receptor, a compound able to inhibit the dimerization or the multimerization

of the netrin-1 receptor, a compound according to the present invention, or selected by the method of the present invention, in said patient in need thereof.

[0042] In another aspect, the present invention pertains to a method for the prevention or for the treatment of cancer in a patient comprising administering a compound according to the present invention, or selected by the method of the present invention, in said patient in need thereof.

[0043] The present invention also comprises the use of a compound according to the present invention, or selected by the method of the present invention, for the manufacture of a medicament for the prevention or the treatment of cancer in mammals, including man. Preferably said cancer is a metastatic or an aggressive cancer.

[0044] More preferably, in the method of treatment or in the use of a compound according to the present invention, said cancer is selected from the group consisting of breast cancer, colorectal cancer, lung cancer, neuroblastoma, glioma, acute myeloid leukemia, sarcoma, melanoma, ovarian adenocarcinoma, renal adenocarcinoma pancreatic adenocarcinoma, uterus adenocarcinoma, stomac adenocarcinoma, kidney adenocarcinoma and rectal adenocarcinoma.

[0045] More preferably, in the method of treatment or in the use of a compound according to the present invention, the primary tumor cells of said cancer express or overexpress netrin-1.

[0046] The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site which specifically binds (immunoreacts with) the netrin-1 protein or its receptor.

[0047] The term "antibody" comprises monoclonal or polyclonal antibodies but also chimeric or humanized antibodies.

[0048] An isolated netrin-1 protein or netrin-1 receptor protein, or a specific fragment thereof can be used as an immunogen to generate antibodies that bind such protein using standard techniques for polyclonal and monoclonal antibody preparation. It may be also possible to use any fragment of these protein which contains at least one antigenic determinant may be used to generate these specific antibodies.

[0049] A protein immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain said protein, or fragment thereof, and further can include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent.

[0050] Thus, antibody for use in accordance with the invention include either polyclonal, monoclonal chimeric or humanized antibodies, antibodies able to selectively bind, or which selectively bind to an epitope-containing a polypeptide comprising a contiguous span of at least 8 to 10 amino acids of an amino acid sequence of the netrin-1 protein or its receptor.

[0051] A preferred agent for detecting and quantifying mRNA or cDNA encoding netrin-1 protein, is a labeled nucleic acid probe or primers able to hybridize this mRNA or cDNA. The nucleic acid probe can be an oligonucleotide of at least 10, 15, 30, 50 or 100 nucleotides in length and sufficient to specifically hybridize under stringent conditions to the mRNA or cDNA. The nucleic acid primer can be an oligonucleotide of at least 10, 15 or 20 nucleotides in length and sufficient to specifically hybridize under stringent conditions to the mRNA or cDNA, or complementary sequence thereof.

[0052] A preferred agent for detecting and quantifying the netrin-1 protein, is an antibody able to bind specifically to this protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or F(ab')2) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.

[0053] For example, in vitro techniques for detection of candidate mRNA include Northern hybridizations and in situ hybridizations. In vitro techniques for detection of the candidate protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. In vitro techniques for detection of candidate cDNA include Southern hybridizations.

[0054] When the invention encompasses kits for quantifying the level of netrin-1 protein, the kit can comprise a labeled compound or agent capable of quantifying these proteins. Said agents can be packaged in a suitable container. The kit can further comprise instructions for using the kit to quantify the level of the netrin-1 protein or of the netrin-1 transcript.

[0055] In certain embodiments of the method of the present invention, the determination of the netrin-1 transcripts involves the use of a probe/primer in a polymerase chain reaction (PCR), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al., 1988, Science 241:23-1080; and Nakazawa et al., 1994, Proc. Natl. Acad. Sci. USA, 91:360-364), or alternatively quantitative real time RT-PCR This method can include the steps of collecting a sample of cells from a patient, isolating nucleic acid (e.g. mRNA) from the cells of the sample, optionally transforming mRNA into corresponding cDNA, contacting the nucleic acid sample with one or more primers which specifically hybridize to the netrin-1 or mRNA or their corresponding cDNA under conditions such that hybridization and amplification of the netrin-1 mRNA or cDNA occurs, and quantifying the presence of the amplification

products. It is anticipated that PCR and/or LCR may be desirable to use as an amplification step in conjunction with any of the techniques used for quantifying nucleic acid detecting.

[0056] The methods described herein may be performed, for example, by utilizing prepackaged diagnostic kits comprising at least one probe nucleic acid or set of primer or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to follow-up or diagnose patients.

[0057] Finally, the present invention is related to the use of antisense or iRNA (interfering RNA) oligonucleotides specific of the nucleic acid encoding netrin-1 protein for the manufacture of a medicament intented to prevent or to treat metastatic or aggressive cancer, preferably said cancer is selected from the group consisting of breast cancer, colorectal cancer, lung cancer, neuroblastoma, glioma, acute myeloid leukemia, sarcoma, melanoma, ovarian adenocarcinoma, renal adenocarcinoma pancreatic adenocarcinoma, uterus adenocarcinoma, stomac adenocarcinoma, kidney adenocarcinoma and rectal adenocarcinoma.

[0058] Interfering RNA (iRNA) is a phenomenon in which a double stranded RNA (dsRNA) specifically suppresses the expression of a gene bearing its complementary sequence. iRNA has since become a useful research tool for many organisms. Although the mechanism by which dsRNA suppresses gene expression is not entirely understood, experimental data provide important insights. This technology has great potential as a tool to study gene function in mammalian cells and may lead to the development of pharmacological agents based upon siRNA (small interfering RNA).

[0059] When administered to a patient, a compound of the present invention is preferably administered as component of a composition that optionally comprises a pharmaceutically acceptable vehicle. The composition can be administered orally, or by any other convenient route, and may be administered together with another biologically active agent. Administration can be systemic or local. Various delivery systems are known, e.g., encapsulation in liposomes, microparticles, microcapsules, capsules, etc., and can be used to administer the selected compound of the present invention or pharmaceutically acceptable salts thereof.

[0060] Methods of administration include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, oral, sublingual, intranasal, intracerebral, intravaginal, transdermal, rectally, by inhalation, or topically. The mode of administration is left to the discretion of the practitioner. In most instances, administration will result in the release of the compound into the bloodstream or directly in the primary tumor.

[0061] Compositions comprising the compound according to the invention or selected by the methods according to the present invention, form also part of the present invention. These compositions can additionally comprise a suitable amount of a pharmaceutically acceptable vehicle so as to provide the form for proper administration to the patient. The term "pharmaceutically acceptable" means approved by a regulatory agency or listed by a national or a recognized pharmacopeia for use in animals, mammals, and more particularly in humans. The term "vehicle" refers to a diluent, adjuvant, excipient, or carrier with which a compound of the invention is administered. Such pharmaceutical vehicles can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. The pharmaceutical vehicles can be saline, gelatin, starch and the like. In addition, auxiliary, stabilizing, thickening, lubricating and coloring agents may be used. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid vehicles, particularly for injectable solutions. Suitable pharmaceutical vehicles also include excipients such as starch, glucose, lactose, sucrose, gelatin, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene, glycol, water and the like. Test compound compositions, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The compositions of the invention can take the form of solutions, suspensions, emulsion, tablets, pills, pellets, capsules, capsules containing liquids, powders, sustained-release formulations, suppositories, emulsions, aerosols, sprays, suspensions, or any other form suitable for use. Said composition is generally formulated in accordance with routine procedures as a pharmaceutical composition adapted to human beings for oral administration or for intravenous administration. The amount of the active compound or a that will be effective in the treatment can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed will also depend on the route of administration, and the seriousness of the disease, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for oral, intranasal, intradermal or intraveneous administration are generally about 0.01 milligram to about 75 milligrams per kilogram body weight per day, more preferably about 0.5 milligram to 5 milligrams per kilogram body weight per day.

[0062] It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the following examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

Figure Legends

[0063]

Figures 1A and 1B: Netrin-1 is over-expressed in human metastatic breast tumors

Figure 1A: Expression profile of netrin-1 examined with quantitative real time reverse transcription PCR. Q-RT PCR was performed using total RNA extracted from 15 metastatic (solid bar) and 15 non-metastatic (open bar) primary tumor biopsies with specific human netrin-1 primers [26] and primers corresponding to the human TBP gene (TATA Binding Protein). TBP was used as a control here, as it shows a weak variability at the mRNA level between normal and breast tumoral tissues, as described in [25]. Netrin-1 expression is given as the ratio between netrin-1 expression in each sample and the average of netrin-1 expression in the non-metastatic samples.
Figure 1B: Q-RT-PCR was performed using total RNA extracted from 67NR and 4T1 mouse cell lines with specific mouse netrin-1 primers and the mouse gene RPLP0 as standard.

Figures 2A-2D: Forced expression of netrin-1 in 67NR mouse cell line leads to metatasis development

Figures 2A and 2B: Mock transfected 67NR cells or 67NR cells stably transfected with netrin-1 (67NR-net) were submitted to netrin-1 expression analysis. Figure 2A, RT-PCR using specific chicken netrin-1 primers was performed using total RNA extracted from 67NR-net1 and 67NR-mock. Figure 2B, Western blot using anti-myc (chick netrin-1) or anti-netrin1 antibodies was performed.
Figure 2C: Photomicrographs of the two 67NR-net1 and 67NR-net2 clones compared to parental 67NR and to 4T1 cell line.
Figure 2D: Metastatic 4T1 cells, two control cell clones bearing puromycine resistance (67NR1 and 67NR2) and two netrin-1 expressing cell clones (67NRnet1 and 67NRnet2) were injected in fat pad of 16 mice (4 mice per cell type) and metastasis was analyzed in the lung environment. Representative photomicrographs of under-pleural and intra-parenchymatous nodules after injection of the respective cell clones (4T1, 67NR1, 67NRnetl, 67NRnet2). IPL: intra-parenchymatous lesion, UPL: under-pleural lesions.

Figures 3A-3D: Induction of mouse metastatic cell death by inhibiting the netrin-1/receptor interaction

Figure 3A: Scheme representing netrin-1 and its receptors, DCC and UNC5H.
Figures 3B, 3C, 3D: Quantitative analysis of cell death in 67NR and 4T1 cells using either DCC-EC-Fc and non specific IL3-EC-Fc as a control (figure 3B), at different concentration (figure 3C) or the more restricted DCC-5Fbn domain (figure 3D) as a competitor for netrin-1/receptor interaction. Cell death was quantified either by the trypan blue exclusion assay (figures 3B, 3D) or by caspase activity assay (figure 3C). Standard deviations are indicated (n=3).

Figures 4A and 4B: Induction of human metastatic cell death by inhibiting netrin-1/receptor interaction

Figure 4A: quantitative measurement of CAL51 cell death treated with different concentration of DCC-EC-Fc by the trypan blue exclusion assay.
Figure 4B: Quantitative analysis of cell death monitored by trypan blue exclusion in the CAL51 parental cell line or in the clonal cell line CAL51-36, treated or not with the DCC-EC-Fc competitor in culture media.

Figure 5: Netrin-1 is over-expressed in human metastatic breast tumors
Expression profile of netrin-1 examined with quantitative real time reverse transcription PCR. Q-RT PCR was performed using total RNA extracted from 51 tumor biopsies. They were obtained from patient with tumors localized to the breast (N0, empty bar); with only axillary node involvement (N+, gray bar) and with distant metastases at diagnosis (M+, solid bar). Specific human netrin-1 primers [39] and primers corresponding to the human PBGD gene (TATA Binding Protein) were used. PBGD was used as a reference here, as it shows a weak variability at the mRNA level between normal and breast tumoral tissues, as described in [38]. The other reference TBP was also used with similar results (not shown). Netrin-1 expression is given as the ratio between netrin-1 expression in each sample and the average of netrin-1 expression in the N0 samples. A non parametric statistical significance test (Mann-Whitney) was used, the p value is indicated.

Figures 6A-6C: Induction of metastatic cell death by inhibiting the netrin-1/receptor interaction

Figure 6A: DCC-EC-Fc displaces DCC/netrin-1 and UNC5H2/netrin-1 interaction. ELISA assay with DCC-EC-Fc (top panel) or UNC5H2-EC-Fc (bottom panel) coated, and quantification of bound netrin-1 using anti-netrin-1 antibody in the presence of increasing concentration of DCC-EC-Fc.
Figures 6B and 6C: Quantitative analysis of cell death in 67NR and 4T1 cells using either DCC-EC-Fc and non specific IL3-EC-Fc as a control. Cell death was quantified either by the trypan blue exclusion assay (figure

6B), or by caspase activity assay (figure 6C). Standard deviations are indicated (n=3).

Figures 7A-7C: Inhibition of metastasis formation in mice by DCC-5Fbn treatment

Figure 7A: Quantitative analysis of cell death in 67NR and 4T1 cells treated with DCC-5Fbn. MTT assay was performed on 67NR or 4T1 cells after treatment with increasing doses of DCC-5Fbn (μg/ml). Percentage of cell survival is presented. Standard deviations are indicated (n=3).

Figures 7B and 7C: 4T1-luc cells were i.v. injected in BALB/c mice at day 0 and PBS or DCC-5Fbn were injected every two days, once i.v., once i.p. starting at day 0. After 13 days, metastasis development was studied by luminescence recording (figures 7B, 7C) or by examination of lungs under a scope.

Figure 7B: A representative image of luminescence recording of PBS treated (right) or DCC-5Fbn treated (left) mice.

Figure 7C: Quantification of the luminescent signal measured by the NightOwLB system. The number of photon/pixel/sec was quantified in each animal and an index of luminescent signal is given at the ratio between the average photon/mouse in PBS treated mice to the average signal detected in DCC-5Fbn treated mice. Two independent experiments are presented (20 mice were analyzed in experiment 1, 8 mice in the experiment 2).

A representative macroscopic photograph of a lung from PBS treated mice or from DCC-5Fbn treated mice has been made (not shown) can be demonstrated in the lung from PBS treated mice.

Figures 8A and 8B: Effect of DCC-5Fbn on netrin-1 expressing human cancer breast cell lines

Figure 8A: Expression of netrin-1 examined by Q-RT PCR using total RNA extracted from 48 different breast tumor cell lines. Netrin-1 expression is given as the ratio between netrin-1 expression and the housekeeping gene HMBS expression (Hydroxymethylbilane synthase) in each sample. TBP was also used as a control here and give similar results. The two cell lines that have been selected for their high level of netrin-1 are indicated by stars.

Figure 8B: Cell death induction by DCC-5Fbn in SKBR7 and T47D cell lines. Cell death was quantified either by MTT assay as described in figure 4A (right panel) or by caspase activity measurement as described in figure 3D (left panel). Standard deviations are indicated (n=3).

Figures 9A and 9B: Human neuroblastoma

Figure 9A: Netrin-1 is a marker of aggressiveness in human neuroblastoma. Expression profile of netrin-1 examined with quantitative real time reverse transcription PCR. Q-RT PCR was performed using total RNA extracted from 101 stage 4 or 4s neuroblastoma biopsies. Tumors were either stage 4 diagnosed in patient who were less than one year old (4<1 year) or stage 4 diagnosed in patient who were more than one year old (4>1 year). It can be noted that bad prognosis cancers (stage 4 >1 year) show a significant over-expression of netrin-1. Student t tests were used and the p values are indicated.

Figure 9B: IMR32 cells that endogenously produce netrin-1 were treated or not with DCC-5Fbn or as a control ILR3 (the interleukin-3 receptor ectodomain) and were analyzed for cell death either by measuring caspase activity (top) or by measuring cell survival via a MTT assay (down). Note that while IL3R has no effect on the death of IMR32 cells, DCC-5Fbn induces a significant IMR32 cell death. Standard deviations are indicated (n=3).

Figures 10A and 10B: Glioma

Figure 10A: Netrin-1 is overexpressed in a large fraction of glioma. Expression profile of netrin-1 examined with quantitative real time reverse transcription PCR. Q-RT PCR was performed using total RNA extracted from stage II and stage III oligodendroglioma and stage IV glioblastoma biopsies and was compared to normal human brain.

Figure 10B: GL26 cells that endogenously produce netrin-1 (not shown) were treated or not with DCC-5Fbn in the presence or not of an excess amount of recombinant netrin-1 and were analyzed for cell death either by measuring caspase activity (top) or by measuring cell survival via a MTT assay (down). Note that DCC-5Fbn induces a significant GL26 cell death and that this effect is fully inhibited by addition of netrin-1, thus demonstrating that the DCC-5Fbn effect is directly related to inhibition of endogenous netrin-1. Standard deviations are indicated (n=3).

Figures 11A-11C: Lung cancer

Figure 11A: Netrin-1 is overexpressed in a sizeable fraction of human lung cancer. Expression profile of netrin-1 examined with quantitative real time reverse transcription PCR. Q-RT PCR was performed using total RNA extracted from lung cancer biopsies and was compared to normal tissu.

Figure 11B: H358 and H460, two NSCLC cell lines, were further used for cell death assays. H358 cells that endogenously express netrin-1 and H460 cells that fail to show detectable netrin-1 expression were treated or not with DCC-5Fbn in the presence or not of an excess amount of recombinant netrin-1 and were analyzed for cell death either by measuring caspase activity (top) or by measuring cell survival via a MTT assay (down). Note that DCC-5Fbn induces a significant H358 cell death but fails to show an effect on H460 cells. Moreover the death effect observed in H358 cells is fully inhibited by addition of netrin-1. Together with the fact that H460 cells are not sensitive to DCC-5Fbn, these data support that netrin-1 expressing lung tumor cells undergo apoptosis in response to DCC-5Fbn.Standard deviations are indicated (n=3).

Figure 11C: DCC-5Fbn inhibits xenografted H358 tumor growth in nude mice. Five-week-old (20-22 g body weight) female athymic nu/nu mice were obtained from Charles River. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. H358 cells were implanted by s.c. injection of 5.10^6 cells in 200 μL of PBS into the left flank of the mice. When tumors were established, PBS or 20 μg of DCC-5Fbn were administered into the tumor (i.t.) everyday (duration of treatment is indicated by arrows). Tumor sizes were measured by a calliper during 41 days. The tumor volume was calculated with the formula v = (0.5*(length*width^2))$\pm$ SE,*) on 6 mice treated with DCC-5Fbn and 4 mice treated with PBS. Note that while PBS treated tumors were shown to grow, DCC-5Fbn treated tumors showed a massive regression.

Figures 12A and 12B: Netrin-1 mediates DCC and UNC5H2 multimerization

Figure 12A: DCC multimerization in the presence of netrin-1 in HEK293T cells. Lysates of HEK293T cells transiently transfected with HA-DCC and/or c-myc-DCC expressing constructs together or not with netrin-1 expressing construct were subjected to myc pull-down (IP α-myc). DCC-HA presence was revealed with an anti-HA antibody.

Figure 12B: UNC5H2 dimerization in the presence of netrin-1 in HEK293T cells. Cell transfection and cell lysate preparation were done as in (A) but with HA-Unc5H2 and/or FlagM2-UNC5H2 expressing constructs. Cell lysates were subjected to FlagM2 pull-down (IP α-Flag). HA-UNC5H2 presence was revealed with an anti-HA antibody. Total: Western blot on lysate before pull-down.

Figures 13A and 13B: Validation of the chemically-inducible system for UNC5H2 dimerization

Figure 13A: Schematic representation of Fv2e-UNC5H2 fusion constructions showing the two constructs (one tagged HA, the other one tagged c-myc) used to validate the artificial dimerization system.

Figure 13B: Lysates of HEK293T cells transiently transfected with Fv2E-UNC5H2 tagged HA or c-myc with or without the dimerization drug (AP20187) were subjected to c-myc pull-down (IP α-myc). Total: Western blot on lysate before pull-down. HA-Fv2E-UNC5H2 presence was revealed with anti-HA antibody.

Figures 14A-14C: Forced DCC dimerization blocks its proapoptotic activity

Figure 14A: DCC-induced cell death is inhibited by dimerization induced by AP20187, as measured by trypan blue exclusion. HEK293T cells were transfected with mock plasmid (Cont.), Fv2E (Fv), Fv2E-DCC-IC (Fv-DCC) with or without AP20187 (AP). In all conditions, cells were also transfected with the surface marker pKk. Transfected cells expressing the marker were magnetically labeled with MACSelect Microbeads and separated using a MACS Separator and Separation Columns. Trypan blue exclusion was assayed on these purified cells.

Figure 14B: UNC5H2-induced cell death is inhibited by dimerization induced by AP20187, as measured by trypan blue exclusion as in (A). Cells were transfected with pMACSKk and Fv2E (Fv), Fv2E-UNC5H2-IC (Fv-UNC5H2) with or without AP20187 (AP).

Figure 14C: UNC5H2-induced caspase activation is inhibited by dimerization induced by AP20187, as measured by relative caspase-3 activity. HEK293T cells were transfected with mock vector pCMV (Cont.), Fv2E (Fv), Fv2E-UNC5H2-IC (Fv-UNC5H2) with or without AP20187 (AP). Index of relative caspase activity is presented as the ratio between the caspase activity of the sample and that measured in HEK293T cells transfected with pCMV. Standard deviations are indicated (n=3).

Figures 15A-15C: The recombinant soluble fifth fibronectin domain of DCC (DCC-5Fbn) inhibits netrin-1-induced DCC-multimerization

Figure 15A: Affinity curve of netrin-1 on DCC-5Fbn measured by ELISA test shows that DCC-5Fbn is able to bind netrin-1. DCC-5Fbn (100 ng) or IL3-R (600 ng) was coated and increasing doses of netrin-1 were added (0 to 800 ng). The IL3 values were substracted to the DCC-5Fbn values. The approximate Kd of DCC-5Fbn/netrin-1 was estimated at 5 nM.

Figure 15B: Competition assay. As in (A) but the complete extracellular domain of DCC (DCC-EC, 125 ng) was coated instead of DCC-5Fbn and netrin-1 was added (50 ng) in the presence of either DCC-5Fbn (625 ng) or the complete DCC-EC (125 ng). Note that DCC-5Fbn fails to compete with DCC/netrin-1 interaction.

Figure 15C: Netrin-1-induced DCC multimerization is inhibited by DCC-5Fbn. Lysates of HEK293T cells transiently transfected with HA-DCC and/or c-myc-DCC expressing constructs with or without netrin-1 (300 ng/mL) and/or DCC-5Fbn (900 ng/mL) were subjected to HA pull-down (IP α-HA). c-myc-DCC presence was revealed with anti-c-myc antibody. Total: Western blot on lysate before pull-down.

Figures 16A and 16B: DCC-5Fbn antagonizes netrin-1-blocking effects on DCC-induced cell death

Figure 16A: HEK293T cells were transiently transfected with a mock (Cont.) or a full length DCC construct and incubated or not with netrin-1 (300 ng/ml) and/or DCC-5Fbn (800 ng/mL). Cell death was assessed by trypan blue staining.

