Combined treatment with netrin-1 interfering drug and chemotherapeutic drug

The present invention relates to novel combined compositions and methods to treat cancer.

Netrin-1, a soluble protein initially discovered as an axon navigation cue (1), was recently proposed to play a crucial role in cancer progression by regulating apoptosis (2, 3). Indeed, netrin-1 receptors DCC and UNC5H, -- i.e., UNC5H1, UNC5H2, UNC5H3 and UNC5H4 also called UNC5A, UNC5B, UNC5C or UNC5D -- belong to the so-called dependence receptor family (4) (5) (6). These dependence receptors, because of their ability to induce cell death when disengaged from their ligands, create cellular states of dependence on their respective ligands (7) and, consequently, may behave as tumor suppressors because they eliminate tumor cells that would develop in settings of ligand unavailability (2, 8). Along this line, mice bearing a DCC receptor inactivated for its pro-apoptotic activity developed spontaneous colorectal cancers and were more prone to intestinal tumor progression (9). Similarly, inactivation of UNC5H3/C in mice in the gastro-intestinal tract is associated with intestinal tumor progression (10).

Thus, according to the dependence receptor paradigm, progression of aggressive human tumors should require inactivation of this death pathway. There are at least three means to achieve this survival advantage: loss of netrin-1 receptors expression, as extensively described in human colorectal cancer for DCC or/and UNC5H (10-13); loss the downstream death signaling induced by DCC or UNC5H; gain autocrine or paracrine expression of the ligand. Interestingly, netrin-1 has been shown to be up-regulated in a sizeable fraction of metastatic breast, lung, ovary and pancreatic cancer, in inflammatory associated colorectal cancer and in neuroblastoma (14-19). Proof-of concept studies, in vitro and in mice or chicken models of cancer, have shown that silencing of netrin-1 by netrin-1 siRNA or interference with netrin-1–receptors interaction are associated with tumor cell death and with inhibition of tumor growth and metastases (14-18). These later studies proposed that disrupting the netrin-1 binding to its receptors could represent an efficient anti-cancer strategy in a large fraction of cancers where netrin-1 is expressed in an autocrine or paracrine fashion. Early drug development has focused on biological agents –biologic- that mimic receptors interaction with netrin-1(20).

Some other works focused on the role of netrin-1 and its receptors in angiogenesis with the hope that regulation of angiogenesis could help inhibiting tumor progression.

US2006/0153840 discloses that modulation of netrin-1 receptor activity may activate or inhibit angiogenesis and proposes strategies to decrease or increase angiogenesis. The document discloses the use of a netrin-1 receptor or a fragment thereof as a pro-angiogenic substance, and a fusion protein comprising netrin-1 receptor and an Fc fragment of an immunoglobulin as a pro-angiogenic polypeptide as well. The document teaches that netrin-1-induced anti-angiogenic effect could be reversed by blocking availability of netrin-1 to its receptor, such as UNC5H2 (also called UNC5B), and inhibiting or blocking the netrin-1 receptor activity can induce strong angiogenesis. WO2010/059821 discloses that UNC5B is down-regulated in quiescent adult vasculature, but re-expressed during sprouting angiogenesis in implanted tumors, that stimulation of UNC5B-expressing neovessels with an agonist (Netrin-1) inhibits sprouting angiogenesis and that genetic loss of function of UNC5B may reduce Netrin-1 mediated angiogenesis inhibition. The document suggests that UNC5B activation inhibits sprouting angiogenesis and that UNC5B would be a potential anti-angiogenic target. The document then proposes the use of an anti-UNC5B antibody inhibiting an activity of UNC5B or inhibiting the binding of netrin-1 to this receptor, as an anti-angiogenic agent and as an agent to treat a disease characterized by abnormal angiogenesis, such as cancer. WO2006/054000 discloses the use of an anti-netrin-1 antibody as an anti-angiogenic agent and its use in a composition for treating cancer. Both last documents further propose to combine the anti-UNC5B or anti-netrin-1 antibody to an existing chemotherapeutic drug. In the absence of consistent experimental results in these contradictory disclosures, it is difficult for the person skilled in the art to reach some clear teaching on the incidence of anti-netrin-1 or anti-UNC5H2 antibodies on angiogenesis let alone on a potential anti-tumour activity. It is also difficult for the person skilled in the art to make a biological link between a chemotherapeutic drug that is known to affect proliferating tumor cells but not quiescent endothelial cells that form the vessels and an anti-angiogenic treatment based on an anti-netrin-1 or anti-UNC5H2 antibodies.