Figure 16B: Metastatic breast cancer cells 4T1 were cultured in the presence (+ DCC-5Fbn) or absence (- DCC-5Fbn) of DCC-5Fbn (300 ng/mL) for 24 hours and cell death was also measured by trypan blue exclusion assay. Standard deviations are indicated (n=3).

Example 1: Materials and Methods

Cell line, cell cultures, transfection procedure, reagents and immunoblots:

[0064] 4T1 and 67NR cells were a kind gift from F. Miller (Detroit, MI, USA). Cal51, MCF7, MDA-MB231, 453, 361, 157, SK-BR3, CAMA-1, T47D were cultured using standard procedure. Human breast cell lines listed in figure 8B and more specifically T47D and SKB7 cell lines were obtained from D. Birnbaun.67NR cells were stably transfected using the lipofectamine reagent (Invitrogen) and puromycine (Sigma) selection. Transient transfections of Human Embryonic Kidney 293T cells (HEK293T) were performed as previously described [10] according to a modified calcium phosphate procedure or using Lipofectamine according to the manufacturer's instructions (Invitrogen). The breast cancer 4T1 cell line was described previously [20]. 4T1-luc cells were obtained by stable transfection of a CMV-luciferase vector bearing hygromycine resistance. Clones were selected by luminescence intensity using the luminoskan Ascent Station (Labsystems). Immunoblots were performed as described previously [6] using anti-c-myc (Sigma; 1/200) anti-FlagM2 (Sigma, 1/200) or anti-HA (Sigma; 1/500). The artificial dimerizing agent AP20187 was from Ariad Pharmaceuticals. The complete extracellular domain of DCC (DCC-EC), DCC-EC-Fc were obtained from R&D system and Netrin-1 from Apotech corp. For cell death analysis, caspase activity measurement and immunoprecipitation, AP20187 was used at a final concentration of 10 nM and netrin-1 was used at a final concentration of 300 ng/mL.

Human breast tumors samples:

[0065] 51 human breast cancer samples were provided by the tumor bank of the Centre Léon Bérard. Fresh tissue of the tumor was obtained during breast surgery prior any systemic therapy and snap-frozen in liquid nitrogen.

Site directed mutagenesis and plasmid constructs:

[0066] PGNET-1 pCMV and pGNET-1 encoding chick netrin-1 were was previously described [6]. pKk was described [22]. The dominant negative mutants for DCC (pCR-DCC-IC-D1290N) and for UNC5H (pCR-UNC5H2-IC-D412N) have been previously described [6,27,7]. HA-DCC was obtained by introducing a HA tag in the template pCMV-DCC [6] by QuikChange site-directed mutagenesis system (Stratagene) using the following primers:

DCC-HA F: 5'-CACAGGCTCAGCCTTTTATCCATATGATGTACCGGATTATGCATA ACATGTATTTCTGAATG-3' (SEQ ID NO:1); DCC-HA R: 5'- CATTCAGAAA TACATGTTATGCATAATCCGGTACATCATATGGATAAAAGGCT-GAGCCTGTG-3' (SEQ ID NO:2).

c-myc-DCC was also obtained by introducing a c-myc tag in the template pCMV-DCC by QuikChange using the following primers: DCC-myc F: 5'-CACAGGCTCAGCCTTTGAGCAGAAGTTGATAAGTGAGGAAGATCTGTAA-CATG TATTTCTGAATG-3' (SEQ ID NO:3). DCC-myc R: 5'- CATTCAGAAATACATGTTAC AGATCTTCCTCACT-TCTCAACTTCTGCTCAAAGGCTGAGCCTGTG-3' (SEQ ID NO:4).

HA-Fv2E encoding expression vector (in pC4M) from the Argent Regulated Homodimerization kit is from Ariad Pharmaceuticals. From this plasmid, the HA-Fv2E-DCC-IC plasmid was constructed. A PCR fragment of the intracellular domain of DCC (1122-1447) was obtained with the primers: F 5'-TATGTCGACCGACGCTCTTCAGCCCAGCAGAGA-3' (SEQ ID NO:5) and R 5'-TATGAATTCTTAGTCGAGTGCGTAGTCTGGTACGTCGTACGGATAAAAGGCTGA GCCTGTGATGGCATTAAG -3' (SEQ ID NO:6).

[0067] The reverse primer fused to the HA tag to C-terminal end of DCC. The PCR fragment was subcloned in HA-Fv2E by SalI and EcoRI restriction digestion. The c-myc-Fv2E-DCC-IC was obtained using the QuikChange site-directed mutagenesis system (Stratagen) with pC4M-Fv2E-DCC-IC-HA as template and the following primers: primer F: 5'-CTTAATGCCATCACAGGCTCAGCCTTTGAACAGAAACTCATCTCTGAAGAGGAT CTGTAAGAATTCATAAAGGGCAAT-3' (SEQ ID NO:7) and primer R: 5'-ATTGCCCTTTATGAATTCTTACAGATCCTCTTCAGAGATGAGTTTCTGTTCAAAG GCTGAGCCTGTGATGGCATTAAG-3' (SEQ ID NO:8).

[0068] HA-UNC5H2 (in pcDNA3.1) has already been described [7] the constructs encoding FlagM2-UNC5H2 was generated by cloning in p3xFlag-CMVTM-7.1 (Sigma) the NotI-EcoRI PCR fragment derived from HA-UNC5H2 as template and the following primers: primer F 5'-GCGCGGCCGCAGGGCCCGGAGCGGG-3' (SEQ ID NO:9) and primer R 5'-CGGAATTCTCAGCAATCGCCATCAGTGGTC-3' (SEQ ID NO:10).

[0069] HA-Fv2E-UNC5H2-IC- and c-myc-Fv2E-UNC5H2-IC in pC4M were generated by PCR amplification of the UNC5H2 intracellular domain using the following primers: UNC5H2-HA F 5'-CGGTCGACGTGTACCGGAGAAACTGC-3' (SEQ ID NO:11) and UNC5H2-HA R 5'-GCGAATTCTCATGCATAATCCGGCACATCATACGGATAGC AATCGCCATCAGTGGTC-3' (SEQ ID NO:12), and UNC5H2-myc F 5'-CGGTCGACGTGTACCGGAGAAACTGC-3' (SEQ ID NO:13) and UNC5H2-myc R 5'-GCGAATTCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCAATCGCCATCA GTGGTC-3' (SEQ ID NO:14) respectively. The PCR fragments were cloned in HA-Fv2E by SalI and EcoRI restriction digestion.

[0070] The cDNA encoding the HA-Fv2E-UNC5H2-IC and c-myc-Fv2E-UNC5H2-IC fusion proteins were then subcloned in pcDNA3.1-TOPO by PCR using the following primers: Fv2E F 5'-CCACCATGGGGAGTAGCA-3' (SEQ ID NO:15) and UNC5H2-HA R 5'-TCATGCATAATCCGGCACATCATACGGATAGCAATCGCCATCAGTGGTC-3' (SEQ ID NO:16), and Fv2E F 5'-CCACCATGGGGAGTAGCA-3' (SEQ ID NO:15) and UNC5H2-myc R 5'-TCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCAATC GCCATCAGTGGTC-3' (SEQ ID NO:17) respectively and HA-Fv2E-UNC5H2-IC and c-myc-Fv2E-UNC5H2-IC in pC4M as respective templates.

[0071] Ps974-DCC-5Fbn allowing bacterial expression of the fifth fibronectin type III domain of DCC was obtained by inserting a Pst1/BamH1 DNA fragment generated by PCR using pDCC-CMV-S as a template.

DCC-5Fbn production:

[0072] DCC-5Fbn production was performed using a standard procedure. Briefly, BL21 cells were forced to express DCC-5Fbn in response to imidazole and the BL21 lysate was subjected to affinity chromatography using Flag-agarose (Sigma).

Immunoprecipitation:

[0073] Coimmunoprecipitations were carried out on HEK293T cells transfected with various tagged constructs as described previously [27] Briefly, HEK293T cells were lysed in 50 mM HEPES pH 7.6, 125 mM NaCl, 5 mM EDTA and 0.1% NP-40 in the presence of protease inhibitor, and further incubated with anti-HA (Sigma), anti-c-myc antibody (Sigma), anti-FlagM2 (Sigma) and protein-A Sepharose (Sigma). Washes were done in 50 mM HEPES pH 7.6, 125 mM NaCl, 5 mM EDTA.

Binding assay and ELISA competition assay:

[0074] DCC-5Fbn (100ng) or IL3-R (R&D systems, 600ng) was coated on maxisorp plate (Nunc) and increasing doses of netrin-1 (Apotech) were added (0 to 800ng) for binding assay. DCC-EC (R&D systems, 125ng) was coated on maxisorp plate for ELISA competition assay. Netrin-1-FlagM2 (50ng) and competitor DCC-EC (125ng) or DCC-5Fbn (625ng) were then added simultaneously. After washes, for both binding assay or ELISA competition assay, residual netrin-1-FlagM2 still fixed was revealed with an anti-FlagM2 antibody (Sigma).

DCC/netrin-1 ELISA assays:

[0075] DCC-EC-Fc (1.25ng/ml) or UNC5H2-EC-Fc (0.5ng/ml) was adsorbed on 96-well maxisorp plate (Nunc) according to manufacturer instruction. Flag-tagged Netrin-1 (0.5ng/ml) was then added together with increased concentrations of DCC-EC-Fc. After a 1 hour incubation, plates were extensively washed and bound netrin-1 was detected by

immunolabelling using an anti-flagM2 antibody (Sigma) and a HRP-goat-anti-mouse (Jackson). Colorimetric measurement was performed on the multilabel Victor station (Wallac).

Cell death assays:

[0076] 67NR, 4T1, CAL51, T47D and SKBR7 were grown in serum-poor medium and were treated (or not) with DCC-EC-Fc or DCC-5Fbn for 24hours. Cell death was analysed using trypan blue staining procedures as described previously [6]. The extent of cell death is presented as the percentage of trypan blue-positive cells in the different cell populations. To select transfected cells, cells were co-transfected with the surface marker pKk and the plasmid encoding genes of interest. Transfected cells expressing the marker were magnetically labeled with MACSelect Microbeads and separated using a MACS Separator and Separation Columns (Miltenyi Biotec). Trypan blue exclusion was assayed on these purified cells. Cell survival was also measured by MTT assay using Vybrant MTT assay kit (Molecular Probes) according to the manufacturer procedures.

Caspase activity measurement:

[0077] Relative Caspase activity was determined by flow cytometric analysis as follows: 2.10^5 treated cells were harvested, washed once in 1 ml PBS, and resuspended in 200 µl staining solution containing FITC-VAD-fmk (CaspACE, Promega). After incubation for 60 min at 37°C, cells were washed in 1 ml PBS and resuspended in 200 µl PBS for flow cytometry analysis. Stained cells were counted using a FACS Calibur (Becton Dickinson) and CellQuest analysis software with excitation and emission settings of 488 nm and 525-550 nm (filter FL1), respectively. Caspase-3 activity was measured by using the Caspase-3 assay from BioVision. Caspase activity is presented as the ratio between the caspase activity of the sample and that measured in HEK293T cells transfected with pCMV. For cell death analysis and caspase activity measurement, AP20187 or/and netrin-1 or/and DCC-5Fbn were added in cell culture medium 20 hours and 1 hour before collecting cells.

Quantitative RT-PCR:

[0078] To assay netrin-1 expression in human breast tumors, total RNA was extracted from biopsies of patients undergoing surgery for breast cancer using Nucleospin RNAII kit (Macherey-Nagel) and 1 µg was reverse-transcribed using the iScript cDNA Synthesis kit (BioRad). Real-time quantitative RT-PCR was performed on a LightCycler 2.0 apparatus (Roche) using the Light Cycler FastStart DNA Master SYBERGreen I kit (Roche). Reaction conditions for all optimal amplification, as well as primer selection of netrin-1, were determined as already described [18]. The ubiquitously expressed human PBGD, TBP and mouse RPLP0 genes showing the less variability in expression between normal and breast tumoral tissues [25 28] were used as internal controls. The following primers were used:

PBGD:

- FOR: 5'-CTGGAGTTCAGGAGTATTCGGGG-3' (SEQ ID NO:18),
- REV: 5'-CAGATCCAAGATGTCCTGGTCCTT-3' (SEQ ID NO:19);

TBP:

- FOR: 5'-CACGAACCACGGCACTGATT-3' (SEQ ID NO:20),
- REV: 5' TTTTCTTGCTGCCAGTCTGGAC 3' (SEQ ID NO:21);

Human netrin-1-NTN1:

- FOR: 5'-TGCAAGAAGGACTATGCCGTC-3' (SEQ ID NO:22),
- REV: 5'-GCTCGTGCCCTGCTTATACAC-3' (SEQ ID NO:23);

UNC5B:

- FOR: 5'-TGCAGGAGAACCTCATGGTC-3' (SEQ ID NO:24),
- REV: 5'-GGGCTGGAGGATTACTGGTG-3' (SEQ ID NO:25);

DCC:

- FOR: 5'-AGCCAATGGGAAAATTACTGCTTAC-3' (SEQ ID NO:26),
- REV: 5'-AGGTTGAGATCCATGATTTGATGAG-3' (SEQ ID NO:27);

UNC5C:

- FOR: 5'-GCAAATTGCTGGCTAAATATCAGGAA-3' (SEQ ID NO:28),
- REV: 5-GCTCCACTGTGTTCAGGCTAAATCTT-3' (SEQ ID NO:29).

Mice, intraveinous and mammary gland injections, measurement of metastasis development:

[0079] Syngenic mice model. Female BALB/cByJ mice of 8-11 weeks of age from Jackson Laboratory were used for surgery. For mammary gland injection of 67NR cells, mice were anesthetized with 2,2,2-tribromoethanol and 10^6 cells in 50 μl PBS were injected into the mammary gland and mice were sacrificed when the tumor exceeded 1.5 cm and caused impediment to the movement of the animal. For intravenous injection, 10^5 tumor 4T1-luc cells in 150 μl PBS were injected into a tail vein and mice were either sacrificed at day 13-15 (after 4T1 cells injection) or at day 20-23 (after 67NR cells injection) or analyzed using luminescence recording. When animals were sacrified, lungs were removed,weighed and compared to the whole weight of the animal, and metastatic nodules counted.

Xenograft in nude mice:

[0080] Five-week-old (20-22 g body weight) female athymic nu/nu mice were obtained from Charles River. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. Human breast cancer cell lines (SKBR7, T47D and H358) were implanted by s.c. injection of 5.10^6 cells in 200 μL of PBS into the left flank of the mice. When tumors were established (5 weeks for T47D, 2 weeks for SKBR7 and 5 days for H358), PBS or 20 μg of DCC-5Fbn were administered into the tumor (i.t.) everyday during 14 days. Tumor sizes were measured by a caliper. The tumor volume was calculated with the formula $v = 0.5*(length*width^2)$.

Tumor analysis:

[0081] 4 μm-thick lung sections were prepared and stained with hematoxylin-eosin-saffron. Histological classification and grading of neoplastic lesions was performed in a blinded fashion and according to standard procedures. For in vivo imaging of metastasis using 4T1-luc cells, the light resulting from the bioluminescent oxidation of the intra-peritonaly injected endotoxin-free luciferin (Promega) (120 mg/kg bodyweight) was detected and quantified (10 minutes after injection) with a NightOWL LB 981 NC 100 system from Berthold Technologies, using an anaesthesia system with gaseous isoflurane from TEM SEGA.

Example 2: Netrin-1 dictates metastasis of breast tumor by inhibiting apoptosis

[0082] We first analysed netrin-1 and its dependence receptors -i.e., DCC and UNC5H expression by Q-RT-PCR in a panel of 30 breast primary tumors, 15 of which were without known metastatic evolution, and 15 that were metastatic at diagnosis. While DCC was barely detectable and UNC5H failed to show significant change between the two types of tumors, netrin-1 appeared to be significantly more expressed in metastatic breast tumors than in non-metastatic breast tumors (figure 1A).

[0083] 60% of tested metastatic breast tumors showed an over-expression of netrin-1 (range from 1.4 to 9.6 fold, $p<0.015$) (Table 1).

Table 1: The percentage of samples showing a netrin-1 expression higher than the average expression in non-metastatic biopsies is indicated, as is the range of the over-expression.

n= 15	metastasis	non metastasis
% of breast tumors that over-express netrin-1	60	33
Range of over-expression of netrin-1	1.4-9.6	1.6-2.9

[0084] In mice, Miller and colleagues developed a powerful model to study the biology of metastatic versus non-metastatic tumors: from a single primary mammary tumor that occurred naturally in a BALB/c mouse, a series of cell lines were obtained that showed different metastatic potentials when injected into syngenic mice. In particular, while

67NR cells form primary mammary tumors but no metastasis, 4T1 cells form primary tumors and metastasis, especially in the lung, the bone marrow and the liver [20]. Interestingly, while netrin-1 failed to be detected in 67NR cells, netrin-1 was highly expressed in 4T1 cells (figure 1B).

[0085] To assay whether the metastatic potential of 4T1 cells, compared to that of 67NR cells, was related to netrin-1 expression, 67NR cells were forced to stably express netrin-1. Mock transfected 67NR cells or 67NR-net cells that express netrin-1 (figures 2A, 2B) were injected in mammary gland or i.v. and metastasis was monitored by anatomopathology examination of lungs. Both cell lines failed to form metastasis when injected in fat pad, suggesting that the presence of netrin-1 in 67NR is not sufficient to allow lung metastasis formation from the primary site. However, when cells were injected i.v., a significant increase of metastasis in the lungs was detected in the netrin-1 expressing 67NR (figure 2C).

[0086] See Table 2 showing the number of under-pleural (metastasis outside the lung) and intra-parenchymatous nodules (metastasis in the lung).

Table 2:

	under-pleural lesions		**Intra parenchymatous Lesions (lung metastasis)**	
mice injected with clone:	Number of lung Affected	Number of noduled per lung (range)	Number of lung affected	Number of nodules per lung (range)
67NR1	3	0-5	0	0
67NR2	2	0-2	0	0
67NR-net1	3	0-5	1	0-4
67NR-net2	1	0-3	2	1-2

[0087] Thus, netrin-1 expression appears to be a crucial event that supports metastasis formation, probably by favoring tumor cells after intravation.

[0088] Because netrin-1 appears to be sufficient for the metastastic potential of 67NR cells after intravation and because netrin-1 was shown to inhibit netrin-1 dependence receptors-induced cell death [6, 7, 18], we next investigated whether autocrine production of netrin-1 provides a selective advantage to 4T1 cells by inhibiting DCC/UNC5H-induced cell death in these cells. A domain located in the N-terminus of netrin-1 (the so-called laminin-VI domain) interacts with both DCC and UNC5H receptors (figure 3A; [21]), so that a soluble extracellular domain of DCC (DCC-EC-Fc) can inhibit both DCC/netrin-1 and UNC5H/netrin-1 interaction (not shown). DCC-EC-Fc was then added to a culture of 4T1 cells and cell death was monitored either by a trypan blue exclusion assay (figure 3B) or by measuring caspase activity (figure 3C). As shown in figure 3C, addition of the competing protein in the culture medium triggers death of 4T1 cells in a dose-dependent manner. Moreover, this effect is specific, as DCC-EC-Fc had no effect on 67NR cell death and IL3R-EC-Fc (the extracellular domain of IL3 receptor) failed to trigger 4T1 cell death (figures 3B, 3C). This effect is due to netrin-1 inhibition, as addition of an excess amount of netrin-1, together with DCC-EC-Fc inhibited the pro-apoptotic activity of DCC-EC-Fc on 4T1 cells (not shown). To restrict the competition to a smaller domain, we produced the fifth fibronectin type III domain of DCC, which is known to interact with netrin-1 [21]. Addition of this domain -DCC-5Fbn- had a similar pro-apoptotic activity on 4T1 cells (figure 3D). Thus, while netrin-1 appears to confer metastatic potential to tumor cells in mice, these netrin-1 expressing metastatic tumor cells can be engaged toward apoptosis by inhibition of the netrin-1/receptors interaction.

[0089] To further analyse whether this holds true in human breast tumor cells, netrin-1 expression was analysed in a panel of human metastatic breast cancer cell lines (see Table 3).

Table 3:

Human breast carcinoma cell line	**Netrin-1 transcriptional expression**	**DCC-EC-Fc sensitivity**
MDA-MB 157	++++	+/-
MCF-7	++++	-
CAMA-1	+++	-
SKBR3	++	*ND*
SAV-NUDE	++	*ND*

(continued)

Human breast carcinoma cell line	Netrin-1 transcriptional expression	DCC-EC-Fc sensitivity
Cal51	++	+++
MDA-MB231	+	++
MDA-MB453	+	+
T47D	-	*ND*
T47D*	++	++
*Carried out on another T47D cell line		

Table 3 showing the different human metastatic breast cell lines analysed for the netrin-1 expression by Q-RT PCR as in figures 1A, 1B, and their sensitivity to DCC-EC-Fc by measurement of cell death by trypan blue exclusion. The relative amount of netrin-1 expression and DCC-EC-Fc sensitivity are indicated by (+), while the absence of these criteria is indicated by (-). In some cell lines the DCC-EC-Fc has not been determined (ND).

[0090] As expected, netrin-1 is expressed in a large number of metastastic cell lines and some of them undergo apoptosis when cultured in the presence of DCC-EC-Fc. As an example, CAL51 cells underwent apoptosis in a dose-dependent manner in response to DCC-Ec-Fc. As above, addition of netrin-1 in excess reverts the effect of DCC-EC-Fc, supporting the view that the competing proteins kill these human cell lines by inhibiting the netrin-1/netrin-1 receptors interaction. Moreover, a clonal selection from CAL51 cells allowed the establishment of a CAL51-36 cell line, that is much more susceptible to cell death in response to DCC-EC-Fc (figure 4B). Because the DCC-EC-Fc or DCC-5Fbn may consequently represent good tools to trigger selective apoptosis of human metastatic tumors cells.

[0091] Here we show that netrin-1 expression may be considered as a marker of breast tumor dissemination. More than half of the breast tumors with metastasis propensity showed elevated netrin-1 expression. Both the mice model described above and the data obtained on human breast cancer cell lines support the view that this elevated netrin-1 level is a selective advantage acquired by the cancer cell to escape netrin-1-dependence receptors induced apoptosis and, consequently, to survive independently of netrin-1 availability. From a mechanistic point of view, this autocrine expression of netrin-1 inhibits cell death induced by UNC5H. Indeed, DCC was barely detectable in the two groups -metastastic and non metastatic- of breast cancers studied, hence suggesting that DCC is either down-regulated early during breast tumorigenesis or is only weakly expressed in breast tissue. Moreover, inhibition of UNC5H-induced apoptosis by co-expression of a dominant negative mutant form of the UNC5H pro-apoptotic activity inhibits CAL51 cell death in response to DCC-EC-Fc (not shown). This may fit with the recent observation that part of UNC5H2 pro-apoptotic activity passes through the activation of the serine/threonine DAPK [22] a protein involved in metastasis regulation [23].

[0092] These observations not only provide evidence for the importance of the ligand/dependence receptor pair in the regulation of tumor development, but also enlighten a new therapeutic strategy. Indeed, as of today, there is no efficient treatment for patients with metastatic breast cancer, a lack of treatment that leads to the death of 400,000 women worldwide per year [24]. Here we propose that a treatment based on inhibition of the interaction between netrin-1 and its dependence receptors could positively affect half of the patients suffering from metastatic breast cancer. These treatments could include chemical drugs, monoclonal antibodies or the DCC-5Fbn protein presented here. Whether this should be considered as a strategy preventing metastasis formation, which would imply a long-term preventive treatment on women diagnosed with primary breast cancer, or as a strategy that could be used to induce metastasis regression remains to be shown. Future clinical trials should also answer this point.

[0093] Here we describe that, unlike human non-metastatic breast tumors, the majority of metastatic breast cancers shows an over-expression of netrin-1. In a mice model, we demonstrate that in non-metastatic mammary tumor cells, forced expression of netrin-1 is associated with metastasis in the lungs. Moreover, mice or human metastatic tumor cell lines, that were shown to highly express netrin-1, undergo apoptosis when the netrin-1/receptors interaction is inhibited by a competing protein. Thus, netrin-1 is a marker for human metastatic cancer such as metastatic breast and inhibition of the netrin-1/receptors interaction represents a therapeutic approach to induce metastatic cell death.

Example 3: Restoration of the netrin-1 dependence receptors pathway trippers apoptosis in metastatic breast tumors

[0094] Netrin-1 and its dependence receptors -i.e., DCC, UNC5H2, UNC5H3 expression were analysed by Q-RT-PCR in a panel of 51 breast tumors. It includes patients whose tumors were either localized to the breast (N0, 16 patients), had nodal involvement (N+, 19 patients) or had distant metastatic disease at the time of diagnosis (M+, 16 patients). While DCC was barely detectable and UNC5H expression failed to display significant changes between the different

types of tumors (not shown), netrin-1 is significantly more expressed in N+ tumors than in N0 tumors (median: 1.8 versus 0.5, p=0.007) with a range of netrin-1 expression higher in N+ tumors (figure 5 and Table 4).

Table 4: The percentage of samples showing a netrin-1 expression higher than the average expression in N0 biopsies, 5 fold higher or 15 fold higher is indicated, as is the range of the over-expression.

n = 51		N0 Localised to breast (n=16)	N+ Nodal involvement (n=19)	M+ Distant metastasis (n=16)
% of breast tumors that over-express netrin-1		31	73.7	93.7
% of breast tumors that over-express netrin-1.	More than 5 fold	0	31.5	62.5
	More that 15 fold	0	0	37.5
Range of over-expression of Netrin-1		0.02-4.6	0.03-12.8	0.6-111.7

[0095] 31.5% of the N+ tumors show at least a 5 fold increase in netrin-1 expression while no such increase was detected in any tested N0 tumors (figure 5 and Table 4). An even more striking difference is observed when comparing netrin-1 expression in M+ versus N0 tumors (median: 7.8 versus 0.5, p<0.0001). Along this line 62.5% of M+ tumors show at least a 5 fold increase in netrin-1 expression. A significant difference in netrin-1 expression also exists between N+ and M+ tumors (median: 1.8 versus 7.8, p=0.009). Moreover, netrin-1 overexpression is higher in M+ tumors than in N+ tumors, as 37.5% of M+ tumors display more than a 15 fold increase in netrin-1 level, while such an increase is not detected in N+ tumors (figure 5 and Table 4). Thus, netrin-1 up-regulation is a marker of nodal involvement and distant metastatic disease in human breast cancer.

[0096] In mice, Miller and colleagues developed a powerful model to study the biology of metastatic versus non-metastatic tumors: from a single primary mammary tumor that occurred naturally in a BALB/c mouse, a series of cell lines were obtained that showed different metastatic potentials when injected into syngenic mice. In particular, while 67NR cells form primary mammary tumors but no metastasis, 4T1 cells form primary tumors and metastasis, especially in the lung, the liver and the bone marrow [20]. Interestingly, while netrin-1 failed to be detected in 67NR cells, netrin-1 was highly expressed in 4T1 cells (figure 1B).

[0097] To first assay whether the metastatic potential of 4T1 cells, compared to that of 67NR cells, was related to netrin-1 expression, 67NR cells were forced to stably express netrin-1. Mock transfected 67NR cells or 67NR cells that express netrin-1 (figures 2A and 2B) were injected into mammary glands and metastasis was monitored by anatomo-pathological examination. Both cell lines failed to efficiently form metastasis in liver or in lungs when injected in mice fat pads (19 mice were injected with netrin-1 expressing 67NR cells and only two suspicions of micro-metastases, one in the lung and one in the liver were detected) (Table 5).

Table 5: Lungs and liver metastasis of fat pad-injected 67NR versus netrin-1 expressing cells. One control cell clone bearing puromycine resistance (67NR-mock), one netrin-1 expressing cell clone (67NRnet1) and one polyclonal population of netrin-1 stably transfected 67NR (67NR-net1-polyclonal) were injected in fat pad of mice and metastasis was analyzed in the lung or liver environment.

Cells injected	Mice (n)	Primary tumors	Metastasis	Comment
67NR-mock	9	9	0	
67NR-net1	7	7	0	Suspicion of 1 micrometastase in liver
67NR-net1 polyclonal	12	12	0	Suspicion of 1 micrometastase in lung

[0098] Thus, netrin-1 expression in tumor cells is not sufficient to enable metastasis formation from the primary site.

[0099] Because netrin-1 was shown to inhibit netrin-1 dependence receptors-induced cell death [6, 7, 18], we next investigated whether the autocrine production of netrin-1 detected in metastatic 4T1 cells confers a selective advantage to these cells, by inhibiting DCC/UNC5H-induced cell death. To assay this, we looked for a compound that may titrate netrin-1. It was reported that a domain located in the N-terminus of netrin-1 (the so-called laminin-VI domain) interacts with both DCC and UNC5H receptors (figure 3A; [21]). We show that a soluble extracellular domain of DCC (DCC-EC-Fc) can inhibit both DCC/netrin-1 and UNC5H2/netrin-1 interaction, as measured by ELISA assay (figure 6A). DCC-EC-Fc was then added to a culture of 4T1 cells and cell death was monitored, either by a trypan blue exclusion assay (figures

3B and 6B) or by measuring caspase activity by flow cytometry (figures 3C and 6C). As shown in figures 3B, 3C, 6B and 6C addition of the competing protein in the culture medium triggers death of 4T1 cells in a dose-dependent manner. This effect is specific, as DCC-EC-Fc had no effect on 67NR cell death (figures 3B, 3C, 6B and 6C) and IL3R-EC-Fc (the extracellular domain of the IL3 receptor) failed to trigger 4T1 cell death (figure 3D). Thus, 4T1 cells survive through autocrine production of netrin-1, which blocks netrin-1 receptors-induced cell death.

[0100] Because the complete extracellular domain of DCC appears as only of modest interest for use in vivo and in therapy (DCC-EC-Fc is about 1100 amino-acid large), we looked for an alternative polypeptide from the DCC extracellular domain, which could trigger apoptosis in 4T1 cells. We consequently produced the fifth fibronectin type III domain of DCC, DCC-5Fbn, which is known to interact with netrin-1 [21] (figure 3A). Interestingly, this 100 amino-acid protein does not interfere with the binding of DCC/netrin-1 or UNC5H/netrin-1, but affects the ability of netrin-1 to trigger multimerization of these receptors (see Example 4). As DCC and UNC5H multimerization is a pre-requisite for the netrin-1 inhibitory activity on DCC/UNC5H-induced cell death, the addition of DCC-5Fbn triggers apoptosis in DCC-expressing cells cultured in the presence of netrin-1 [22] and triggers death of both 4T1 (figure 7A).

[0101] We next investigated whether the cell death effect observed in vitro may be extended in vivo. To do so, 4T1 cells were stably transfected with a luciferase-based vector and 4T1-luc cells were intraveinously (i.v.) injected into syngenic BALB/c mice. Mice were then intraperitonaly (i.p.) and i.v injected (1 injection every two days, once i.v., once i.p.) from day 0 to day 13 with either PBS buffer or Flag-tagged-DCC-5Fbn (1.25 μg/mouse g/injection). Metastasis formation was then analyzed using luminescence recording. As shown in figures 7B and 7C, when i.v. injected, 4T1-luc cells efficiently colonize lungs. On the opposite, mice treated with DCC-5Fbn show a dramatic reduction of lung metastasis (figures 7B and 7C). This inhibition of metastasis formation was then confirmed by anatomo-pathological examination of lungs, (not shown, see Table 6).

Table 6: Total number of lung metastatic nodules in individual mice were counted under a dissection scope in the two treated populations (+PBS, +DCC-5Fbn)

Treatment	Mice (n)	Average of Metastatis per mouse	Range of metastasis per mouse
PBS	10	42.4	0-75
DCC-5Fbn	10	2.6	0-6

[0102] Similar results were obtained when we performed daily i.p. injection of GST-tagged-DCC-5Fbn instead of Flag-tagged-DCC-5Fbn and GST-FADD instead of PBS (not shown). Thus, in mice, the inhibition by DCC-5Fbn of the pro-survival activity conferred by netrin-1 autocrine expression is associated with metastasis prevention.

[0103] The acquired survival advantage through netrin-1 autocrine expression is not restricted to murine tumor cells, as it is detected in human breast cancer cell lines. Indeed, netrin-1 was shown to be expressed in a sizeable fraction of human breast cancer lines (figure 8A) and addition of DCC-EC-Fc or DCC-5Fbn to naturally netrin-1 expressing human breast adenocarcinoma T47D or SKBR7 cell cultures triggers cell death induction measured either by caspase-3 activity assay or MTT assay (figure 8B and not shown). This effect is due to netrin-1 inhibition, as addition of an excess amount of netrin-1, inhibited the pro-apoptotic activity of DCC-EC-Fc/DCC-5Fbn (not shown). To monitor the anti-tumor effect of DCC-5Fbn, xenografts of T47D cells were implanted in nude mice. When tumors reached a palpable size, mice were daily treated with either PBS or DCC-5Fbn and tumor volume was determined for 18 days. Similarly to the data obtained in the syngenic model above, DCC-5Fbn fully inhibits tumor growth (Table 7).

Table 7: showing the number and behavior of xenografted T47D tumors that have been treated either with PBS or DCC-5Fbn. The number of tumors that has grown in size more than 40% and the number of tumors that has a reduced size (more than 30%) are indicated

Treatment	Number of mice	Tumor growth (> 40 %)	Tumor regression (> 30 %)
PBS	4	3	0
DCC-5Fbn	5	0	3

[0104] Here we show that netrin-1 expression may be considered as a marker of breast tumor ability to disseminate. Most of the breast tumors with metastasis propensity showed elevated netrin-1 expression. Both the data obtained on human/mice breast cancer cell lines and the syngenic/human xenograft mice models described above and support the view that this elevated netrin-1 level is a selective advantage acquired by the cancer cell to escape netrin-1-dependence receptors induced apoptosis and, consequently, to survive independently of netrin-1 availability. From a mechanistic

point of view, in the human pathology, this autocrine expression of netrin-1 probably inhibits UNC5H-induced cell death. Indeed, DCC was barely detectable in the different groups (N0, N+, M+) of breast cancers studied, hence suggesting that DCC is either down-regulated early during breast tumorigenesis or is only weakly expressed in breast tissue. Moreover, inhibition of UNC5H-induced apoptosis by co-expression of a dominant negative mutant form of the UNC5H pro-apoptotic activity inhibits human breast cancer cell death in response to DCC-EC-Fc (not shown).

[0105] Thus, as predicted by the dependence receptor model, we have now shown that a tumor cell can escape dependence receptor dependency in at least three manners. First, expression of the dependence receptor can be down-regulated, as extensively described for DCC and more recently for UNC5H [15, 17, 19, 29]. Second, the downstream death signalling can be shut down. Along this line, we have recently shown that UNC5H2 pro-apoptotic activity relies on the binding of UNC5H2 to the serine/threonine DAPK [22], a protein that was demonstrated to be involved in metastasis regulation and down-regulated in human malignancy [23]. Similarly, a recent report by Stupack and colleagues shows that, in the case of some integrins that act as dependence receptors, caspase-8, which triggers the cell death mediated by these integrins, is crucial for neuroblastoma metastasis [30]. Here we show that a third selective advantage for the tumor cell is the self-production of the dependency ligand. One intriguing question remains as to why breast tumors with metastatic propensity seem to have preferably selected netrin-1 self-production rather than receptor loss, while colorectal tumors have mostly selected loss of the receptors rather than gain of netrin-1 expression - indeed, only 7% of colorectal cancers show an increase of netrin-1 expression [18]. A possible explanation is that netrin-1 expression not only confers a gain in survival to the migrating cells, but also possibly a gain in the non-apoptotic/positive signalling of netrin-1 receptors. Along this line, it is important to note that netrin-1 was originally described as a guidance cue [31], which, even though completely unproven, could play a role in the tropism of metastatic cells. Other proposed roles of netrin-1 include adhesion and morphogenesis regulation [32-34], both mechanisms that may be of importance for metastasis development. Similarly, netrin-1 was recently proposed to play a role during embryonic angiogenesis and even though conflicting results have been published [36-38], we cannot at this stage discard the role of netrin-1 as an angiogenic factor that somehow could favor metastasis development at the secondary site. However, the gain of "positive" signalling by netrin-1 autocrine expression is probably not sufficient per se to promote metastasis, as forced expression of netrin-1 in non-metastatic cells failed to be associated with metastasis formation.

[0106] These observations not only provide evidence for the importance of ligand/dependence receptors pairs in the regulation of tumor development, but also enlighten a new therapeutic strategy. Indeed, as of today, there is no efficient treatment for patients with metastatic breast cancer, a lack of treatment that leads to the death of 400,000 women worldwide per year [24]. Here we propose that a treatment based on inhibition of the interaction between netrin-1 and its dependence receptors could positively affect a large fractions of the patients suffering from metastatic cancer, such as breast cancer -i.e. patients who would shown high netrin-1 expression in primary tumors-. These treatments could include chemical drugs, monoclonal antibodies or the DCC-5Fbn protein presented here.

Example 4: Netrin-1 expression and inhibition of netrin-1 activity in other human tumors

[0107]

A) Netrin-1 is a marker of aggressiveness in human neuroblastoma (see figure 9A, its legend and table 8) and inhibiting netrin-1 activity promotes neuroblastoma cell death (see figure 10B ant its legend).

Table 8: 26 neuroblastoma cell lines (either obtained directly from patient tumors at Centre Léon Bérard (CLB-X) or classic neuroblastoma cell lines (IMR32, SHEP, SHSY or SKNAS)) were tested as in (a) for netrin-1 expression by Q-RT-PCR. Netrin-1 level is indicated as (-) no netrin-1, (+, ++, +++) low to high netrin-1 level. It can be noted that a significant fraction of cell lines have high expression of netrin-1.

Cell line	**netrin-1**
CLB-BAB	-
CLB-BAC	-
CLB-BAR	-
CLB-BARREC	-
CLB-BEL	+
CLB-BOULT	++
CLB-BER2	-
CLB-BERLUD	-
CLB-CAR	-
CLB-ESP	-/+
CLB-GAR	-

(continued)

Cell line	**netrin-1**
CLB-GHE MO	-
CLB-GHE PCT	-
CLB-HUT	+++
CLB-MAR MO	-/+
CLB-MAR LT	+
CLB-PEC	-
CLB-REM	+++
CLB-SED	+
CLB-TRA	-
CLB-VOL	++++
IGRN91	-/+
IMR32	+++
SHEP	-/+
SHSY 5Y	-/+
SKNAS	++++

B) Netrin-1 is overexpressed in a large fraction of glioma (see figure 10A and its legend) and inhibition of netrin-1 activity promotes glioma cell death (see figure 10B and its legend).
C) Netrin-1 is overexpressed in human lung cancer (see figure 11A, its legend and table 9) and inhibition of netrin-1 activity promotes lung cancer cell death and prevents lung cancer development (see figures 12C and 12D and their legends).

Table 9: Lung cancer cell lines either derived from small-cell-lung cancer (SCLC) or non-small-cell-lung cancer (NSCLC) were tested as in (a) for netrin-1 expression by Q-RT-PCR. Netrin-1 level is indicated as (-) no netrin-1, (+, ++) low to high netrin-1 level. It can be noted that a significant fraction of cell lines have high expression of netrin-1.

Cell line	**netrin-1**
NSCLC	
A549	+
H322	++
H358	++
H460	-
H1299	+
SCLC	
H69	-
H146	+
H196	-

D) Netrin-1 expression in other human tumors.
Expression of netrin-1 examined by Q-RT PCR using total RNA extracted from different human tumors as in figures 10A-10B, 11A-11C, 12A-12B. The table 10 indicates (n) the number of tumors tested and the percentage of tumors showing an overexpression of netrin-1 in each pathology.

Table 10:

Cancers	**n**	**over expression of netrin-1**
Renal adenocarcinoma	5	40%
Acute myeloid leukaemia	55	62%
Sarcoma	10	30%
Melanoma	6	50%

(continued)

Cancers	n	over expression of netrin-1
Ovarian adenocarcinoma	14	93 %*
Pancreatic adenocarcinoma	7	57%
Uterus adenocarcinoma	42	19%
Stomac adenocarcinoma	27	26%
Kidney adenocarcinoma	20	50%
Rectal adenocarcinoma	18	17 %
*100 % of the 7 metastatic samples		

Example 5:

[0108] To analyze whether DCC was under its monomeric form unless netrin-1 was present, we transiently co-expressed an HA-tagged full-length DCC together with c-myc-tagged full-length DCC in HEK293T cells. Immunoprecipitation was then performed using an anti-c-myc antibody and as shown in figure 12A, despite a good expression of both HA and c-myc tagged-DCC, HA-DCC was only modestly included in the c-myc-DCC pull-down in absence of ligand, suggesting that DCC, was mainly present as a monomer when expressed in HEK293T in the absence of netrin-1. In the same experimental conditions, when netrin-1 was added to the culture medium (not shown and figure 15C) or when a netrin-1 expression construct was co-expressed with the DCC-expressing constructs (figure 11A), HA-DCC was clearly included in the c-myc DCC pull-down, hence demonstrating that netrin-1 triggers dimerisation or multimerization of DCC. This result is in agreement with data from Tessier-Lavigne and colleagues who first reported netrin-1-induced multimerization [46], even though in our culture and immunoprecipitation conditions, DCC displays a modest albeit detectable level of multimerization in the absence of netrin-1. This constitutive, low multimerization level could either be attributed to the low affinity of DCC receptors for themselves in the absence of ligand or to the system used, which is based on forced expression of high levels of transmembrane receptors.

[0109] We then investigated whether the other UNC5H netrin-1 receptors share a similar behaviour. HEK293T cells were transiently transfected with an HA-tagged full-length UNC5H2 together with Flag-tagged full-length UNC5H2 in the presence or absence of netrin-1. Immunoprecipitation was then performed using an anti-FlagM2 antibody. As shown in figure 11B, the presence of netrin-1 triggers an efficient immunoprecipitation of HA-UNC5H2 with Flag-UNC5H2. Thus, while in the absence of netrin-1, DCC and UNC5H2 are mainly under monomeric forms, both DCC and UNC5H2 show an increased propensity to multimerize in the presence of netrin-1.

[0110] To determine whether netrin-1-induced multimerization is the crucial step for inhibiting DCC/UNC5H2 pro-apoptotic cell death, we developed a chimeric system in which protein dimerization can be induced by a chemical agent. This system was successfully used to show both the role of caspase-8 dimerization in caspase-8 activation [49] and the importance of p75ntr-multimerization in p75ntr pro-apoptotic activity [48]. This system is derived from the ability of the Fk1012 compound to cross-dimerize the FkBP motif. DCC and UNC5H2 intracellular domains were fused in their N-terminus to derived Fv2e FkBP motives and dimerization was induced using the AP20187 chemical compound (figure 13A). We first analyzed whether the developed system recapitulates netrin-1-induced multimerization of the UNC5H2 intracellular domain. HEK293T cells were co-transfected with an HA-tagged Fv2e-UNC5H2-IC together with c-myc-tagged Fv2e-UNC5H2-IC and co-immunoprecipitations were performed using an anti-c-myc antibody. As shown in fiure 13B, without addition of AP20187, HA-Fv2e-UNC5H2-IC was barely detectable in the c-myc-Fv2e-UNC5H2-IC pull-down, hence supporting that Fv2e-UNC5H2-IC is expressed in HEK293T cells mainly as a monomer. As expected, addition of AP20187 led to the efficient pull-down of HA-Fv2e-UNC5H2-IC with c-myc-Fv2e-UNC5H2-IC. Similar results were obtained with Fv2e-DCC-IC (not shown). Thus, this dimerization system recapitulates dimerization of the intracellular domain of the netrin-1 receptors DCC and UNC5H2.

[0111] Because this chemically-inducible DCC/UNC5H2 dimerization system appears to work adequately to mimic netrin-1-induced DCC/UNC5H2 multimerization, we then assessed whether the dimerization of DCC/UNC5H2 was sufficient to inhibit DCC/UNC5H2 pro-apoptotic activity. HEK293T cells were forced to express Fv2e-DCC-IC in the presence or absence of AP20187 and cell death was assessed by trypan blue staining, as previously described, to measure DCC-induced cell death [6, 27]. As shown in figure 14A, expression of Fv2e-DCC-IC was associated with increased cell death compared to expression of the Fv2e motives without the DCC-IC fusion. Interestingly, when AP20187 was added, cell death induced by Fv2e-DCC-IC was dramatically reduced (figure 14A). Similarly, while Fv2e-UNC5H2-IC triggers cell death (figure 14B) or caspase activation (figure 14C) when expressed in HEK293T in the absence of

AP20187, the addition of the dimerizing drug is sufficient to reduce significantly Fv2e-UNC5H2-IC-induced cell death (figure 14B) or caspase activation (figure 14C). Thus, while monomeric DCC-IC and UNC5H2-IC are pro-apoptotic, the multimeric forms of DCC-IC or UNC5H2-IC no longer display pro-apoptotic activity. Therefore, the ability of netrin-1 to inhibit DCC/UNC5H2-pro-apoptotic activity is intrinsically linked to the ability of netrin-1 to multimerize DCC or UNC5H2, as this multimerization process is sufficient to shut down DCC and UNC5H2 pro-apoptotic activity.