The present invention provides however a biological rationale for combining a treatment based on netrin-1 interference and a chemotherapeutic drug that increases, as a result of a stress response by the tumor cell, the dependency for tumor cell survival on netrin-1.

Indeed, the search of the fraction of cancer patients who would be eligible to a netrin-1 interference-based treatment during early clinical evaluation led the present inventors to examine the effect of conventional chemotherapeutic treatments on netrin-1 and netrin-1

receptors expression. Doxorubicin, 5-Fluorouracil (5FU), paclitaxel (Taxol) and Cisplatin are indeed "classic" chemotherapies and are still widely used in the management of patients with breast, lung, colorectal, as well as other types of solid tumors both in patients with localized and advanced tumors. However, despite their efficacy, the use of conventional agents is limited by toxicity and the emergence of resistance. The present inventors show here that these chemotherapeutic treatments, even though they act on different cellular mechanisms, triggers significant increase of netrin-1 and its receptors. The present inventors show that this increase is associated with an increased cell death induction upon netrin-1 interference *in vitro*. As a consequence the present inventors show that combination of Doxorubicin with a netrin-1 interfering drug candidate potentiates tumor growth inhibiting effect in an animal model.

The pre-clinical data showed here support the view that combining conventional drugs plus netrin-1 interference may lead to an unexpected increased efficacy with reduced concentration of conventional drugs. Together these data support the rationale that netrin-1 interference based therapy in combination with conventional chemotherapies is associated with synergic anti-cancer effect. It is deemed the netrin-1 interference based therapy has two positive effects, the first is the induction of apoptosis or cell death owing the inhibition of the netrin-1/receptor binding (the so-called promotion of netrin-1 receptors-induced apoptosis), the second is that the potentially deleterious effect of a-chemotherapy-induced increase in netrin-1 and/or receptor expression would be counter-balanced by the inhibition of netrin-1/receptor binding and its anti-apoptotic effect.

In the present invention, the compositions and methods are for the treatment of cancers expressing or over-expressing netrin-1, wherein this expression or over-expression is linked to the cancer itself, or is induced by the chemotherapeutic drug treatment alone, or both.

An object of the invention is a method of combined anti-cancer treatment comprising the administration to a patient in need thereof of a chemotherapeutic drug and of a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*. The chemotherapeutic drug and the netrin-1 interfering drug are in effective amount.

Another object of the invention is a composition comprising a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo* for use as an anti-cancer medicament to be used in combination with a chemotherapeutic drug in a patient. The invention also relates to a composition comprising a netrin-1 interfering drug or a vector

capable of expressing a netrin-1 interfering drug *in vivo* for use as an anti-cancer medicament in a patient who is treated with a chemotherapeutic drug.

Another object of the invention is a composition comprising a chemotherapeutic drug for use as an anti-cancer medicament to be used in a patient in combination with a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*. The invention also relates to a composition comprising a chemotherapeutic drug for use as an anti-cancer medicament in a patient who is treated with a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*.

Another object of the invention is a composition or kit of parts comprising a chemotherapeutic drug and a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*, for a simultaneous, separate or sequential administration to a patient.

Another object of the invention is a composition or kit of parts comprising a chemotherapeutic drug and a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*, for a simultaneous, separate or sequential administration to a patient, for use as an anti-cancer medicament or anti-cancer treatment.

Another object of the invention is a composition comprising a chemotherapeutic drug and a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*, in a pharmaceutically acceptable carrier or vehicle.

Another object of the invention is a composition comprising a chemotherapeutic drug and a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*, in a pharmaceutically acceptable carrier or vehicle, for use as an anti-cancer medicament.

Still another object is the use of a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo* for the preparation of an anti-cancer medicament intended for a combined treatment of a patient with a chemotherapeutic drug.

Still another object is the use of a chemotherapeutic drug for the preparation of an anti-cancer medicament intended for a combined treatment of a patient with a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*.

Still another object is the use of a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo* and a chemotherapeutic drug for the preparation of a combined anti-cancer medicament.

Still another object is the use of a netrin-1 interfering drug or a vector capable of

expressing a netrin-1 interfering drug *in vivo* and a chemotherapeutic drug for the preparation of a combined anti-cancer medicament composition or kit of parts, for a simultaneous, separate or sequential administration to a patient.

In accordance with an important feature of the invention and as further explained below, the chemotherapeutic drug is a drug which induces an over-expression of netrin-1 in cancer cells and the netrin-1 interfering drug promotes netrin-1 receptors-induced apoptosis or cell death.

A patient may be a mammal, and more particularly a human.

Therapeutic treatment encompasses prophylaxy and therapy.

More detailed embodiments for these objects will now be described.

The chemotherapeutic drug is in particular a drug which induces an over-expression of netrin-1 in cancer cells. The determination that a drug induces a netrin-1 over-expression may be easily performed on any cancerous cell, such as cell line or cells from a biopsy. In an embodiment, the assay is performed on cells from the cancer to be treated, for example from a biopsy. In another embodiment, the assay is performed on a cell, such as a cell line, which is representative for the cancer to be treated. In another embodiment, the assay is made on a A549 or H460 cell line. The assay may comprise comparing the netrin-1 gene expression between the cells treated with the chemotherapeutic drug and the cells not treated. The expression may be measured by PCR, especially quantitative RT-PCR, for example using the primers disclosed and provided herein (SEQ ID NO: 11 and 12). The classification of a drug in the family of those inducing this over-expression may simply be performed in accordance with the method described in the following Material and Method on a A549 or H460 cell line, by reference to Figure 1.

The chemotherapeutic drug is especially a cytotoxic drug.

In some preferred embodiments, the drug is doxorubicin, 5-fluorouracil (5FU), paclitaxel (e.g. Taxol), or cisplatin.

In an embodiment, the drug is a cytotoxic antibiotic. The cytotoxic antibiotic may be actinomycin, an anthracycline, bleomycin, plicamycin or mitomycin. The anthracycline may be doxorubicin, daunorubicin, valrubicin, idarubicine or epirubicine.

In an embodiment, the drug is an alkylating agent. The alkylating agent may be a platinum derivative, such as cisplatin, carboplatin, oxaliplatine or other alkylating agents such as cyclophosphamide, ifosfamide, melphalan, thiotepa. Other classes include-epipodophylotoxines, e.g. etoposide, topoisomerase inhibitors (camptotecines), e.g.

irinotecan, topotecan , alkylating agents of the minor groove of DNA , e.g. Trabectedine (YONDELIS), methotrexate, pemetrexed, raltitrexed.

In an embodiment, the drug is a taxane or other tubulin targeting agents. The taxane may be paclitaxel or docetaxel, or eribuline (recently approved for breast cancer).

In an embodiment, the drug is an antineoplastic agent such as:

- breast hormonotherapy agents: e.g. tamoxifene, letrozole, anastrozole, exemestane, faslodex;
- prostate hormonotherapy agents:e.g. LHRH agonists, bicalutamide, abiraterone;
- monoclonal antibodies : e.g. cetuximab, panitumumab, bevacizumab;
- kinase inhibitors : e.g. imatinib, nilotinib, dasatinib, erlotinib, gefitinib, afatinib, sunitinib, sorafenib, pazopanib, crizotinib, axitinib.

The invention does or may not imply a change of the dose regimen of the chemotherapeutic drug. However, the synergy that occurs with the netrin-1 interfering drug may allow to using lower dose regimen in a patient. The skill practitioner is able to determine the optimum dose regimen in the context of the combined treatment provided by the present invention.

The invention also concerns a combined treatment of a patient wherein the chemotherapy is already a combined chemotherapy, in the sense that at least two chemotherapeutic drugs are incorporated in the treatment protocol. That is to say that the methods, compositions, kit of parts and uses according to the different objects of the invention, combine at least one netrin-1 interfering drug and at least two (e.g. 2, 3, 4 or 5) chemotherapeutic drugs.

The netrin-1 interfering drug is a drug which interferes with the netrin-1 ability to interact with a netrin-1 receptor, or which interferes with the ability of netrin-1 to induce dimerisation or multimerization of netrin-1 receptor, or more generally which promotes netrin-1 receptors-induced apoptosis. The person skilled in the art may refer to WO2007/099133 which discloses interference between netrin-1 and its receptors, either a decrease or an inhibition of interaction or binding between netrin-1 and receptors, or a decrease or an inhibition of the ability of netrin-1 to induce dimerisation or multimerization of netrin-1 receptor, whereby netrin-1 receptors-induced apoptosis is promoted.