[0112] A tempting model would be that the monomeric form of DCC or UNC5H2 has a spatial conformation that is easily subjected to the initial caspase cleavage of the receptor's intracellular domain. On the opposite, presence of the ligand would lead to multimerization of the intracellular domain, which somehow becomes less accessible to caspase cleavage. Along this line, Arakawa and colleagues have shown that the caspase cleavage of UNC5H2 is inhibited by netrin-1 presence [47]. Yet, because of technical limitations, we have failed to detect DCC or UNC5H2 cleavage in cells forced to express the Fv2e fusion proteins. An alternative model to the cleavage inhibition would be that netrin-1-induced receptor mutimerization triggers a survival signal, that somehow inhibits a constitutive pro-apoptotic activity of DCC or UNC5H2 related to constitutive caspase cleavage. However, we failed to show that the known positive signalling pathways activated by DCC upon netrin-1 binding are involved in the inhibitory activity of netrin-1 on DCC pro-apoptotic activity. For example, netrin-1 induces DCC-mediated activation of ERK-1/2 [3], kinases known to display an anti-apoptotic effect. However, classic inhibitors of the ERK-1/2 pathway, while affecting netrin-1-induced ERK-1/2 phosphorylation, failed to block the netrin-1-inhibitory effect on DCC pro-apoptotic activity (Forcet and Mehlen, unpublished). Thus, it is probable that netrin-1-induced DCC multimerization affects DCC intracellular accessibility. However, it remains to be demonstrated whether this is a matter of simple stochiometry, or whether the bringing closer of the extracellular domains induces a change of conformation within the intracellular compartments.

[0113] If the mechanisms underlying netrin-1-induced receptor multimerization are yet to be described, the observation that netrin-1-induced DCC/UNC5H2 multimerization is sufficient to inhibit DCC/UNC5H2-induced cell death may represent an interesting tool to turn on DCC or UNC5H pro-apoptotic activity in vivo, in tumors in which netrin-1 is expressed in an autocrine manner. Indeed, we have demonstrated that netrin-1 overexpression in mice gut is associated with intestinal tumor development because of apoptosis inhibition [18] and we have recently observed that netrin-1 is overexpressed in the majority of human metastastic breast cancers. Moreover, the mechanism of netrin-1 overexpression appears to be an acquired selective advantage of metastatic tumor cells for survival in settings of environmental absence of netrin-1 (see examples 2 and 3). Thus, to inhibit DCC/UNC5H dimerization would putatively represent an interesting way to trigger tumor cell apoptosis.

[0114] Along this line, the fifth fibronectin domain of DCC has been shown to be a domain of interaction with netrin-1 (figure15A and [21]), even though conflicting data have also been reported [43]. We thus first assessed whether a recombinant soluble fifth fibronectin domain of DCC (DCC-5Fbn) could bind to recombinant netrin-1. ELISA assay demonstrates that DCC-5Fbn specifically binds to netrin-1, as opposed to the extracellular domain of an unrelated receptor, IL3-R (figure 15A). The approximate Kd for DCC-5Fbn/netrin-1 was roughly estimated at 5 nM, in keeping with the order of magnitude of the described DCC/netrin-1 Kd. We next investigated whether this domain was sufficient to displace DCC/netrin-1 interaction. As shown in figure 15B, using an ELISA assay in which the extracellular domain of DCC was coated and netrin-1/DCC interaction was detected by netrin-1 immunoreactivity, we observed that, while as a positive control the complete extracellular domain of DCC (DCC-EC) was sufficient to displace DCC/netrin-1 interaction, DCC-5Fbn failed to interfere. Thus, DCC-5Fbn interacts with netrin-1 but is not sufficient to inhibit DCC/netrin-1 interaction. We next investigated whether DCC-5Fbn could influence DCC multimerization. We performed co-immunoprecipitation in HEK293T transiently transfected with HA-tagged full-length DCC together with c-myc-tagged full-length DCC in the presence or absence of netrin-1. As also described in Figure 1A, presence of netrin-1 triggers the immunoprecipitation of HA-DCC with c-myc-DCC, demonstrating netrin-1-induced DCC multimerization (figure 15C). However, when the cells incubated with netrin-1 were also simultaneously treated with DCC-5Fbn, the HA-DCC/c-myc-DCC interaction returns to netrin-1 non-treated levels. Thus, DCC-5Fbn interacts with netrin-1 in a region responsible for the netrin-1-mediated coming closer of two or more DCC molecules and is able to inhibit netrin-1-induced DCC-multimerization.

[0115] We then tested whether DCC-5Fbn could consequently trigger netrin-1 receptors-induced cell death. To this purpose, HEK293T cells were forced to express DCC in the presence or absence of netrin-1, with or without DCC-5Fbn, and cell death was determined by trypan blue exclusion assay (figure 16A). As shown in figure 16A, while DCC triggers apoptosis in the absence of netrin-1, a pro-apoptotic activity blocked by the presence of netrin-1, the presence of DCC-5Fbn is sufficient to block the inhibitory activity of netrin-1, thus leading to DCC-induced cell death. Because the HEK293T cell system uses ectopic expression of netrin-1, we next tested DCC-5Fbn in a more biologically relevant model. We recently demonstrated that compared to non-metastatic breast cancers, the majority of human metastatic breast cancers overexpresses netrin-1. We have also shown that titrating overexpressed netrin-1 triggers tumor cell apoptosis in vitro and metastasis inhibition in mice (see Examples 2 and 3). Many breast tumor cell lines appear to express netrin-1 and we have shown that titrating netrin-1 in mice breast carcinoma 4T1 cells triggers apoptosis (see Examples 2 and 3). As shown in figure 16B, addition of DCC-5Fbn to a 4T1 cell culture is associated with increased cell death.

[0116] Taken together, we have shown here that the multimerization of the dependence receptors DCC and UNC5H

is a sufficient mechanism to block their pro-apoptotic activity. Interestingly, this inhibitory mechanism appears to mirror what is observed with death receptors. Indeed, it is known that TNFr or Fas requires trimerization to induce apoptosis [45]. This intrinsic difference may therefore represent an added-value for therapeutic strategies using dependence receptors. Indeed, the search of therapeutic molecules in the past has mainly led to hits that act on the inhibition of cellular processes -e.g., kinases inhibitors, IAP inhibitors- rather than activators. As a consequence, inhibition of netrin-1 receptors multimerization via the use of recombinant DCC-5Fbn or via any compound screened to interfere with receptor multimerization appears as a tempting strategy for the treatment of cancers in which netrin-1 autocrine expression has been acquired.

[0117] Here, we show that netrin-1 triggers the multimerization of both DCC and UNC5H receptors. By using a system in which dimerization is chemically-induced, we demonstrate that multimerization of the intracellular domain of netrin-1 receptors, such as DCC and UNC5H2, is the critical step to inhibit their pro-apoptotic activity. We therefore propose a model in which monomeric netrin-1-dependence receptors are pro-apoptotic, while their multimerization, induced by netrin-1, abolishes their pro-apoptotic activity. Using this property, we propose the use of a recombinant specific domain of the DCC extracellular region that (i) interacts with netrin-1 and (ii) inhibits netrin-1-induced multimerization, in order to trigger apoptosis of tumor cells.

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[0118]

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52. WO 2005/074556.

SEQUENCE LISTING

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<220>
<223> Primer used for expressed human UNC5C as internal control

<400> 28
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<400> 29
gctccactgt gttcaggcta aatctt 26

Claims

1. Method for selecting a compound for the prevention or the treatment of cancer, said cancer to be prevented or treated being selected from the group consisting of breast cancer, lung cancer, neuroblastoma, glioma, leukemia, sarcoma, melanoma and adenocarcinoma, wherein said cancer to be prevented or treated is a cancer wherein tumoral cells express or overexpress netrin-1, wherein said method comprises the following steps of:

   a) having a medium containing netrin-1, or a fragment thereof, and a netrin-1 receptor, or a fragment thereof, wherein:

      - said netrin-1, or a fragment thereof, and said netrin-1 receptor, or a fragment thereof, is able to specifically interact together to form a binding pair, and/or
      - said netrin-1, or a fragment thereof, is able to induce the dimerization or multimerization of the intracellular domain of said netrin-1 receptor;

   b) contacting said medium with the compound to be tested;
   c)

      - measuring the inhibition of the interaction between netrin-1, or a fragment thereof, and said netrin-1 receptor, or a fragment thereof, and/or
      - determining whether said compound inhibits the dimerization or multimerization of said netrin-1 receptor, or a fragment thereof, particularly the dimerization of the intracellular domain of said netrin-1 receptor; and

   d) selecting said compound if:

      - the measuring in step c) demonstrates a significant inhibition of the interaction between netrin-1, or a fragment thereof, and netrin-1 receptor, or a fragment thereof, in presence of said compound, and/or
      - the determination in step c) demonstrates a significant inhibition of the dimerization or multimerization, in presence of said compound, of the intracellular domain of said netrin-1 receptor.

2. The method according to claim 1, wherein said adenocarcinoma to be prevented or treated is selected from the group consisting of ovarian adenocarcinoma, renal adenocarcinoma, pancreatic adenocarcinoma, uterus adenocarcinoma, stomac adenocarcinoma, kidney adenocarcinoma and rectal adenocarcinoma.

3. The method according to claim 1 or 2, wherein said cancer to be prevented or treated is a metastatic cancer.

4. The method according to claims 1 to 3, wherein at step a) said netrin-1 receptor is selected from the group of Deleted in Colorectal Cancer (DCC), Unc-5 homolog (UNC5H), neogenin and the adenosine A2b receptors.

5. The method according to claim 4, wherein at step a) said netrin-1 receptor is selected from the group of DCC, UNC5H1, UNC5H2 and UNC5H3.

6. The method according to claims 1 to 5, wherein at step a):

   - said netrin-1 receptor fragment comprises or is the extracellular domain of the netrin-1 receptor, or part thereof able to bind to netrin-1; and/or

- said netrin-1 receptor fragment comprises or is the intracellular domain of the netrin-1 receptor, or part thereof able to dimerize or multimerize in presence of netrin-1.

7. The method according to claims 1 to 6, wherein at step a) said netrin-1 or/and said netrin 1 receptor are from mammal.

8. The method according to claims 1 to 7, wherein at step a) said netrin-1 is from chicken.

9. The method according to claims 1 to 8, wherein said netrin-1 or/and said netrin-1 receptor and/or the compound to be tested is labeled by a marker able to be directly or indirectly measured.

10. The method according to claims 1 to 9, wherein at step c):

- the measure of the inhibition of the interaction between netrin-1, or a fragment thereof, and said netrin-1 receptor, or a fragment thereof, is carried out by immunoassay (particularly by Enzyme-linked Immunosorbent Assay (ELISA) or by Immunoradiometric Assay (IRMA)), by Scintillation Proximity Assay (SPA) or by Fluorescence Resonance Energy Transfer (FRET); and/or
- the dimerization or multimerization, or its inhibition, of said netrin-1 receptor, or fragment thereof, particularly the intracellular domain, is carried out by immunoprecipitation or FRET.

11. The method according to claims 1 to 10, wherein at step a) said medium contains cells which express at their surface membrane an endogenous netrin-1 receptor or a recombinant netrin-1 receptor, particularly at least the extracellular domain of a recombinant netrin-1 receptor.

12. The method according to claim 11, wherein at step a) said medium contains cells which express recombinant netrin-1 receptor.

13. The method according to claim 11, wherein at step a) said medium contains tumoral cells which express endogenously said netrin-1 receptor at their membrane surface and which express or overexpress netrin-1, and wherein at step c) the inhibition of the interaction between netrin-1 and its netrin-1 receptor in presence of the compound to be tested, is measured by the apoptosis or cells death induced by the presence of the compound to be tested.

14. The method according to claim 13, wherein at step a) said medium contains metastatic tumoral cells.

15. A method according to claims 1 to 14 for selecting a compound for the prevention or the treatment of cancer, wherein said method comprises the following steps of:

a) having a medium containing a mammal cell expressing an endogenous or a recombinant netrin-1 receptor, or a fragment thereof comprising at least its intracellular domain;
b) contacting said medium with the compound to be tested;
c) determining whether the dimerization or multimerization of said netrin-1 receptor intracellular domain is inhibited in presence of said compound to be tested; and
d) selecting said compound if the determination in step c) demonstrates a significant inhibition of the dimerization or multimerization of the intracellular domain of said netrin-1 receptor.

16. The method according to claim 15, wherein said cell of step (a) is a tumor cell.

17. The method according to claim 15, wherein said cell of step (a) is a cell presenting dimerization or multimerization of its netrin-1 receptor intracellular domain or a cell wherein its netrin-1 receptor intracellular domain is able to dimerize or multimerize in presence of netrin-1.

18. The method according to any one of claims 15 to 17, wherein at step (b) the medium further containing netrin-1, or a fragment thereof able to bind to the extracellular domain of the netrin-1 receptor.

19. The method according to any one of claims 15 to 18, comprising further determining whether the presence of the compound to be tested induces the cell death of said mammal cell; and step (d) comprises the determination of the cell death of said mammal cell.

20. An in vitro method for predicting the presence of a metastatic or an aggressive cancer in a patient having a primary

tumor from a biopsy of said patient containing primary tumors cells, said cancer being selected from the group consisting of breast cancer, lung cancer, neuroblastoma, glioma, leukemia, sarcoma, melanoma and adenocarcinoma, wherein said cancer to be prevented or treated is a cancer wherein tumoral cells express or overexpress netrin-1, comprising the following step of:

(a) measuring of a netrin-1 expression product level in said biopsy, wherein said product is (i) RNA encoding netrin-1 or (ii) netrin-1 protein; and
(b) comparing said expression product level with expression of netrin-1 expression product level in a non-metastatic primary tumor biopsy or in a non-aggressive cancer biopsy ;

wherein an increase with a ratio superior to 2 between netrin-1 expression product levels in the biopsy to be tested and in the non-metastatic or non-aggressive reference biopsy is significant of the presence of a metastatic cancer or an aggressive cancer.

21. A method for determining *in vitro* the efficiency of an anti-cancer treatment for a patient or for *in vitro* selecting patients who respond to a specific anti-cancer treatment, said cancer being selected from the group consisting of breast cancer, lung cancer, neuroblastoma, glioma, leukemia, sarcoma, melanoma and adenocarcinoma, wherein said cancer to be prevented or treated is a cancer wherein tumoral cells express or overexpress netrin-1, said method comprising the following step of:

- measuring of a netrin-1 expression product level in a biopsy of said patient, wherein said product is (i) RNA encoding netrin-1 or (ii) netrin-1 protein;

wherein the efficiency of said anti-cancer treatment is correlated with the decrease by a ratio of at least 2 of the amount of the netrin-1 expression product level measured in said biopsy, or
wherein the selected patients who respond to a specific anti-cancer treatment are patients where the amount of the netrin-1 expression product level measured in their biopsy has been decreased by a ratio of at least 2 after said specific treatment.

22. A method according to claim 21, wherein said cancer induced an overexpression of netrin-1 and/or is a metastatic cancer.

23. The method according to claims 20 to 22, wherein said RNA encoding netrin-1 is measured by a quantitative real time reverse polymerase chain reaction (PCR) method.

24. The method according to claims 20 to 22, wherein said netrin-1 protein is measured using specific antibodies able to specifically recognize said netrin-1 protein.

25. The method according to claims 20 to 24, wherein the primary tumor is a primary tumor of a cancer selected from the group consisting of breast cancer, lung cancer, neuroblastoma, glioma, leukemia, sarcoma, melanoma and adenocarcinoma.

26. The method according to claim 25, wherein said adenocarcinoma is selected from the group consisting of ovarian adenocarcinoma, renal adenocarcinoma, pancreatic adenocarcinoma, uterus adenocarcinoma, stomac adenocarcinoma, kidney adenocarcinoma and rectal adenocarcinoma.

27. Use for the selection of a compound for the prevention or the treatment of cancer, said cancer being selected from the group consisting of breast cancer, lung cancer, neuroblastoma, glioma, leukemia, sarcoma, melanoma and adenocarcinoma, of a kit comprising tumoral cells which express netrin-1 receptor and express or overexpress netrin-1.

28. Use according to claim 27, wherein the tumoral cells are cells from a tumoral cell line.

29. Use according to claim 27 or 28, further comprising a netrin-1 protein, or a fragment thereof able to specifically interact with said netrin-1 receptor protein to form a binding pair.

30. A compound selected from the group consisting of:

- a compound comprising an extracellular domain of netrin-1 receptor or fragment thereof able to specifically inhibit the interaction between the netrin-1 and said netrin-1 receptor, and/or able to inhibit the dimerization or multimerization of the intracellular domain of said netrin-1 receptor; and
- a monoclonal or polyclonal antibody directed specifically against netrin-1 or the extracellular domain of netrin-1 receptor or to a netrin-1 fragment able to interact with the extracellular domain of said netrin-1 receptor,

for use in an in vivo method to prevent or treat breast cancer, lung cancer, neuroblastoma, glioma, leukemia, sarcoma, melanoma and adenocarcinoma.

31. A compound for the use according to claim 30, wherein said extracellular domain of netrin-1 receptor or fragment thereof is selected from the group of DCC; UNC5H , neogenin and the adenosine A2b.

32. A compound for the use according to claim 30 or 31, wherein said compound comprising an extracellular domain of netrin-1 receptor is from DCC.

33. A compound for the use according to claim 32, wherein said compound is DCC-EC-Fc or DCC-5Fbn.

34. A compound for the use according to any one of claims 30 to 33, for use in a method to prevent or treat a cancer wherein tumoral cells express or overexpress netrin-1.

35. The compound according to claims 30 to 34 or an antisense or iRNA (interfering RNA) oligonucleotide which is specific and complementary to the nucleic acid encoding netrin-1 protein for use in an in vivo method to treat or prevent cancer in mammals, including man, said cancer being selected from the group consisting of breast cancer, lung cancer, neuroblastoma, glioma, leukemia, sarcoma, melanoma and adenocarcinoma, wherein said cancer to be prevented or treated is a cancer wherein tumoral cells express or overexpress netrin-1.

36. The use of the compound according to claim 35, **characterized in that** said cancer is a metastatic cancer.

37. The use of the compound according to claim 35 or 33, **characterized in that** said adenocarcinoma is selected from the group consisting of ovarian adenocarcinoma, renal adenocarcinoma, pancreatic adenocarcinoma, uterus adenocarcinoma, stomac adenocarcinoma, kidney adenocarcinoma and rectal adenocarcinoma.

38. The use of the compound according to claims 35 to 37, **characterized in that** the primary tumor cells of said cancer express or overexpress netrin-1.

Patentansprüche

1. Verfahren zum Auswählen einer Verbindung für die Prävention oder die Behandlung von Krebs, wobei der Krebs, dem vorgebeugt oder der behandelt werden soll, ausgewählt ist aus der Gruppe bestehend aus Brustkrebs, Lungenkrebs, Neuroblastom, Gliom, Leukämie, Sarkom, Melanom und Adenokarzinom, wobei der Krebs, dem vorgebeugt oder der behandelt werden soll, ein Krebs ist, bei dem tumoröse Zellen Netrin-1 exprimieren oder überexprimieren, wobei das Verfahren die folgenden Schritte umfasst:

a) Bereitstellen eines Mediums enthaltend Netrin-1 oder ein Fragment davon und einen Netrin-1-Rezeptor oder ein Fragment davon, wobei:

- das Netrin-1 oder ein Fragment davon und der Netrin-1-Rezeptor oder ein Fragment davon dazu in der Lage ist, spezifisch miteinander zu interagieren, um ein bindendes Paar zu bilden, und/oder
- das Netrin-1 oder ein Fragment davon dazu in der Lage ist, die Dimerisierung oder Multimerisiereung der intrazellulären Domäne des Netrin-1-Rezeptors zu induzieren;

b) In-Kontakt-bringen des Mediums mit der zu testenden Verbindung;
c)

- Messen der Inhibierung der Interaktion zwischen Netrin-1 oder einem Fragment davon und dem Netrin-1-Rezeptor oder einem Fragment davon und/oder
- Bestimmen, ob die Verbindung die Dimerisierung oder Multimerisiereung des Netrin-1-Rezeptors oder

eines Fragments davon, insbesondere die Dimerisierung der intrazellulären Domäne des Netrin-1-Rezeptors, inhibiert; und

d) Auswählen der Verbindung, wenn:

- das Messen in Schritt c) eine signifikante Inhibierung der Interaktion zwischen Netrin-1 oder einem Fragment davon und dem Netrin-1-Rezeptor oder einem Fragment davon in Anwesenheit der Verbindung zeigt und oder
- das Bestimmen in Schritt c) eine signifikante Inhibierung der Dimerisierung oder Multimerisierung der intrazellulären Domäne des Netrin-1-Rezeptors in Anwesenheit der Verbindung zeigt.

2. Das Verfahren gemäß Anspruch 1, wobei das Adenokarzinom, dem vorgebeugt oder das behandelt werden soll, ausgewählt ist aus der Gruppe bestehend aus Eierstock-Adenokarzinom, Nieren-Adenokarzinom, Pankreas-Adenokarzinom, Gebärmutter-Adenokarzinom, Magen-Adenokarzinom, Nieren-Adenokarzinom und Mastdarm-Adenokarzinom.

3. Das Verfahren gemäß Anspruch 1 oder 2, wobei der Krebs, dem vorgebeugt oder der behandelt werden soll, ein metastatischer Krebs ist.

4. Das Verfahren gemäß den Ansprüchen 1 bis 3, wobei in Schritt a) der Netrin-1-Rezeptor ausgewählt ist aus der Gruppe von Deleted in Colorectal Cancer (DCC), Unc-5-Homolog (UNC5H), Neogenin und den Adenosin-A2b-Rezeptoren.

5. Das Verfahren gemäß Anspruch 4, wobei in Schritt a) der Netrin-1-Rezeptor ausgewählt ist aus der Gruppe von DCC, UNC5H1, UNC5H2 und UNC5H3.

6. Das Verfahren gemäß den Ansprüchen 1 bis 5, wobei in Schritt a):

- das Netrin-1-Rezeptor-Fragment die extrazelluläre Domäne des Netrin-1-Rezeptors oder Teil davon, dazu in der Lage, Netrin-1- zu binden, umfasst oder ist und/oder
- das Netrin-1-Rezeptor-Fragment die extrazelluläre Domäne des Netrin-1-Rezeptors oder Teil davon, dazu in der Lage, in Anwesenheit von Netrin-1-zu dimerisieren oder multimerisieren, umfasst oder ist.