In an embodiment, it is a small interfering RNA or siRNA which is a double stranded RNA (dsRNA) (that may have namely from 10 to 50 nucleotides in length) and which reduces expression of the gene coding for netrin-1. Portions of the first strand are complementary to

the target gene, i.e. it has sufficient complementarity to hybridize to the target gene, for example there is at least 80% identity to the target gene or to a portion thereof. AP: human Netrin-1 mRNA sequence accession number: NM_004822. siRNA sequence that may be used: SEQ ID NO: 10 AAGCUGGACGCAGCAUGAUGC (sense), corresponding to position 94-114 of sequence NM_004822.

In a second embodiment, the interfering drug is one which binds to netrin-1 and netrin-1 is rendered unable to bind to its receptors due to the binding of the interfering drug or to induce dimerization/multimerization of the netrin-1 receptors, especially DCC and/or UNC5. In an embodiment, this drug is an antibody binding to netrin-1. It is preferably a polyclonal or monoclonal antibody specifically binding to netrin-1. In another embodiment, this drug is a compound comprising an extracellular domain of a netrin-1 receptor or a fragment of said extracellular domain. For example, the amino acid sequence of the extracellular domain of a netrin-1 receptor or a fragment of said extracellular domain are given in UniProt Sequence ID [extracellular domain position range]: UNC5A : Q6ZN44 [aas 26-306, or fragment 34-240]; UNC5B : Q8IZJ1 [aas 27-377 or fragment 29-244]; UNC5C : O95185 [aas 41-380 or fragment 61-258]; UNC5D : Q6UXZ4 [aas 33-379]; DCC : P43146 [aas 26-1097].This drug is able to bind to netrin-1. The netrin-1 receptors may be DCC, UNC5A, UNC5B, UNC5C or UNC5D. The method of the invention may make use of two or more compounds each comprising an extracellular domain or part thereof, from a different netrin-1 receptor. For example, the drug comprises two compounds comprising an extracellular domain or part thereof, from DCC and from anUNC5, e.g. UNC5A.

In a third embodiment, the interfering drug is one which binds to a netrin-1 receptor. The netrin-1 receptors may be DCC, UNC5A, UNC5B, UNC5C or UNC5D. The method of the invention may makes use of two or more interfering drugs each one binding to a different netrin-1 receptor. For example, the drug comprises two interfering drugs, one binding to DCC and the other to an UNC5, e.g. UNC5A. In an embodiment, this drug is an antibody binding to a netrin-1 receptor. It is preferably a polyclonal or monoclonal antibody specifically binding to a netrin-1 receptor. In another embodiment, this drug is a compound, especially a compound comprising a peptidic moiety, or a small molecule, which is able to bind to a netrin-1 receptor, this binding being able to prevent netrin-1 ability to block apoptosis induction by a netrin-1 receptor, in particular to induce the dimerization or the multimerization of the receptor.

"Antibody" is used in the broadest sense to designate any antibody that may bind to netrin-1 wherein this binding impedes the binding between netrine-1 and a netrin 1 receptor.

"Antibody" includes monoclonal antibodies, polyclonal antibodies, single-chain antibodies and antigen binding fragments of these antibodies which exhibit the desired biological activity. The monoclonal antibodies may be murine, chimeric or humanized. The term "antibody" refers to any full-length antibody or functional fragment of an antibody (obtained by genetic engineering or not), comprising, or consisting of, at least one antigenic combination site, allowing said antibody to bind to at least one antigenic determinant of an antigenic compound. By way of example of antibody fragments, there may be mentioned the fragments Fab, Fab', $F(ab')_2$ and the single-chain variable fragments (scFv chains). The antibodies used in the present invention are antibodies specific for the antigen. They are preferably monoclonal antibodies or monospecific polyclonal antibodies, that is to say that they specifically recognize only one epitope. The production of monoclonal antibodies or of monospecific polyclonal sera, or of antibodies obtained by screening genomic libraries, useful in the context of the invention are conventional techniques.

An anti-netrin 1 polyclonal antibody may, inter alia, be obtained by immunizing an animal such as a rabbit, a mouse and the like with the aid of the selected amino acid sequence, collecting and then depleting the antiserum obtained on, for example, an immunoadsorbent containing the receptor according to methods known per se to a person skilled in the art.