7. Das Verfahren gemäß den Ansprüchen 1 bis 6, wobei in Schritt a) das Netrin-1 oder/und der Netrin-1-Rezeptor von einem Säugetier sind.

8. Das Verfahren gemäß den Ansprüchen 1 bis 7, wobei in Schritt a) das Netrin-1 aus dem Huhn ist.

9. Das Verfahren gemäß den Ansprüchen 1 bis 8, wobei das Netrin-1 oder/und der Netrin-1-Rezepor und/oder die zu testende Verbindung mit einem Marker markiert ist, der direkt oder indirekt gemessen werden kann.

10. Das Verfahren gemäß den Ansprüche 1 bis 9, wobei in Schritt c):

- das Messen der Inhibierung der Interaktion zwischen Netrin-1 oder einem Fragment davon und dem Netrin-1-Rezeptor oder einem Fragment davon mittels eines Immunassays (insbesondere mittels Enzyme-linked Immunosorbent Assay (ELISA) oder mittels Immunoradiometric Assay (IRMA)), mittels Scintillation Proximity Assay (SPA) oder mittels Fluoreszenz-Resonanz-Energie-Transfer (FRET) durchgeführt wird; und/oder
- die Dimerisierung oder Multimerisierung oder ihre Inhibierung des Netrin-1-Rezptors oder Fragments davon, insbesondere der intrazellulären Domäne, mittels Immunpräzipitation oder FRET durchgeführt wird.

11. Das Verfahren gemäß den Ansprüchen 1 bis 10, wobei in Schritt a) das Medium Zellen enthält, die die Expression an ihrer Oberflächenmembran einen endogenen Netrin-1-Rezeptor oder einen rekombinanten Netrin-1-Rezeptor, insbesondere zumindest die extrazelluläre Domäne des rekombinanten Netrin-1-Rezeptors, exprimieren.

12. Das Verfahren gemäß Anspruch 11, wobei in Schritt a) das Medium Zellen enthält, die einen rekombinanten Netrin-1-Rezeptor exprimieren.

13. Das Verfahren gemäß Anspruch 11, wobei in Schritt a) das Medium tumoröse Zellen enthält, die den Netrin-1-

Rezeptor endogen an ihrer Membranoberfläche exprimieren und die Netrin-1 exprimieren oder überexprimieren, und wobei in Schritt c) die Inhibierung der Interaktion zwischen Netrin-1 und seinem Netrin-1-Rezeptor in Anwesenheit der zu testenden Verbindung über die Apoptose oder Zelltode, die in Anwesenheit der zu testenden Verbindung induziert werden, gemessen wird.

14. Das Verfahren gemäß Anspruch 13, wobei in Schritt a) das Medium metastatische, tumoröse Zellen enthält.

15. Ein Verfahren gemäß den Ansprüchen 1 bis 14 zum Auswählen einer Verbindung für die Prävention oder die Behandlung von Krebs, wobei das Verfahren die folgende Schritte umfasst:

a) Bereitstellen eines Mediums enthaltend eine Säugetierzelle, die einen endogenen oder einen rekombinanten Netrin-1-Rezeptor oder ein Fragment davon umfassend zumindest seine intrazelluläre Domäne exprimiert;
b) In-Kontakt-bringen des Mediums mit der zu testenden Verbindung;
c) Bestimmen, ob die Dimerisierung oder Multimerisiereung der intrazellulären Netrin-1-Rezeptor-Domäne in Anwesenheit der zu testenden Verbindung inhibiert ist; und
d) Auswählen der Verbindung, wenn das Bestimmen in Schritt c) eine signifikante Inhibierung der Dimerisierung oder Multimerisierung der intrazellulären Domäne des Netrin-1-Rezeptors zeigt.

16. Das Verfahren gemäß Anspruch 15, wobei die Zelle von Schritt (a) eine Tumorzelle ist.

17. Das Verfahren gemäß Anspruch 15, wobei die Zelle von Schritt (a) eine Zelle ist, die Dimerisierung oder Multimerisierung ihrer intrazellulären Netrin-1-Rezeptor-Domäne aufweist, oder eine Zelle, in der ihre intrazelluläre Netrin-1-Rezeptor-Domäne dazu in der Lage ist, in Anwesenheit von Netrin-1- zu dimerisieren oder multimerisieren.

18. Das Verfahren gemäß einem der Ansprüche 15 bis 17, wobei in Schritt (b) das Medium des Weiteren Netrin-1- oder ein Fragment davon, dazu in der Lage, an die extrazelluläre Domäne des Netrin-1-Rezeptors zu binden, enthält.

19. Das Verfahren gemäß einem der Ansprüche 15 bis 18, umfassend des Weiteren das Bestimmen, ob die Anwesenheit der zu testenden Verbindung den Zelltod der Säugetierzelle induziert; und Schritt (d) umfasst das Bestimmen des Zelltods der Säugetierzelle.

20. Ein in vitro Verfahren zur Vorhersage der Anwesenheit eines metastatischen oder eines aggressiven Krebses in einem Patienten, der einen primären Tumor hat, von einer Biopsie des Patienten enthaltend primäre Tumorzellen, wobei der Krebs ausgewählt ist aus der Gruppe bestehend aus Brustkrebs, Lungenkrebs, Neuroblastom, Gliom, Leukämie, Sarkom, Melanom und Adenokarzinom, wobei der Krebs, dem vorgebeugt oder der behandelt werden soll, ein Krebs ist, bei dem tumoröse Zellen Netrin-1 exprimieren oder überexprimieren, umfassend die folgenden Schritte:

(a) Messen eines Netrin-1-Expressionsproduktlevels in der Biopsie, wobei das Produkt (i) RNA kodierend für Netrin-1 oder (ii) Netrin-1-Protein ist; und
(b) Vergleichen des Expressionsproduktlevels mit der Expression des Netrin-1-Expressionsproduktlevels in einer nicht-metastatischen, primären Tumorbiopsie oder in einer nicht-aggressiven Krebsbiopsie;

wobei eine Erhöhung mit einem Verhältnis von mehr als 2 zwischen Netrin-1-Expressionsproduktleveln in der zu testenden Biopsie und in der nicht-metastatischen oder nicht-aggressiven Referenzbiopsie charakteristisch für die Anwesenheit eines metastatischen Krebses oder eines aggressiven Krebses ist.

21. Ein Verfahren zur *in vitro* Bestimmung der Wirksamkeit einer anti-Krebs-Behandlung für einen Patienten oder zum *in vitro* Auswählen von Patienten, die auf eine spezifische anti-Krebs-behandlung ansprechen, wobei der Krebs ausgewählt ist aus der Gruppe bestehend aus Brustkrebs, Lungenkrebs, Neuroblastom, Gliom, Leukämie, Sarkom, Melanom und Adenokarzinom, wobei der Krebs, dem vorgebeugt oder der behandelt werden soll, ein Krebs ist, bei dem tumoröse Zellen Netrin-1 exprimieren oder überexprimieren, wobei das Verfahren die folgenden Schritte umfasst:

- Messen des Netrin-1-Expressionsproduktlevels in einer Biopsie des Patienten, wobei das Produkt (i) RNA kodierend für Netrin-1 oder (ii) Netrin-1-Protein ist;

wobei die Wirksamkeit der anti-Krebs-Behandlung in Verbindung mit der Abnahme um ein Verhältnis von mindestens

2 der Menge des Netrin-1-Expressionsproduktlevels gemessen in der Biopsie steht, oder
wobei die ausgewählten Patienten, die auf eine spezifische anti-Krebs-Behandlung ansprechen, Patienten sind, bei denen die Menge des Netrin-1-Expressionsproduktlevels gemessen in ihrer Biopsie um ein Verhältnis von mindestens 2 nach der spezifischen Behandlung verringert wurde.

22. Ein Verfahren gemäß Anspruch 21, wobei der Krebs eine Überexpression von Netrin-1 induziert hat und/oder ein metastatischer Krebs ist.

23. Das Verfahren gemäß den Ansprüchen 20 bis 22, wobei die für Netrin-1 kodierende RNA mittels eines quantitativen, reversen Echtzeit-Polymerase-Kettenreaktion (PCR) -Verfahrens gemessen wird.

24. Das Verfahren gemäß den Ansprüchen 20 bis 22, wobei das Netrin-1-Protein unter Verwendung spezifischer Antikörper, die in der Lage sind, das Netrin-1-Protein spezifisch zu erkennen, gemessen wird.

25. Das Verfahren gemäß den Ansprüchen 20 bis 24, wobei der primäre Tumor ein primärer Tumor eines Krebses ausgewählt aus der Gruppe bestehend aus Brustkrebs, Lungenkrebs, Neuroblastom, Gliom, Leukämie, Sarkom, Melanom und Adenokarzinom ist.

26. Das Verfahren gemäß Anspruch 25, wobei das Adenokarzinom ausgewählt ist aus der Gruppe bestehend aus Eierstock-Adenokarzinom, Nieren-Adenokarzinom, Pankreas-Adenokarzinom, Gebärmutter-Adenokarzinom, Magen-Adenokarzinom, Nieren-Adenokarzinom und Mastdarm-Adenokarzinom.

27. Verwendung, zur Auswahl einer Verbindung für die Prävention oder die Behandlung von Krebs, wobei der Krebs ausgewählt ist aus der Gruppe bestehend aus Brustkrebs, Lungenkrebs, Neuroblastom, Gliom, Leukämie, Sarkom, Melanom und Adenokarzinom, eines Kits umfassend tumoröse Zellen, die Netrin-1-Rezeptor exprimieren und Netrin-1 exprimieren oder überexprimieren.

28. Verwendung gemäß Anspruch 27, wobei die tumorösen Zellen Zellen von einer tumorösen Zelllinie sind.

29. Verwendung gemäß Anspruch 27 oder 28, des Weiteren umfassend ein Netrin-1-Protein oder ein Fragment davon, das dazu in der Lage ist, mit dem Netrin-1-Rezeptor-Protein zu interagieren, um ein bindendes Paar zu bilden.

30. Eine Verbindung ausgewählt aus der Gruppe bestehend aus:

- einer Verbindung umfassend eine extrazelluläre Domäne eines Netrin-1-Rezeptors oder Fragments davon, dazu in der Lage, spezifisch die Interaktion zwischen Netrin-1 und dem Netrin-1-Rezeptor zu inhibieren, und/oder dazu in der Lage, die Dimerisierung oder Multimerisierung der intrazellulären Domäne des Netrin-1-Rezeptors zu inhibieren; und
- einem monoklonalen oder polyklonalen Antikörper, der spezifisch gegen Netrin-1 oder die extrazelluläre Domäne des Netrin-1-Rezeptors oder ein Netrin-1-Fragment gerichtet ist, dazu in der Lage, mit der extrazellulären Domäne des Netrin-1-Rezeptors zu interagieren,

zur Verwendung in einem in vivo Verfahren zum Vorbeugen oder Behandeln von Brustkrebs, Lungenkrebs, Neuroblastom, Gliom, Leukämie, Sarkom, Melanom und Adenokarzinom.

31. Eine Verbindung zur Verwendung gemäß Anspruch 30, wobei die extrazelluläre Domäne des Netrin-1-Rezeptors oder Fragments davon ausgewählt ist aus der Gruppe von DCC, UNC5H, Neogenin und Adenosin-A2b.

32. Eine Verbindung zur Verwendung gemäß Anspruch 30 oder 31, wobei die Verbindung umfassend eine extrazelluläre Domäne des Netrin-1-Rezeptors von DCC ist.

33. Eine Verbindung zur Verwendung gemäß Anspruch 32, wobei die Verbindung DCC-EC-Fc oder DCC-5Fbn ist.

34. Eine Verbindung zur Verwendung gemäß einem der Ansprüche 30 bis 33, zur Verwendung in einem Verfahren zum Vorbeugen oder Behandeln von Krebs, bei dem tumoröse Zellen Netrin-1 exprimieren oder überexprimieren.

35. Die Verbindung gemäß den Ansprüchen 30 bis 34 oder ein Antisense- oder iRNA-(interfering RNA) Oligonukleotids, das spezifisch für oder komplementär zu der Nukleinsäure ist, die Netrin-1-Protein kodiert, zur Verwendung in einem

in vivo Verfahren zum Behandeln oder Vorbeugen von Krebs in Säugetieren, einschließlich des Menschen, wobei der Krebs ausgewählt ist aus der Gruppe bestehend aus Brustkrebs, Lungenkrebs, Neuroblastom, Gliom, Leukämie, Sarkom, Melanom und Adenokarzinom, wobei der Krebs, dem vorgebeugt oder der behandelt werden soll, ein Krebs ist, bei dem tumoröse Zellen Netrin-1 exprimieren oder überexprimieren.

36. Die Verwendung der Verbindung gemäß Anspruch 35, **dadurch gekennzeichnet, dass** der Krebs ein metastatischer Krebs ist.

37. Die Verwendung der Verbindung gemäß Anspruch 35 oder 33, **dadurch gekennzeichnet, dass** das Adenokarzinom ausgewählt ist aus der Gruppe bestehend aus Eierstock-Adenokarzinom, Nieren-Adenokarzinom, Pankreas-Adenokarzinom, Gebärmutter-Adenokarzinom, Magen-Adenokarzinom, Nieren-Adenokarzinom und Mastdarm-Adenokarzinom.

38. Die Verwendung der Verbindung gemäß den Ansprüche 35 bis 37, **dadurch gekennzeichnet, dass** die primären Tumorzellen des Krebses Netrin-1 exprimieren oder überexprimieren.

Revendications

1. Méthode de sélection d'un composé destiné à la prévention ou au traitement d'un cancer, ledit cancer à prévenir ou à traiter étant choisi dans le groupe constitué par un cancer du sein, un cancer du poumon, un neuroblastome, un gliome, une leucémie, un sarcome, un mélanome et un adénocarcinome, ledit cancer à prévenir ou à traiter étant un cancer dans lequel des cellules tumorales expriment ou surexpriment la nétrine-1, ladite méthode comprenant les étapes suivantes consistant à :

a) avoir un milieu contenant de la nétrine-1, ou un fragment de celle-ci, et un récepteur de la nétrine-1, ou un fragment de celui-ci, dans lequel :

- ladite nétrine-1, ou un fragment de celle-ci, et ledit récepteur de la nétrine-1, ou un fragment de celui-ci, sont capables d'interagir spécifiquement l'un avec l'autre pour former une paire de liaison, et/ou
- ladite nétrine-1, ou un fragment de celle-ci, est capable d'induire la dimérisation ou la multimérisation du domaine intracellulaire dudit récepteur de la nétrine-1 ;

b) mettre en contact ledit milieu avec le composé à tester ;
c) mesurer l'inhibition de l'interaction entre la nétrine-1, ou un fragment de celle-ci, et ledit récepteur de la nétrine-1, ou un fragment de celui-ci, et/ou

- déterminer si ledit composé inhibe la dimérisation ou la multimérisation dudit récepteur de la nétrine-1, ou d'un fragment de celui-ci, en particulier la dimérisation du domaine intracellulaire dudit récepteur de la nétrine-1 ; et

d) sélectionner ledit composé si :

- la mesure dans l'étape c) démontre une inhibition significative de l'interaction entre la nétrine-1, ou un fragment de celle-ci, et un récepteur de la nétrine-1, ou un fragment de celui-ci, en présence dudit composé, et/ou
- la détermination dans l'étape c) démontre une inhibition significative de la dimérisation ou de la multimérisation, en présence dudit composé, du domaine intracellulaire dudit récepteur de la nétrine-1.

2. Méthode selon la revendication 1, dans laquelle ledit adénocarcinome à prévenir ou à traiter est choisi dans le groupe constitué par un adénocarcinome de l'ovaire, un adénocarcinome rénal, un adénocarcinome du pancréas, un adénocarcinome de l'utérus, un adénocarcinome de l'estomac, un adénocarcinome du rein et un adénocarcinome rectal.

3. Méthode selon la revendication 1 ou 2, dans laquelle ledit cancer à prévenir ou traiter est un cancer métastatique.

4. Méthode selon les revendications 1 à 3, dans laquelle à l'étape a), ledit récepteur de la nétrine-1 est choisi dans le groupe du gène délété dans le cancer colorectal (DCC), l'homologue de Unc-5 (UNC5H), la néogénine et les

récepteurs de l'adénosine A2b.

5. Méthode selon la revendication 4, dans laquelle à l'étape a), ledit récepteur de la nétrine-1 est choisi dans le groupe constitué par DCC, UNC5H1, UNC5H2 et UNC5H3.

6. Méthode selon les revendications 1 à 5, dans laquelle à l'étape a) :

- ledit fragment du récepteur de la nétrine-1 comprend ou est le domaine extracellulaire du récepteur de la nétrine-1, ou une partie de celui-ci, capable de se lier à la nétrine ; et/ou
- ledit fragment du récepteur de la nétrine-1 comprend ou est le domaine extracellulaire du récepteur de la nétrine-1, ou une partie de celui-ci, capable de se dimériser ou de se multimériser en présence de nétrine-1.

7. Méthode selon les revendications 1 à 6, dans laquelle à l'étape a), ladite nétrine-1 et/ou ledit récepteur de la nétrine-1 proviennent d'un mammifère.

8. Méthode selon les revendications 1 à 7, dans laquelle à l'étape a), ladite nétrine-1 provient de poulet.

9. Méthode selon les revendications 1 à 8, dans laquelle ladite nétrine-1 et/ou ledit récepteur de la nétrine-1 et/ou le composé à tester sont marqués par un marqueur capable d'être mesuré de manière directe ou indirecte.

10. Méthode selon les revendications 1 à 9, dans laquelle à l'étape c) :

- la mesure de l'inhibition de l'interaction entre la nétrine-1, ou un fragment de celle-ci, et ledit récepteur de la nétrine-1, ou un fragment de celui-ci, est réalisée par immunodosage (en particulier par dosage d'immunoabsorption par enzyme liée (ELISA) ou par dosage immunoradiométrique (IRMA)), par dosage de scintillation par proximité (SPA) ou par transfert d'énergie par résonance en fluorescence (FRET) ; et/ou
- la dimérisation ou la multimérisation, ou son inhibition, dudit récepteur de la nétrine-1, ou d'un fragment de celui-ci, en particulier du domaine intracellulaire, est réalisée par immunoprécipitation ou par FRET.

11. Méthode selon les revendications 1 à 10, dans laquelle à l'étape a) ledit milieu contient des cellules qui expriment, au niveau de leur membrane de surface, un récepteur de la nétrine-1 endogène ou un récepteur de la nétrine-1 recombinant, en particulier au moins le domaine extracellulaire d'un récepteur de la nétrine-1 recombinant.

12. Méthode selon la revendication 11, dans laquelle à l'étape a) ledit milieu contient des cellules qui expriment le récepteur de la nétrine-1 recombinant.

13. Méthode selon la revendication 11, dans laquelle à l'étape a) ledit milieu contient des cellules tumorales qui expriment de manière endogène ledit récepteur de la nétrine-1 au niveau de leur surface membranaire et qui expriment ou surexpriment la nétrine-1, et dans laquelle à l'étape c) l'inhibition de l'interaction entre la nétrine-1 et son récepteur de la nétrine-1 en présence du composé à tester est mesurée par l'apoptose ou la mort des cellules induite par la présence du composé à tester.

14. Méthode selon la revendication 13, dans laquelle à l'étape a) ledit milieu contient des cellules tumorales métastatiques.

15. Méthode selon les revendications 1 à 14, de sélection d'un composé destiné à la prévention ou au traitement d'un cancer, ladite méthode comprenant les étapes suivantes consistant à :

a) avoir un milieu contenant une cellule de mammifère exprimant un récepteur de la nétrine-1 endogène ou recombinant, ou un fragment de celui-ci, comprenant au moins son domaine intracellulaire ;
b) mettre en contact ledit milieu avec le composé à tester ;
c) déterminer si la dimérisation ou la multimérisation dudit domaine intracellulaire du récepteur de la nétrine-1 est inhibée en présence dudit composé à tester ; et
d) sélectionner ledit composé si la détermination dans l'étape c) démontre une inhibition significative de la dimérisation ou de la multimérisation du domaine intracellulaire dudit récepteur de la nétrine-1.

16. Méthode selon la revendication 15, dans laquelle ladite cellule de l'étape (a) est une cellule tumorale.

17. Méthode selon la revendication 15, dans laquelle ladite cellule de l'étape (a) est une cellule présentant une dimérisation ou une multimérisation de son domaine intracellulaire du récepteur de la nétrine-1, ou une cellule dans laquelle son domaine intracellulaire du récepteur de la nétrine-1 est capable de se dimériser ou de se multimériser en présence de nétrine-1.

18. Méthode selon l'une quelconque des revendications 15 à 17, dans laquelle à l'étape (b) le milieu contenant en outre de la nétrine-1, ou un fragment de celle-ci, est capable de se lier au domaine extracellulaire du récepteur de la nétrine-1.

19. Méthode selon l'une quelconque des revendications 15 à 18, consistant en outre à déterminer si la présence du composé à tester induit la mort cellulaire de ladite cellule de mammifère ; et l'étape (d) comprend la détermination de la mort cellulaire de ladite cellule de mammifère.

20. Méthode in vitro de prédiction de la présence d'un cancer métastatique ou agressif chez un patient présentant une tumeur primaire à partir d'une biopsie dudit patient contenant des cellules de tumeurs primaires, ledit cancer étant choisi dans le groupe constitué par un cancer du sein, un cancer du poumon, un neuroblastome, un gliome, une leucémie, un sarcome, un mélanome et un adénocarcinome, ledit cancer à prévenir ou à traiter étant un cancer dans lequel des cellules tumorales expriment ou surexpriment la nétrine-1, comprenant les étapes suivantes consistant à :

(a) mesurer un taux de produit d'expression de nétrine-1 dans ladite biopsie, ledit produit étant (i) un ARN codant pour la nétrine-1 ou (ii) une protéine nétrine-1 ; et
(b) comparer ledit taux de produit d'expression avec l'expression du taux de produit d'expression de nétrine-1 dans une biopsie de tumeur primaire non métastatique ou dans une biopsie de cancer non agressif ;
dans laquelle une augmentation présentant un rapport supérieur à 2 entre les taux de produit d'expression de nétrine-1 dans la biopsie à tester et dans une biopsie de référence non métastatique ou non agressive est significative de la présence d'un cancer métastatique ou d'un cancer agressif.