The netrin-1 amino acid sequence (without the signal peptide) is as depicted on SEQ ID NO:13 and netrin-1 may be used in whole or in part to design antibodies.

Generally, monoclonal antibodies may be obtained according to the conventional method of lymphocyte fusion and hybridoma culture described by Köhler and Milstein, (1975). Other methods for preparing monoclonal antibodies are also known (Harlow et al., ed., 1988 "Antibodies: a laboratory manual"). The monoclonal antibodies may be prepared by immunizing a mammal (for example a mouse, a rat, a rabbit or even a human being, and the like) and using the lymphocyte fusion technique leading to hybridoma (Köhler and Milstein, 1975).

Alternative techniques to this customary technique exist. It is possible, for example, to produce monoclonal antibodies by expressing a nucleic acid cloned from a hybridoma. It is also possible to produce antibodies by the phage display technique by introducing cDNAs for antibodies into vectors, which are typically filamentous phages which exhibit gene libraries V

at the surface of the phage (for example fUSE5 for *E. coli*, Scott, 1990). Protocols for constructing these antibody libraries are described in Marks et al. (1991). The cDNA corresponding to full length netrin-1 with signal sequence (SEQ ID NO: 14) or to a suitable fragment thereof is used to produce monoclonal antibodies according to these methods.

In a preferred embodiment, the interfering drug comprises an extracellular domain of a netrin-1 receptor or a fragment of said extracellular domain. The netrin-1 receptors may be DCC, UNC5A, UNC5B, UNC5C or UNC5D.

In an embodiment, the extracellular domain or part thereof is bound to an antibody Fc part. In a preferred embodiment, the Fc part is the Fc or part thereof of a human IgG. The human IgG may be namely IgG1, IgG2A, IgG2B, IgG3. In a preferred embodiment, the IgG is IgG1.

In an embodiment, the fusion protein is single chain, which means that the protein is made of a DCC or a UNC5 fragment comprising or constituted of respectively the fourth or fifth fibronectin-like domain of DCC or the two Ig-like domains of UNC5 and of a peptidic or protein sequence improving the pharmaceutical parameters of the compound.

In another preferred embodiment, the fusion protein is double chain, which means that the fusion protein is made of two chains each comprising or constituted of respectively the fourth or fifth fibronectin-like domain of DCC or the two Ig-like domains of UNC5 and of an antibody Fc part, wherein both chains are linked together, preferably by one or more, e.g. two, disulfide bonds.

In an embodiment, the drug comprises the fifth fibronectin domain (Fn5 or 5Fbn) of DCC. Preferably, the drug comprises a DCC-fusion protein comprising this Fn5 fused to an antibody Fc part. In a preferred embodiment, the Fc part is the Fc or part thereof of a human IgG. The human IgG may be namely IgG1, IgG2A, IgG2B, IgG3. In a preferred embodiment, the IgG is IgG1. The DCC gene is available for example from NCBI, under ID 1630 (as updated on July 14, 2012), it encodes the DCC receptor protein as Uniprot P43146, updated July 11, 2012. A DCC-fusion protein useful in the invention and comprising the Fn5 is described in WO2012025618. In an embodiment, the fusion protein has the amino acid sequence SEQ ID NO: 2, 3 or 4 in WO2012025618. In an embodiment, the fusion protein is encoded by the DNA sequence SEQ ID NO: 1 in WO2012025618. Other examples of fusion proteins comprising the Fn5 are the DCC-5-fibronectin fusion protein with Glutathione-S-transferase (DCC-5Fbn-GST) described in WO2007099133, Fitamant et al. (14) and Delloye-Bourgeois (16).

In an embodiment, the drug comprises the two Ig-like domains of a UNC5. Preferably, the drug comprises an UNC5-fusion protein comprising the two Ig-like domains of a UNC5 fused to an antibody Fc part. The human IgG may be namely IgG1, IgG2A, IgG2B, IgG3. In a preferred embodiment, the IgG is IgG1. In an embodiment, UNC5 is UNC5A. In another embodiment, UNC5 is UNC5B. In another embodiment, UNC5 is UNC5C. In still another embodiment, UNC5 is UNC5D.

In an embodiment, the UNC5A protein in UNC5A-fusion comprises or consists of the amino acids 20 to 217 of SEQ ID NO: 1. This fusion protein may further comprise the IgG1 Fc comprising or consisting of amino acids 220 to 446 of SEQ ID NO: 1. This Fc is fused to the UNC5A protein, for example through a linker, such as GT. In an embodiment, the present invention relates to an UNC5A-fusion of UNC5A protein comprising or consisting of the amino acid sequence of SEQ ID NO: 1: Kappa2 signal peptide sequence: aas 1 to 19; Ig-like domains of UNC5A: aas 20 to 217; Linker: aas 218-219; Human IgG1 Fc: aas 220 to 446. In an embodiment, the mature fusion protein does not comprise the Kappa2 signal peptide sequence. In a preferred embodiment, the fusion protein is double chain. The present invention also encompasses variant sequences having a percentage of identity which is equal or more than 90%, preferably than 96, 95, 94, 93, 92 or 91%, on the whole length of the 20-217 amino acid sequence, or of amino acids 20-446 of SEQ ID NO: 1. Amino acid substitutions may for example occur at one or several of positions 9, 72, 74, 87, 144, 164, 170, 193 and/or 210 on the whole length of the 20-217 amino acid sequence, or of SEQ ID NO: 1.

In another embodiment, the UNC5B protein in UNC5B-fusion comprises or consists of the amino acids 20 to 215 of SEQ ID NO: 2. This fusion protein may further comprise the IgG1 Fc comprising or consisting of amino acids 218 to 444 of SEQ ID NO: 2. This Fc is fused to the UNC5A protein, for example through a linker, such as GT. In an embodiment, the present invention relates to an UNC5B-fusion of UNC5B protein comprising or consisting of the amino acid sequence of SEQ ID NO: 2: Kappa2 signal peptide sequence: aas 1 to 19; Ig-like domains of UNC5B: aas 20 to 215; Linker: aas 216-217; Human IgG1 Fc: aas 218 to 444. In an embodiment, the mature fusion protein does not comprise the Kappa2 signal peptide sequence. In a preferred embodiment, the fusion protein is double chain. The present invention encompasses variant sequences having a percentage of identity which is equal or more than 90%, preferably than 96, 95, 94, 93, 92 or 91%, on the whole length of the 20-215 amino acid sequence, or of amino acids 20-444 of SEQ ID NO: 2. Amino acid

substitutions may for example occur at one or several of positions 29, 74, 100, 109, 113, 146, 149, 155, 172, 184, 189, 201, 213 and/or 214 on the whole length of the 20-215 amino acid sequence, or of SEQ ID NO: 2.

In still another embodiment, the UNC5C protein in UNC5C-fusion comprises or consists of the amino acids 20 to 217 of SEQ ID NO: 3. This fusion protein may further comprise the IgG1 Fc comprising or consisting of amino acids 220 to 446 of SEQ ID NO: 3. This Fc is fused to the UNC5A protein, for example through a linker, such as GT. In an embodiment, the present invention relates to an UNC5C-fusion of UNC5C protein comprising or consisting of the amino acid sequence of SEQ ID NO: 3: Kappa2 signal peptide sequence: aas 1 to 19; Ig-like domains of UNC5C: aas 20 to 217; Linker: aas 218-219; Human IgG1 Fc: aas 220 to 446. In an embodiment, the mature fusion protein does not comprise the Kappa2 signal peptide sequence. In a preferred embodiment, the fusion protein is double chain. The present invention encompasses variant sequences having a percentage of identity which is equal or more than 90%, preferably than 96, 95, 94, 93, 92 or 91%, on the whole length of the 20-217 amino acid sequence, or of amino acids 20-446 of SEQ ID NO: 3. Amino acid substitutions may for example occur at one or several of positions 33, 66, 109, 129, 136, 178, 189 and/or 211 on the whole length of the 20-217 amino acid sequence, or of SEQ ID NO: 3.