21. Méthode de détermination *in vitro* de l'efficacité d'un traitement anticancéreux pour un patient ou de sélection *in vitro* de patients qui répondent à un traitement anticancéreux spécifique, ledit cancer étant choisi dans le groupe constitué par un cancer du sein, un cancer du poumon, un neuroblastome, un gliome, une leucémie, un sarcome, un mélanome et un adénocarcinome, ledit cancer à prévenir ou à traiter étant un cancer dans lequel des cellules tumorales expriment ou surexpriment la nétrine-1, ladite méthode comprenant l'étape suivante consistant à :

- mesurer un taux de produit d'expression de nétrine-1 dans une biopsie dudit patient, ledit produit étant (i) un ARN codant pour la nétrine-1 ou (ii) une protéine nétrine-1 ;

dans laquelle l'efficacité dudit traitement anticancéreux est en corrélation avec la diminution par un rapport d'au moins 2 de la quantité du taux de produit d'expression de nétrine-1 mesuré dans ladite biopsie,
ou
dans laquelle les patients choisis qui répondent à un traitement anticancéreux spécifique sont des patients pour lesquels la quantité du taux de produit d'expression de nétrine-1 mesuré dans leur biopsie a été diminuée par un rapport d'au moins 2 après ledit traitement spécifique.

22. Méthode selon la revendication 21, dans laquelle ledit cancer induit une surexpression de la nétrine-1 et/ou est un cancer métastatique.

23. Méthode selon les revendications 20 à 22, dans laquelle ledit ARN codant pour la nétrine-1 est mesuré par une méthode de réaction en chaîne par polymérase inverse (PCR) en temps réel.

24. Méthode selon les revendications 20 à 22, dans laquelle ladite protéine nétrine-1 est mesurée en utilisant des anticorps spécifiques capables de reconnaitre de manière spécifique ladite protéine nétrine-1.

25. Méthode selon les revendications 20 à 24, dans laquelle la tumeur primaire est une tumeur primaire d'un cancer choisi dans le groupe constitué par un cancer du sein, un cancer du poumon, un neuroblastome, un gliome, une leucémie, un sarcome, un mélanome et un adénocarcinome.

26. Méthode selon la revendication 25, dans laquelle ledit adénocarcinome à prévenir ou à traiter est choisi dans le

groupe constitué par un adénocarcinome de l'ovaire, un adénocarcinome rénal, un adénocarcinome du pancréas, un adénocarcinome de l'utérus, un adénocarcinome de l'estomac, un adénocarcinome du rein et un adénocarcinome rectal.

27. Utilisation, pour la sélection d'un composé destiné à la prévention ou au traitement d'un cancer, ledit cancer étant choisi dans le groupe constitué par un cancer du sein, un cancer du poumon, un neuroblastome, un gliome, une leucémie, un sarcome, un mélanome et un adénocarcinome, d'un kit comprenant des cellules tumorales qui expriment le récepteur de la nétrine-1 et qui expriment ou surexpriment la nétrine-1.

28. Utilisation selon la revendication 27, dans laquelle les cellules tumorales sont des cellules provenant d'une lignée de cellules tumorales.

29. Utilisation selon la revendication 27 ou 28, comprenant en outre une protéine nétrine-1, ou un fragment de celle-ci, capable d'interagir de manière spécifique avec ladite protéine récepteur de la nétrine-1 pour former une paire de liaison.

30. Composé choisi dans le groupe constitué par :

- un composé comprenant un domaine extracellulaire du récepteur de la nétrine-1 ou un fragment de celui-ci capable d'inhiber de manière spécifique l'interaction entre la nétrine-1 et ledit récepteur de la nétrine-1, et/ou capable d'inhiber la dimérisation ou la multimérisation du domaine intracellulaire dudit récepteur de la nétrine-1 ; et
- un anticorps monoclonal ou polyclonal dirigé spécifiquement contre la nétrine-1 ou le domaine extracellulaire du récepteur de la nétrine-1 ou un fragment de la nétrine-1 capable d'interagir avec le domaine extracellulaire dudit récepteur de la nétrine-1,

pour une utilisation dans une méthode *in vivo* destinée à prévenir ou traiter un cancer du sein, un cancer du poumon, un neuroblastome, un gliome, une leucémie, un sarcome, un mélanome et un adénocarcinome.

31. Composé pour une utilisation selon la revendication 30, dans lequel ledit domaine extracellulaire du récepteur de la nétrine-1 ou fragment de celui-ci est choisi dans le groupe constitué par DCC, UNC5H, la néogénine et l'adénosine A2b.

32. Composé pour une utilisation selon la revendication 30 ou 31, dans lequel ledit composé comprenant un domaine extracellulaire du récepteur de la nétrine-1 provient de DCC.

33. Composé pour une utilisation selon la revendication 32, dans lequel ledit composé est DCC-EC-Fc ou DCC-5Fbn.

34. Composé pour une utilisation selon l'une quelconque des revendications 30 à 33, pour une utilisation dans une méthode destinée à prévenir ou traiter un cancer dans lequel les cellules tumorales expriment ou surexpriment la nétrine-1.

35. Composé selon les revendications 30 à 34 ou un oligonucléotide antisens ou ARNi (ARN interférent) qui est spécifique et complémentaire à l'acide nucléique codant pour la protéine nétrine-1 pour une utilisation dans une méthode *in vivo* destinée à prévenir ou traiter un cancer des mammifères, notamment l'homme, ledit cancer étant choisi dans le groupe constitué par un cancer du sein, un cancer du poumon, un neuroblastome, un gliome, une leucémie, un sarcome, un mélanome et un adénocarcinome, ledit cancer à prévenir ou à traiter étant un cancer dans lequel des cellules tumorales expriment ou surexpriment la nétrine-1.

36. Utilisation du composé selon la revendication 35, **caractérisée en ce que** ledit cancer est un cancer métastatique.

37. Utilisation du composé selon la revendication 35 ou 33, **caractérisée en ce que** ledit adénocarcinome est choisi dans le groupe constitué par un adénocarcinome de l'ovaire, un adénocarcinome rénal, un adénocarcinome du pancréas, un adénocarcinome de l'utérus, un adénocarcinome de l'estomac, un adénocarcinome du rein et un adénocarcinome rectal

38. Utilisation du composé selon les revendications 35 à 37, **caractérisée en ce que** les cellules de la tumeur primaire dudit cancer expriment ou surexpriment la nétrine-1.

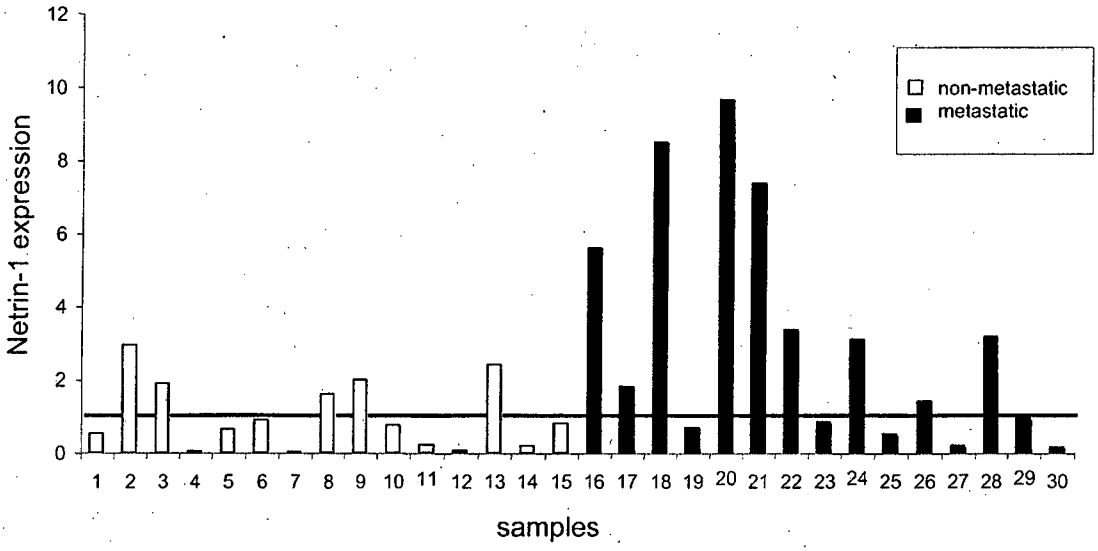


FIGURE 1A

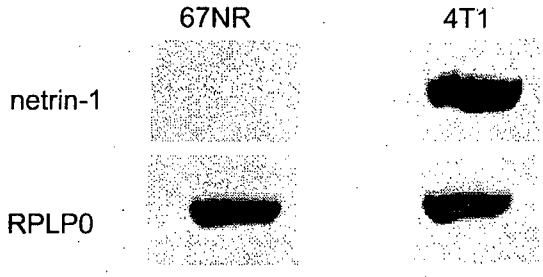


FIGURE 1B

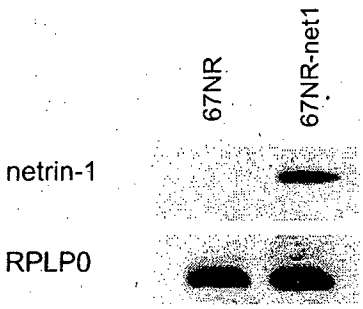


FIGURE 2A

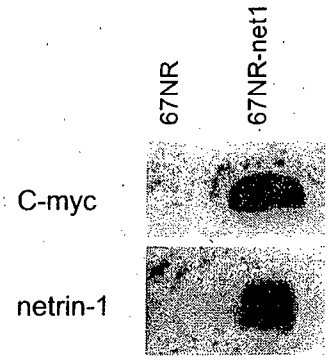


FIGURE 2B

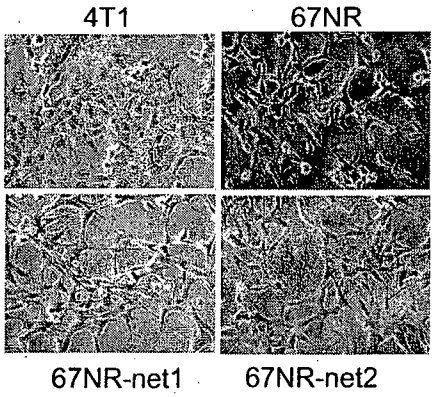


FIGURE 2C

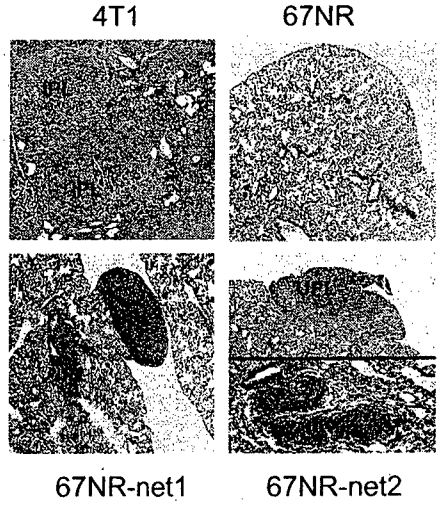


FIGURE 2D

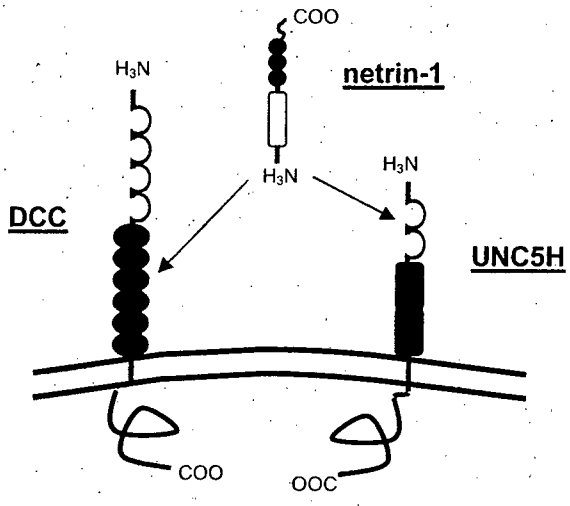


FIGURE 3A

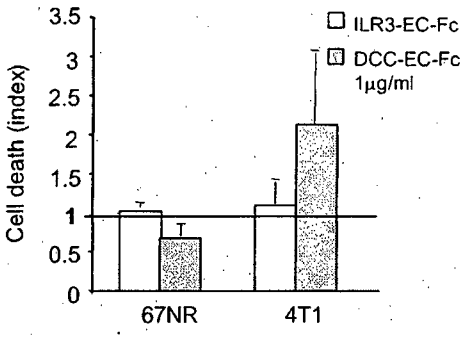


FIGURE 3B

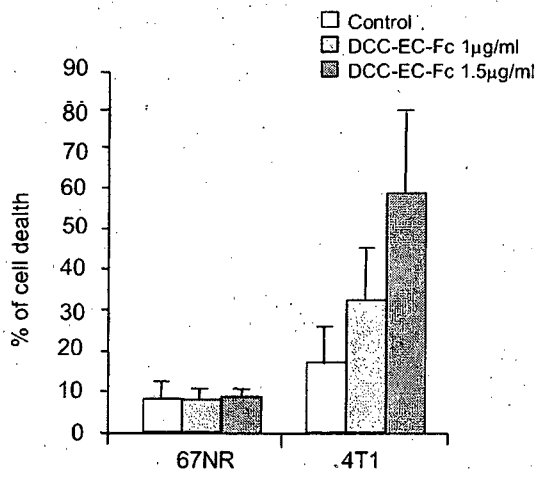


FIGURE 3C

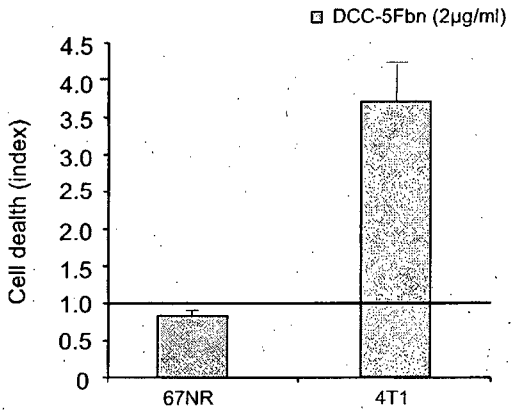


FIGURE 3D

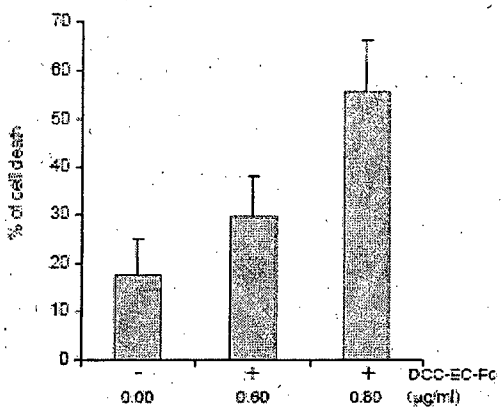


FIGURE 4A

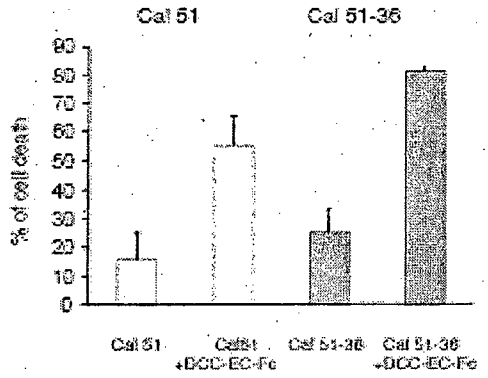


FIGURE 4B

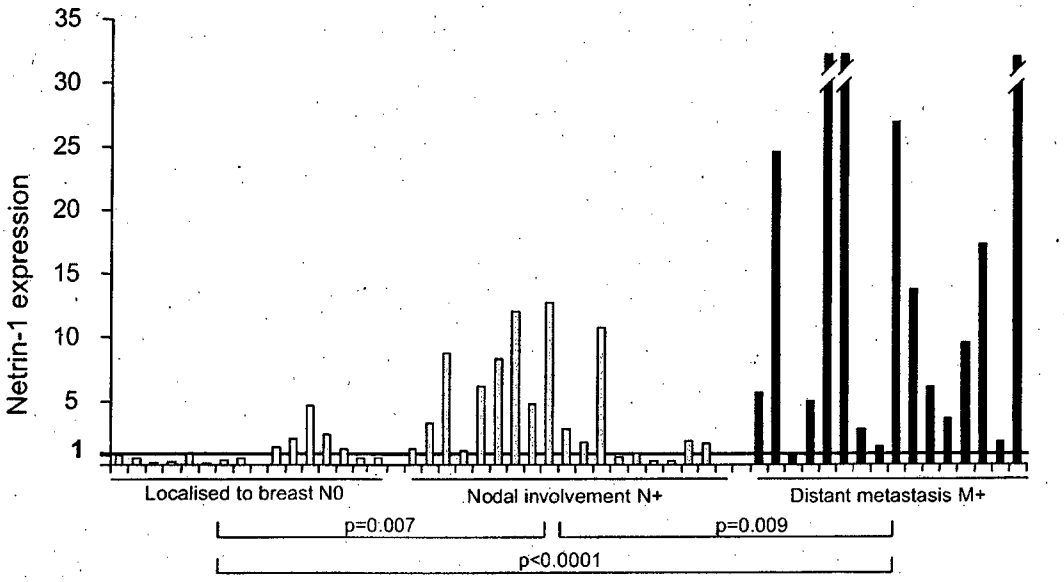


FIGURE 5

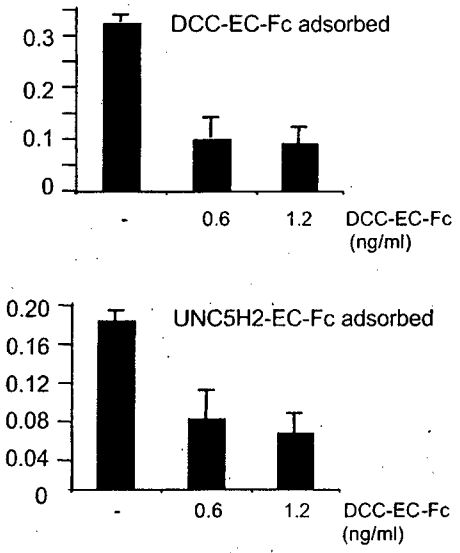


FIGURE 6A

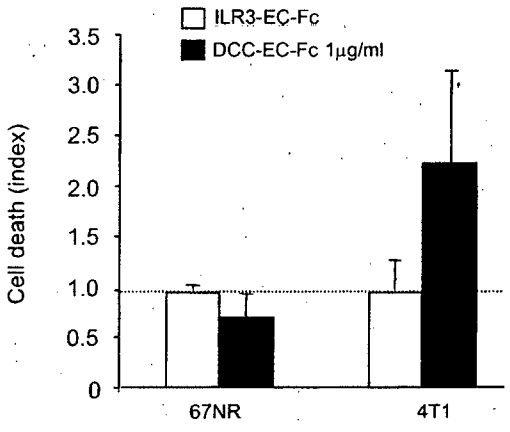


FIGURE 6B

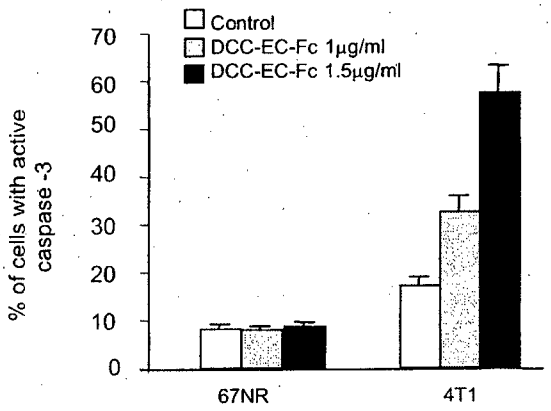


FIGURE 6C

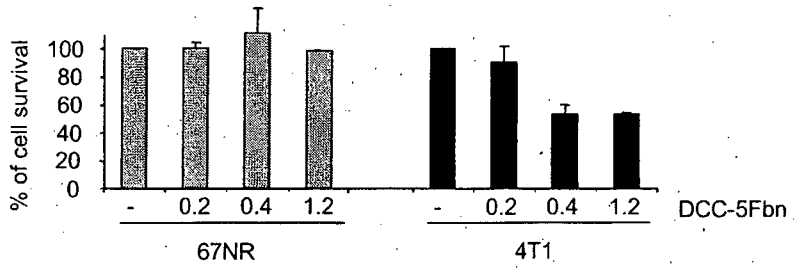


FIGURE 7A

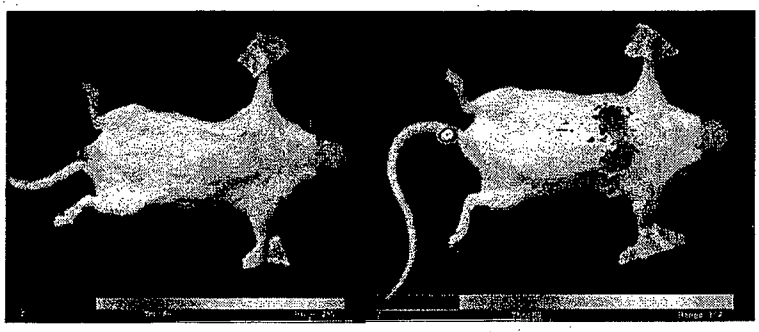


FIGURE 7B

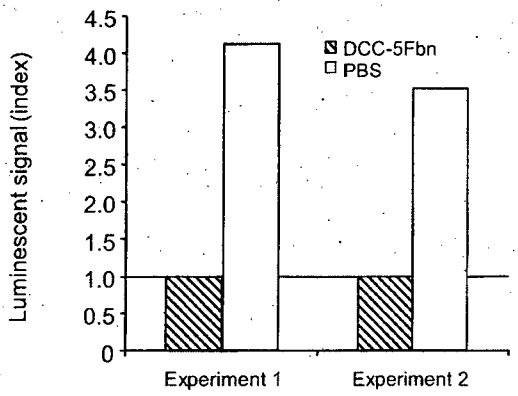


FIGURE 7C

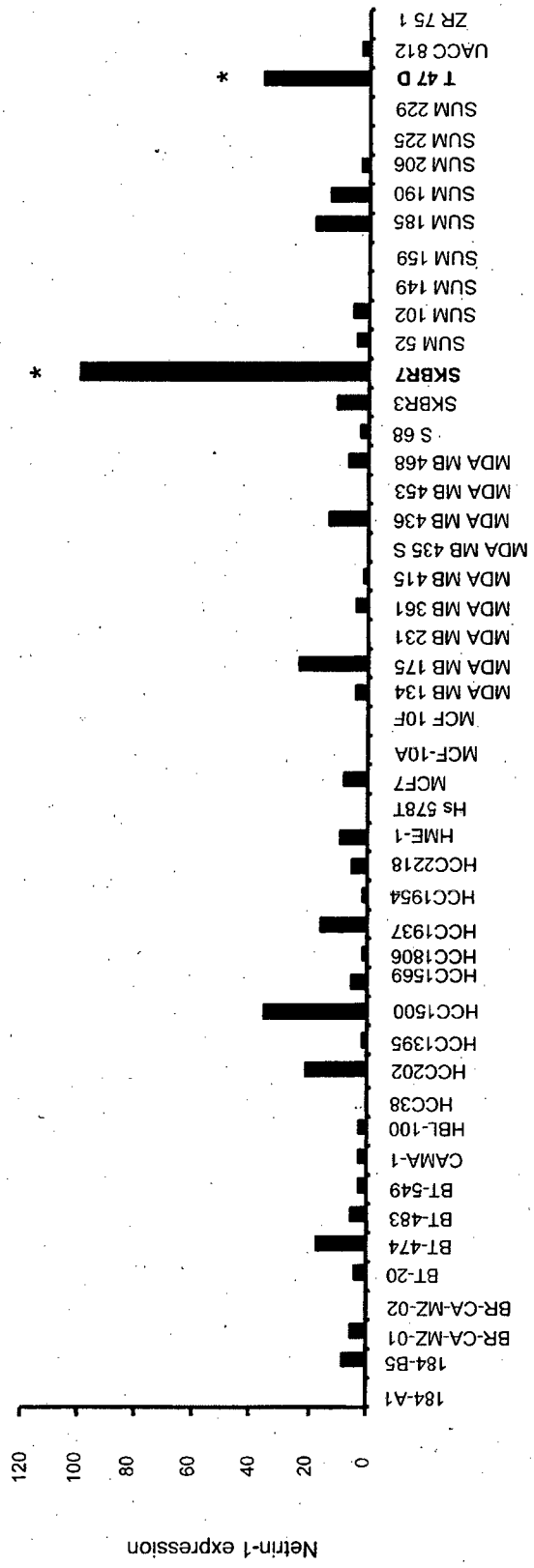

Netrin-1 expression
120
100
80
60
40
20
0
184-A1
184-B5
BR-CA-MZ-01
BR-CA-MZ-02
BT-20
BT-474
BT-483
BT-549
CAMA-1
HBL-100
HCC38
HCC202
HCC1395
HCC1500
HCC1569
HCC1806
HCC1937
HCC1954
HCC2218
HME-1
Hs 578T
MCF7
MCF-10A
MCF 10F
MDA MB 134
MDA MB 175
MDA MB 231
MDA MB 361
MDA MB 415
MDA MB 435 S
MDA MB 436
MDA MB 453
MDA MB 468
S 68
SKBR3
SKBR7
SUM 52
SUM 102
SUM 149
SUM 159
SUM 185
SUM 190
SUM 206
SUM 225
SUM 229
T 47 D
UACC 812
ZR 75 1
*
*

FIGURE 8A

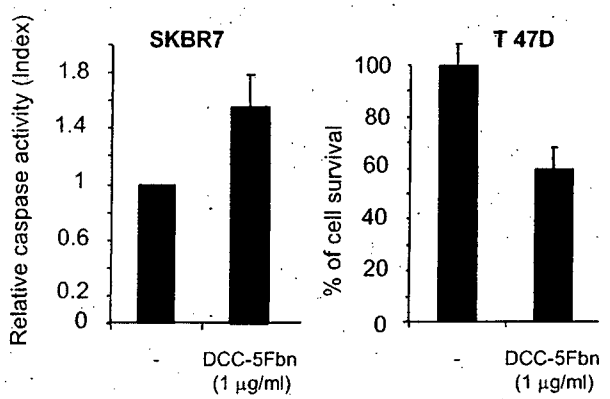


FIGURE 8B

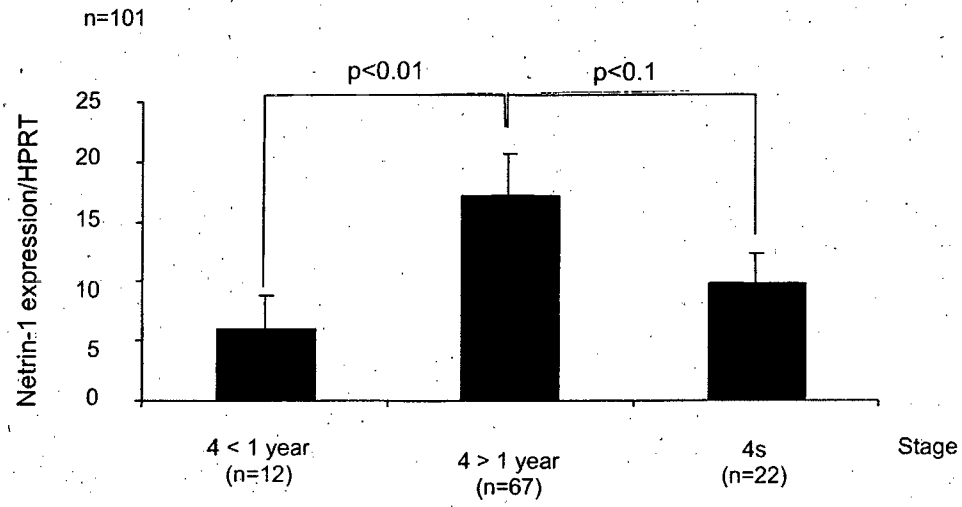


FIGURE 9A

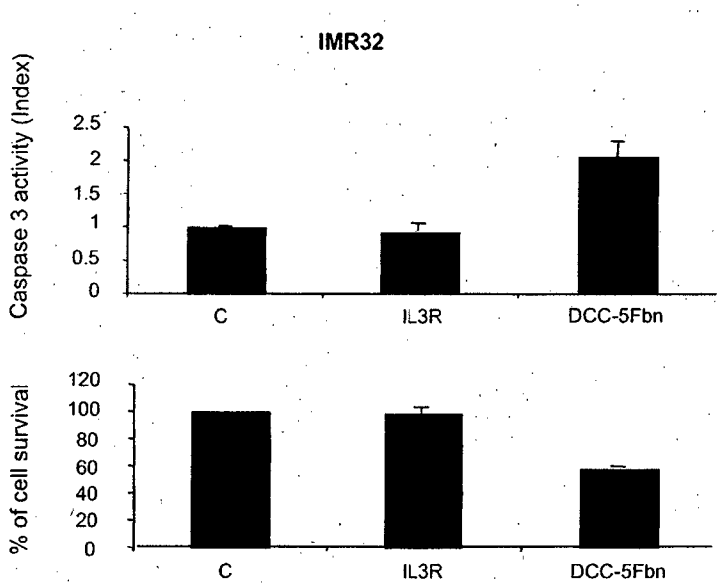

IMR32
Caspase 3 activity (Index)
2.5
2
1.5
1
0.5
0
C
IL3R
DCC-5Fbn
% of cell survival
120
100
80
60
40
20
0
C
IL3R
DCC-5Fbn

FIGURE 9B

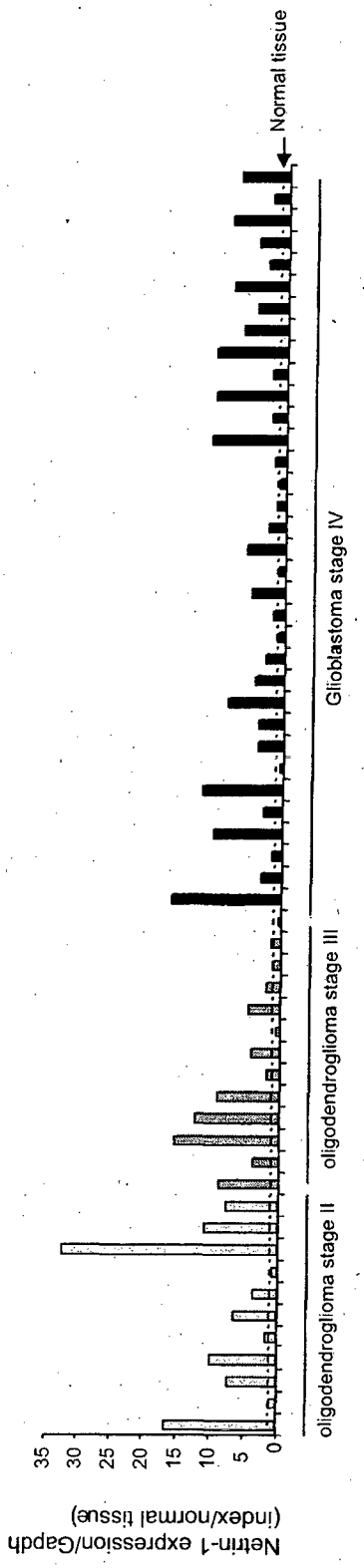


FIGURE 10A

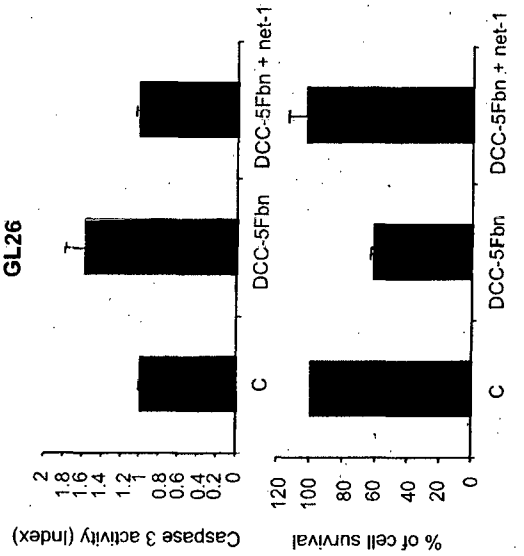


FIGURE 10B

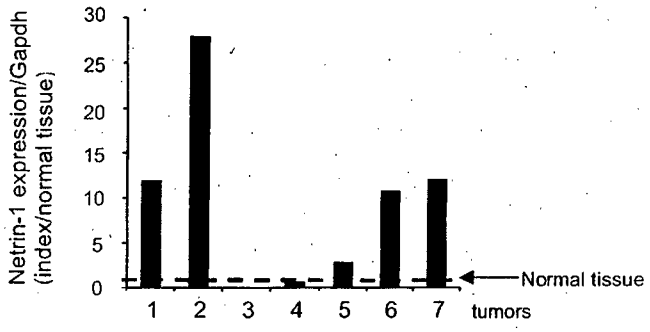


FIGURE 11A

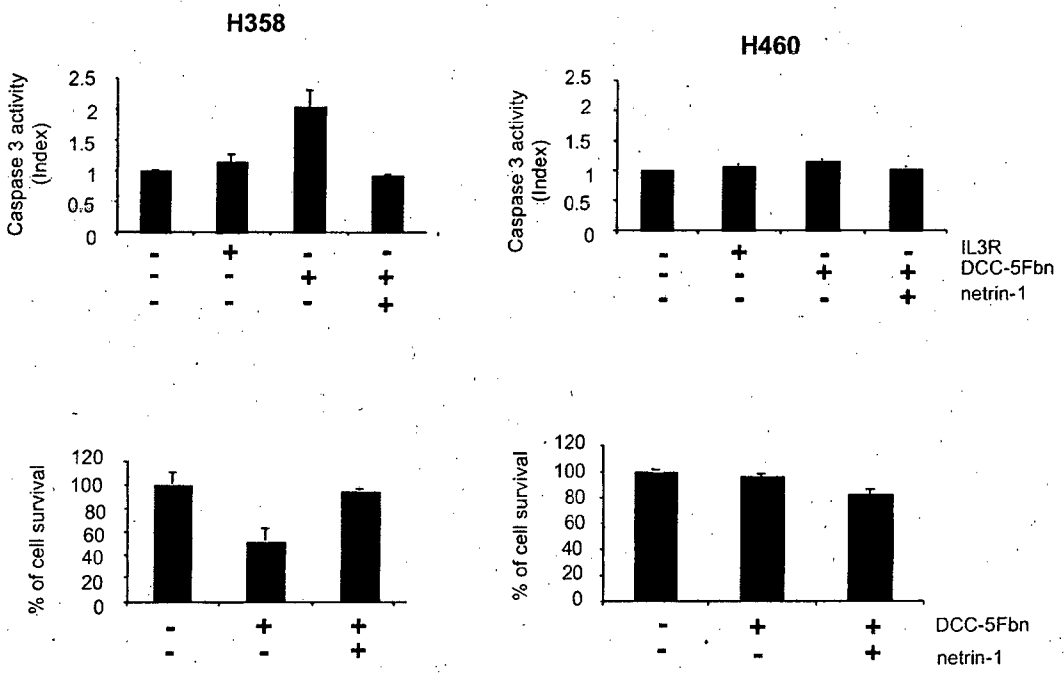


FIGURE 11B

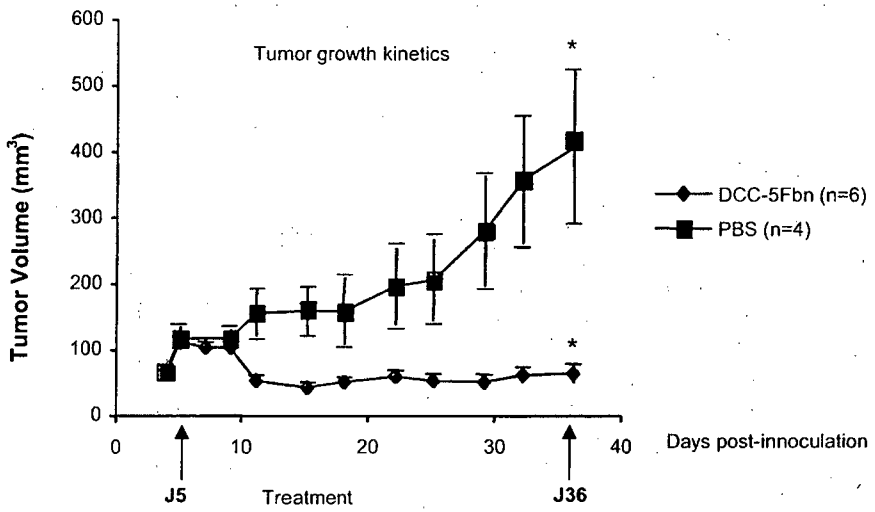


FIGURE 11C

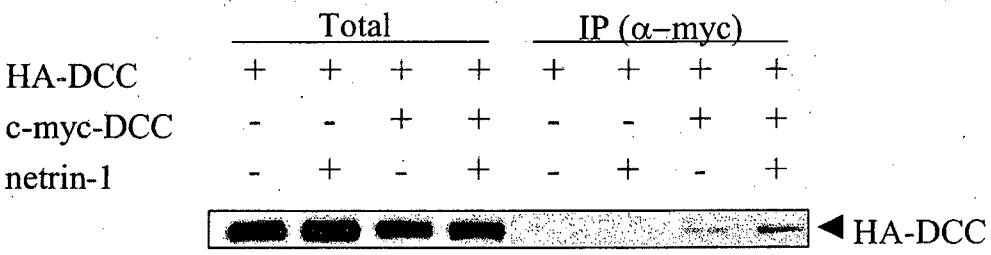


FIGURE 12A

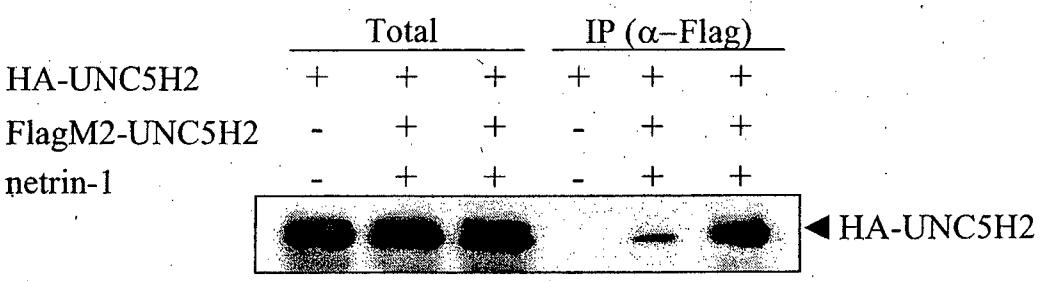


FIGURE 12B

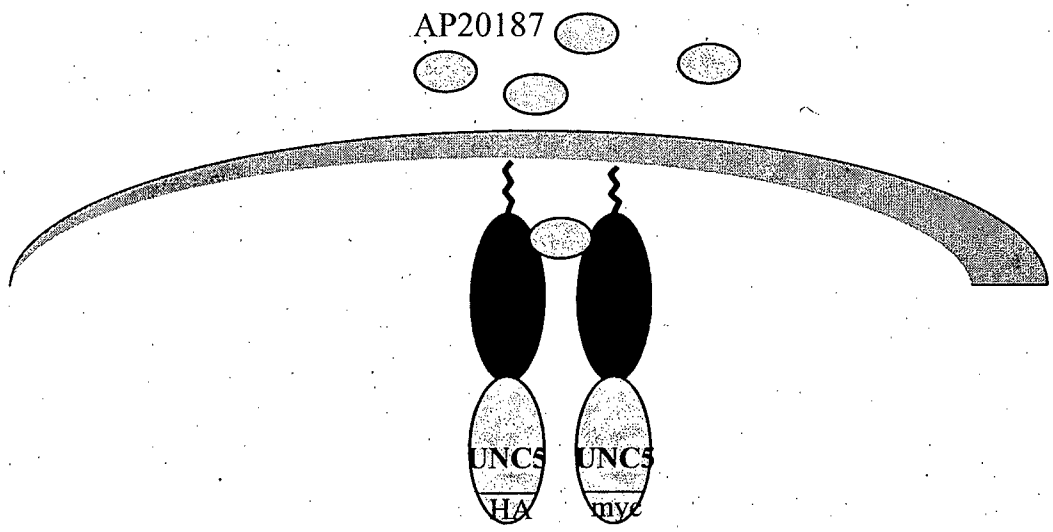


FIGURE 13A

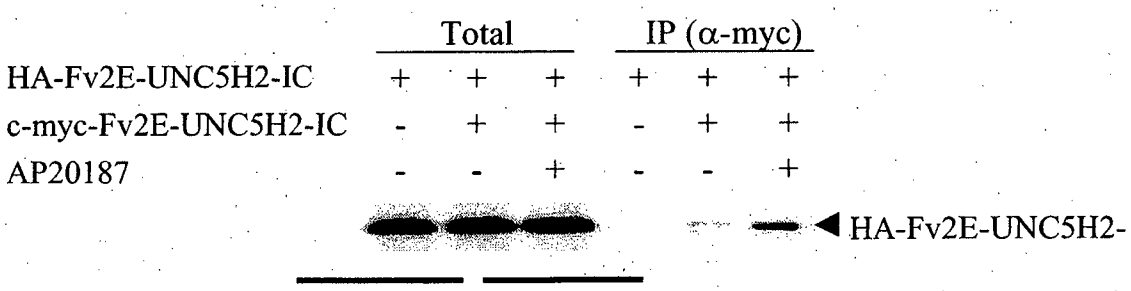


FIGURE 13B

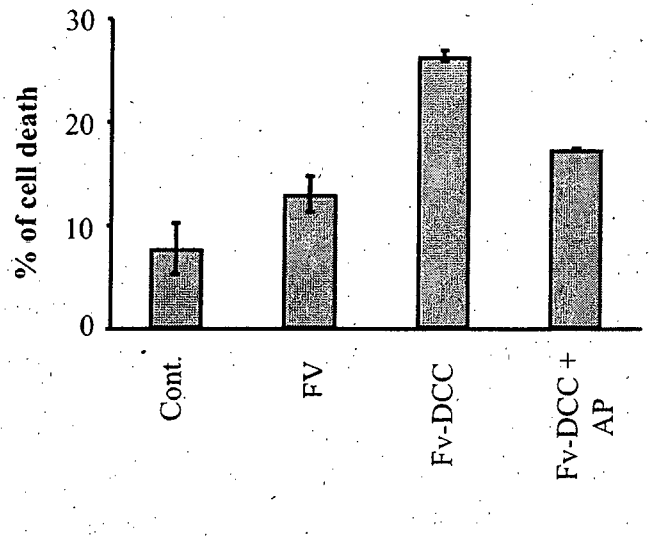


FIGURE 14A

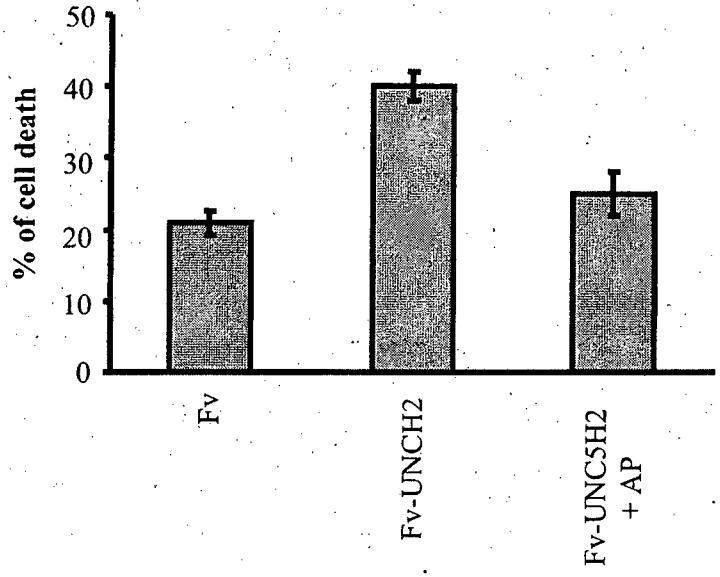


FIGURE 14B

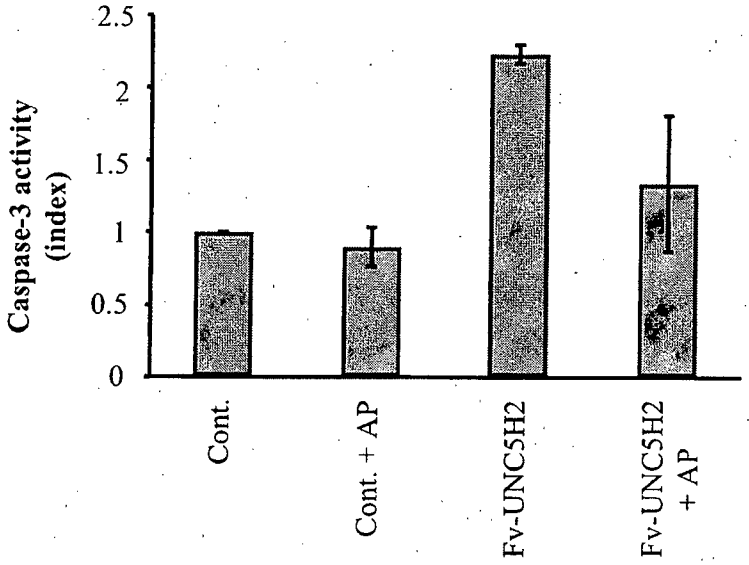


FIGURE 14C

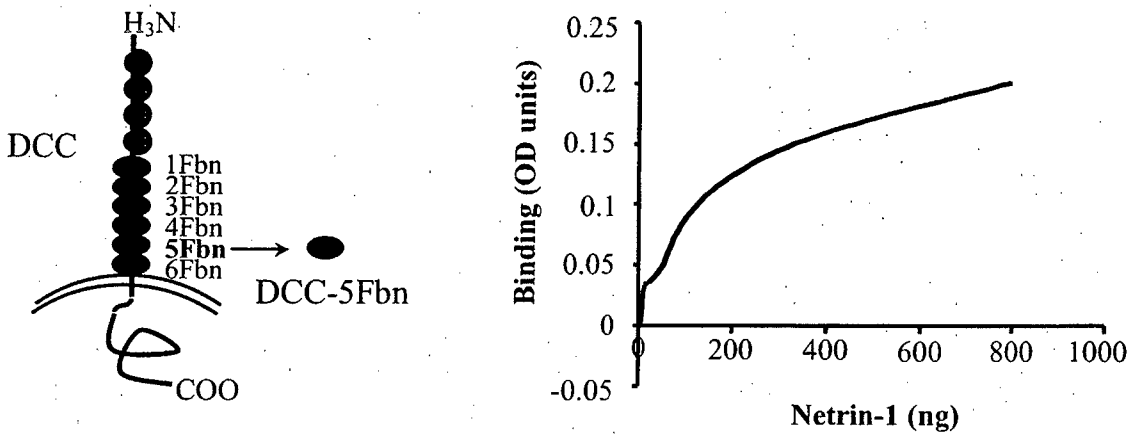


FIGURE 15A

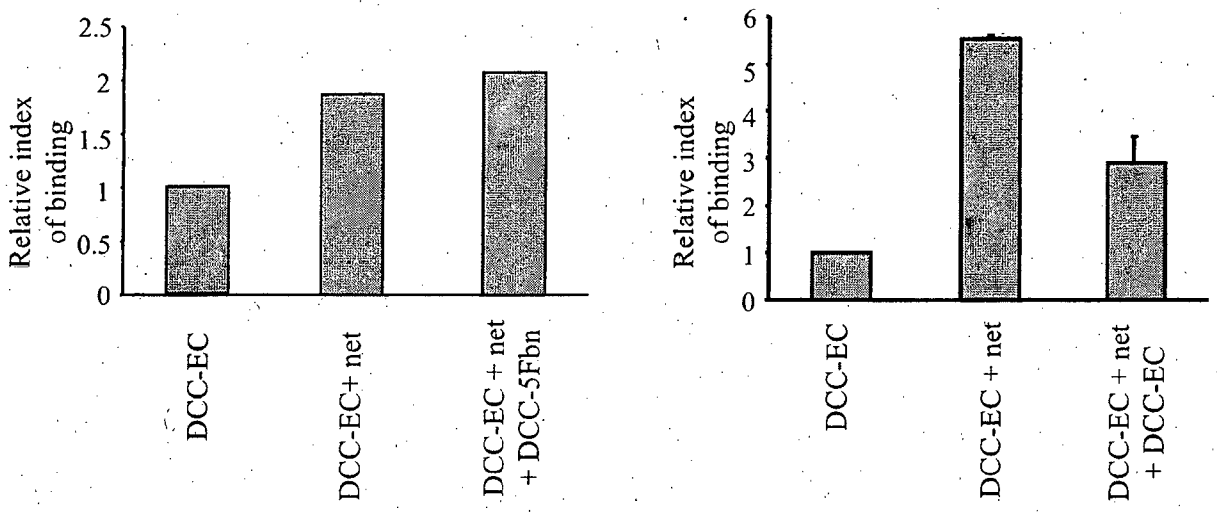


FIGURE 15B

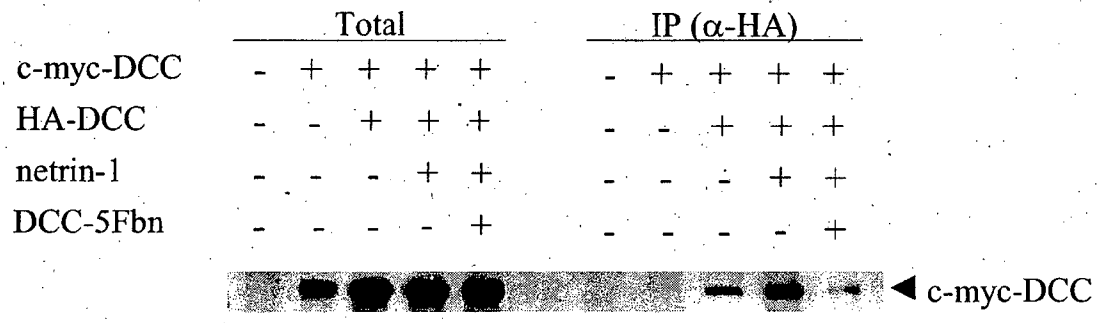


FIGURE 15C

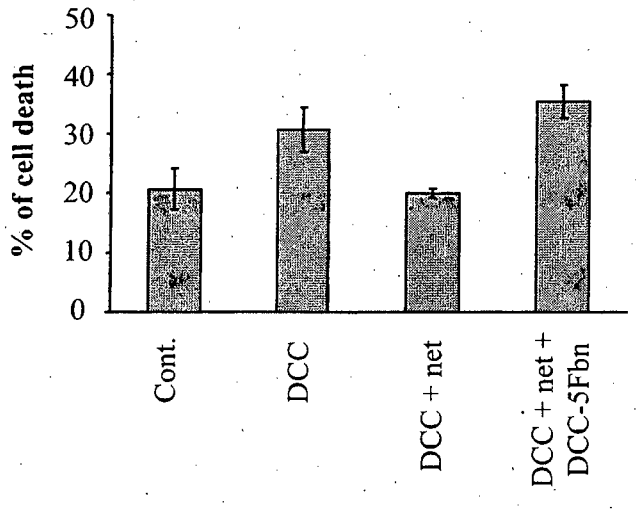


FIGURE 16A

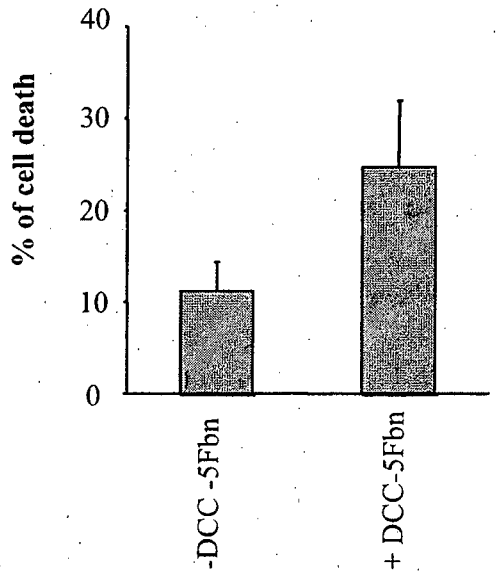


FIGURE 16B

REFERENCES CITED IN THE DESCRIPTION

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SZŰRÉS NETRIN-1 AKTIVITÁSÚ, RÁK ELLENI VEGYÜLETEKRE

Szabadalmi igénypontok

1. Eljárás rák megelőzésére vagy kezelésére szolgáló vegyület kiválasztására, ahol a megelőzendő vagy kezelendő rák a következők alkotta csoportból választott: mellrák, tüdőrák, neuroblasztóma, glióma, leukémia, szarkóma, melanóma és adenokarcinóma, ahol a megelőzendő vagy kezelendő rák olyan rák, amelyben a tumoros sejtek netrin-1-et expresszálnak vagy túlexpresszálnak, ahol az eljárás tartalmazza a következő lépéseket:

a) netrin-1-et vagy fragmensét és netrin-1-receptort vagy fragmensét tartalmazó közeg biztosítása, ahol

- a netrin-1 vagy fragmense és a netrin-1-receptor vagy fragmense képes egymással specifikusan kölcsönhatva kötőpárt kialakítani, és/vagy
- a netrin-1 vagy fragmense képes a netrin-1-receptor intracelluláris doménjének dimerizációját vagy multimerizációját indukálni;

b) a közeg érintkeztetése a vizsgálandó vegyülettel;

c)

- a netrin-1 vagy fragmense és a netrin-1-receptor vagy fragmense közötti kölcsönhatás gátlásának mérése, és/vagy
- annak meghatározása, hogy a vegyület gátolja-e a netrin-1-receptor vagy fragmense dimerizációját vagy multimerizációját, különösen a netrin-1-receptor intracelluláris doménjének dimerizációját; és

d) a vegyület kiválasztása, ha:

- a c) lépésben a mérés a netrin-1 vagy fragmense és a netrin-1-receptor vagy fragmense közötti kölcsönhatás jelentős gátlását mutatja ki a vegyület jelenlétében, és/vagy
- a c) lépésben a meghatározás a netrin-1-receptor intracelluláris doménje dimerizációjának vagy multimerizációjának jelentős gátlását mutatja ki a vegyület jelenlétében.

2. Az 1. igénypont szerinti eljárás, ahol a megelőzendő vagy kezelendő adenokarcinóma a következők alkotta csoportból választott: petefészek-adenokarcinóma, renális adenokarcinóma, hasnyálmirigy-adenokarcinóma, méh-adenokarcinóma, gyomor-adenokarcinóma, vese-adenokarcinóma és rektális adenokarcinóma.

3. Az 1. vagy 2. igénypont szerinti eljárás, ahol a megelőzendő vagy kezelendő rák metasztázisos rák.

4. Az 1-3. igénypont szerinti eljárás, ahol az a) lépésben a netrin-1-receptor a következők alkotta csoportból választott: Kolorektális Rákban Deletált (Deleted in Colorectal Cancer, DCC), Unc-5-homológ (UNC5H), neogenin és adenozin-A2b receptorok.

5. A 4. igénypont szerinti eljárás, ahol az a) lépésben a netrin-1-receptor a következő csoportból választott: DCC, UNC5H1, UNC5H2 és UNC5H3.

6. Az 1-5. igénypontok bármelyike szerinti eljárás, ahol az a) lépésben

- a netrin-1-receptor-fragmens a netrin-1-receptor extracelluláris doménjét vagy annak netrin-1-hez kötődni képes részét tartalmazza vagy azzal azonos; és/vagy
- a netrin-1-receptor-fragmens a netrin-1-receptor extracelluláris doménjét vagy annak netrin-1 jelenlétében dimerizálódni vagy multimerizálódni képes részét tartalmazza vagy azzal azonos.

7. Az 1-6. igénypontok bármelyike szerinti eljárás, ahol az a) lépésben a netrin-1 vagy/és a netrin-1-receptor emlősből származnak.

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8. Az 1-7. igénypont szerinti eljárás, ahol az a) lépésben a netrin-1 baromfiból származik.

9. Az 1-8. igénypontok bármelyike szerinti eljárás, ahol a netrin-1 vagy/és a netrin-1-receptor és/vagy a tesztelendő vegyület olyan markerrel van jelölve, amely közvetlenül vagy közvetetten mérhető.

10. Az 1-9. igénypontok bármelyike szerinti eljárás, ahol a c) lépésben:

- netrin-1 vagy fragmense és a netrin-1-receptor vagy fragmense közötti kölcsönhatás gátlásának mérése immunológiai vizsgálati eljárással történik (különösen enzimkapcsolt immunoszorbens vizsgálati eljárással (ELISA) vagy immunoradiometriás vizsgálati eljárással (IRMA)), szcintillációs közelségi vizsgálati eljárással (SPA) vagy fluoreszcens rezonancia energiatranszferrel (FRET); és/vagy

- a netrin-1-receptor vagy fragmense, különösen az intracelluláris domén, dimerizációja vagy multimerizációja, vagy ennek gátlása, immunprecipitációval vagy FRET-tel történik.

11. Az 1-10. igénypontok bármelyike szerinti eljárás, ahol az a) lépésben a közeg tartalmaz sejteket, amelyek felszíni membránjukon endogén netrin-1-receptort vagy rekombináns netrin-1-receptort expresszálnak, különösen legalább a rekombináns netrin-1-receptor extracelluláris doménjét expresszálják.

12. A 11. igénypont szerinti eljárás, ahol az a) lépésben a közeg tartalmaz sejteket, amelyek rekombináns netrin-1-receptort expresszálnak.

13. A 11. igénypont szerinti eljárás, amelyben az a) lépésben a közeg tumoros sejteket tartalmaz, amelyek endogén módon expresszálják a netrin-1-receptort membránfelszínükön, és amelyek expresszálják vagy fokozottan expresszálják a netrin-1-et, és ahol a c) lépésben a netrin-1 és netrin-1-receptora közötti kölcsönhatás gátlásának mérése, a vizsgálandó vegyület jelenlétében, a vizsgálandó vegyület jelenlétében indukált apoptózis vagy sejthalál mérésével történik.

14. A 13. igénypont szerinti eljárás, ahol az a) lépésben a közeg metasztázisos tumoros sejteket tartalmaz.

15. Az 1-14. igénypont szerinti eljárás rák megelőzésére vagy kezelésére szolgáló vegyület kiválasztására, ahol az eljárás tartalmazza a következő lépéseket:

a) endogén vagy rekombináns netrin-1-receptort vagy legalább intracelluláris doménjét tartalmazó fragmensét expresszáló emlőssejtet tartalmazó közeg biztosítása;

b) a közeg érintkeztetése a vizsgálandó vegyülettel;

c) annak meghatározása, hogy a netrin-1-receptor intracelluláris domén dimerizációja vagy multimerizációja gátolt a vizsgálandó vegyület jelenlétében; és

d) a vegyület kiválasztása, amennyiben a c) lépésben a meghatározás a netrin-1-receptor intracelluláris doménje dimerizációjának vagy multimerizációjának jelentős gátlását mutatja ki.

16. A 15. igénypont szerinti eljárás, ahol az (a) lépés szerinti sejt tumorsejt.

17. A 15. igénypont szerinti eljárás, ahol az (a) lépés szerinti sejt netrin-1-receptora intracelluláris doménjének dimerizációját vagy multimerizációját prezentáló sejt vagy olyan sejt, ahol a sejt netrin-1-receptorának intracelluláris doménje képes dimerizálódni vagy multimerizálódni netrin-1 jelenlétében.

18. A 15-17. igénypontok bármelyike szerinti eljárás, ahol a (b) lépésben a közeg tartalmaz továbbá netrin-1-et vagy a netrin-1-receptor extracelluláris doménjéhez kötődni képes fragmensét.

19. A 15-18. igénypontok bármelyike szerinti eljárás, amely tartalmazza továbbá annak meghatározását, hogy a tesztelendő vegyület jelenléte az emlőssejt sejthalálát indukálja-e; és a (d) lépés tartalmazza továbbá az emlőssejt sejthalálának meghatározását.

20. *In vitro* eljárás metasztázisos vagy agresszív rák jelenlétének predikciójára primer tumorban szenvedő páciensben a páciens primer tumorsejteket tartalmazó biopsziájából, ahol a rák a következők alkotta csoportból választott: mellrák, tüdőrák, neuroblasztóma, glióma, leukémia, szarkóma, melanóma és adenokarcinóma, ahol a megelőzendő vagy kezelendő rák olyan rák, amelyben a tumoros sejtek netrin-1-et expresszálnak vagy túlexpresszálnak, ahol az eljárás tartalmazza a következő lépéseket:

a) a biopsziában netrin-1-expresszió terméke szintjének mérése, ahol a termék (i) netrin-1-et kódoló RNS vagy (ii) netrin-1-fehérje; és

b) az expresszió terméke szintjének összehasonlítás netrin-1-expresszió terméke szintjével nem metasztázisos primertumor-biopsziában vagy nem agresszív rák biopsziájában;

ahol a netrin-1-expresszió terméke vizsgálandó biopsziabeli szintje és a netrin-1-expresszió terméke nem metasztázisos vagy nem agressziv, referencia biopsziabeli szintje arányának 2 feletti növekedése metasztázisos rákra vagy agresszív rákra jellemző.

20. Eljárás páciens rák elleni terápiája hatékonyságának meghatározására *in vitro* vagy adott rák elleni kezelésre válaszoló páciensek kiválasztására *in vitro*, ahol a rák a következők alkotta csoportból választott: mellrák, tüdőrák, neuroblasztóma, glióma, leukémia, szarkóma, melanóma és adenokarcinóma, ahol a megelőzendő vagy kezelendő rák olyan rák, amelyben a tumoros sejtek netrin-1-et expresszálnak vagy túlexpresszálnak, ahol az eljárás tartalmazza a következő lépéseket:

- a páciens biopsziájában netrin-1-expresszió terméke szintjének mérése, ahol a termék (i) netrin-1-et kódoló RNS vagy (ii) netrin-1-fehérje;

ahol a rák elleni kezelés hatékonysága korrelál a netrin-1-expresszió terméke biopsziában mért mennyiségének legalább 2-szeres arányú csökkenésével, vagy

ahol az adott rák elleni terápiára válaszoló, kiválasztott páciensek azok, akikben a netrin-1-expresszió terméke biopsziájukban mért mennyisége a kezelés után legalább 2-szeres arányban csökkent.

22. A 21. igénypont szerinti eljárás, ahol a rák netrin-1 túlexpresszálását indukálta és/vagy metasztázisos rák.

23. A 20-22. igénypontok bármelyike szerinti eljárás, ahol a netrin-1-et kódoló RNS-s mérése kvantitatív, valós idejű reverz polimeráz láncreakcióval (PCR) történik.

24. A 20-22. igénypontok bármelyike szerinti eljárás, ahol a netrin-1-fehérje mérése a netrin-1-fehérjét specifikusan felismerni képes, specifikus antitestekkel történik.

25. A 20-24. igénypontok bármelyike szerinti eljárás, ahol a primer tumor a következők alkotta csoportból választott rák: mellrák, tüdőrák, neuroblasztóma, glióma, leukémia, szarkóma, melanóma és adenokarcinóma.

26. A 25. igénypont szerinti eljárás, ahol az adenokarcinóma a következők alkotta csoportból választott: petefészek-adenokarcinóma, renális adenokarcinóma, hasnyálmirigy-adenokarcinóma, méh-adenokarcinóma, gyomor-adenokarcinóma, vese-adenokarcinóma és rektális adenokarcinóma.

27. Reagenskészlet alkalmazása rák megelőzésére vagy kezelésére szolgáló vegyület kiválasztására, ahol a rák a következők alkotta csoportból választott: mellrák, tüdőrák, neuroblasztóma, glióma, leukémia, szarkóma, melanóma és adenokarcinóma, és a reagenskészet netrin-1-receptort expresszáló és netrin-1-et expresszáló vagy túlexpresszáló tumoros sejteket tartalmaz.

28. A 27. igénypont szerinti alkalmazás, ahol a tumoros sejtek tumorsejtvonalból származó sejtek.

29. A 27. vagy 28. igénypont szerinti alkalmazás, amely tartalmaz továbbá netrin-1-fehérjét vagy annak a netrin-1-receptorfehérjével kötőpárt kialakító módon, specifikusan kölcsönható fragmensét.

30. A következők alkotta csoportból választott vegyület:

- vegyület, amely tartalmazza netrin-1-receptor extracelluláris doménjét vagy annak a netrin-1 és a netrin-1-receptor közötti kölcsönhatást specifikusan gátolni képes fragmensét, és/vagy képes gátolni a netrin-1-receptor intracelluláris doménjének dimerizációját vagy multimerizációját; és
- monoklonális vagy poliklonális antitest, amely specifikusan közvetlenül netrin-1 vagy netrin-1-receptor extracelluláris doménje vagy a netrin-1-receptor extracelluláris doménjével kölcsönhatni képes netrin-1-fragmens elleni antitest

mellrák, tüdőrák, neuroblasztóma, glióma, leukémia, szarkóma, melanóma és adenokarcinóma megelőzésére vagy kezelésére szolgáló *in vivo* eljárásban történő alkalmazásra.

31. A 30. igénypont szerinti vegyület az ott meghatározott alkalmazásra, ahol a netrin-1-receptor vagy fragmense extracelluláris doménje a következők alkotta csoportból választott: DCC; UNC5H, neogenin és az adenozin-A2b.

32. A 30. vagy 31. igénypont szerinti vegyület az ott meghatározott alkalmazásra, ahol a netrin-1-receptor extracelluláris doménjét tartalmazó vegyület DCC-ből származik.

33. A 32. igénypont szerinti vegyület az ott meghatározott alkalmazásra, ahol a vegyület DCC-EC-Fc vagy DCC-5Fbn.

34. A 30-33. igénypontok bármelyike szerinti vegyület az ott meghatározott alkalmazásra, olyan rák megelőzésében vagy kezelésében történő alkalmazásra, amelyben tumoros sejtek netrin-1-et expresszálnak vagy túlexpresszálnak.

35. A 30-34. igénypontok bármelyike szerinti vegyület vagy antiszensz vagy iRNS (interferáló RNS) oligonukleotid, amely netrin-1-fehérjét kódoló nukleinsavra komplementer, emlősben, beleértve az embert, rák kezelésére vagy megelőzésére szolgáló *in vivo* eljárásban történő alkalmazásra, ahol a rák a következők alkotta csoportból választott: mellrák, tüdőrák, neuroblasztóma, glióma, leukémia, szarkóma, melanóma és adenokarcinóma, ahol a megelőzendő vagy kezelendő rákban tumoros sejtek netrin-1-et expresszálnak vagy túlexpresszálnak.

36. A 35. igénypont szerinti vegyület alkalmazása, **azzal jellemezve, hogy** a rák metasztázisos rák.

37. A 35. vagy 33. igénypont szerinti vegyület alkalmazása, **azzal jellemezve, hogy** az adenokarcinóma a következők alkotta csoportból választott: petefészek-adenokarcinóma, renális adenokarcinóma, hasnyálmirigy-adenokarcinóma, méh-adenokarcinóma, gyomor-adenokarcinóma, vese-adenokarcinóma és rektális adenokarcinóma.

38. Vegyület 35-37. igénypontok bármelyike szerinti alkalmazása, **azzal jellemezve, hogy** a rák primer tumorsejtjei netrin-1-et expresszálnak vagy túlexpresszálnak.