In still another embodiment, the UNC5D protein in UNC5D-fusion comprises or consists of the amino acids 20 to 217 of SEQ ID NO: 4. This fusion protein may further comprise the IgG1 Fc comprising or consisting of amino acids 220 to 446 of SEQ ID NO: 4. This Fc is fused to the UNC5A protein, for example through a linker, such as GT. In an embodiment, the present invention relates to an UNC5D-fusion of UNC5D protein comprising or consisting of the amino acid sequence of SEQ ID NO: 4: Kappa2 signal peptide sequence: aas 1 to 19; Ig-like domains of UNC5D: aas 20 to 217; Linker: aas 218-219; Human IgG1 Fc: aas 220 to 446. In an embodiment, the mature fusion protein does not comprise the Kappa2 signal peptide sequence. In a preferred embodiment, the fusion protein is double chain.The present invention encompasses variant sequences having a percentage of identity which is equal or more than 90%, preferably than 96, 95, 94, 93, 92 or 91%, on the whole length of the 20-217 amino acid sequence, or of amino acids 20-446 of SEQ ID NO: 4. Amino acid substitutions may for example occur at one or several of positions 38, 79, 80, 115, 131, 178, 186, 201 and/or 212 on the whole length of the 20-217 amino acid sequence, or of SEQ ID NO: 4.

The present invention provides for the following nucleic acid molecules:

- SEQ ID NO: 5 encoding an UNC5A protein; nt (nucleotides); nt 1-6 HindIII restriction site, nt 7-15 kozak sequence, nt 16-72 kappa2 signal sequence, nt 73-666 UNC5A coding sequence, nt 667-672 XpnI restriction site;
- SEQ ID NO: 6 encoding an UNC5B protein; nt (nucleotides); nt 1-6 HindIII restriction site, nt 7-15 kozak sequence, nt 16-72 kappa2 signal sequence, nt 73-660 UNC5B coding sequence, nt 661-666 XpnI restriction site;
- SEQ ID NO: 7 encoding an UNC5C protein; nt (nucleotides); nt 1-6 HindIII restriction site, nt 7-15 kozak sequence, nt 16-72 kappa2 signal sequence, nt 73-666 UNC5C coding sequence, nt 667-672 XpnI restriction site;
- SEQ ID NO: 8, encoding an UNC5D protein; nt (nucleotides); nt 1-6 HindIII restriction site, nt 7-15 kozak sequence, nt 16-72 kappa2 signal sequence, nt 73-666 UNC5C coding sequence, nt 667-672 XpnI restriction site;
- SEQ ID NO: 9 encoding a human IgG1 Fc (hinge + CH2 + CH3 DNA sequence, nt 7-693), with KpnI restriction site at positions 1-6 nt and XbaI restriction site at position 694-699.

The nucleic acid molecules of the present invention may be DNA molecules or RNA molecules. They may also be nucleic acid analogues, such as oligonucleotide thiophosphates, substituted ribo-oligonucleotides, LNA (Locked nucleic acid) molecules, PNA (Peptide nucleic acid) molecules, GNA (glycol nucleic acid) molecules, TNA (threose nucleic acid) molecules, morpholino polynucleotides, or antagomir (cholesterol-conjugated) nucleic acid molecules or any modification thereof as known in the art (see., e.g. US 5,525,711, US 4,711,955, US 5,792,608 or EP 302 175 for examples of modifications). Nucleic acid molecules in context of the present invention may be naturally occurring nucleic acid residues or artificially produced nucleic acid residues. Examples for nucleic acid residues are adenine (A), guanine (G), cytosine (C), thymine (T), uracil (U), xanthine (X), and hypoxanthine (HX). In context of the present invention, thymine (T) and uracil (U) may be used interchangeably depending on the respective type of nucleic acid molecule. For example, as the skilled person is well aware of, a thymine (T) as part of a DNA corresponds to an uracil (U) as part of the corresponding transcribed mRNA. The nucleic acid molecule of the present invention may be single- or double-stranded, linear or circular, natural or

synthetic, and, if not indicated otherwise, without any size limitation. The nucleic acid molecule may also comprise a promoter as further detailed herein below. The promoter may be homologous or heterologous. In a particular embodiment, the nucleic acid molecule provided herein is under the control of this promoter.

Generally, as used herein, a polynucleotide comprising the nucleic acid sequence of a sequence provided herein may also be a polynucleotide consisting of said nucleic acid sequence.

The nucleic acid molecule of the present invention may be cloned into a vector. The person skilled in the art may refer to WO2007/099133 which describes vectors and methods of preparing vectors and their use, which can be used in carrying out the present invention. The term "vector" as used herein particularly refers to plasmids, cosmids, viruses, bacteriophages and other vectors commonly used in genetic engineering. In a preferred embodiment, these vectors are suitable for the transformation of cells, eukaryotic cells like fungal cells, cells of microorganisms such as yeast or prokaryotic cells. In a particular preferred embodiment, such vectors are suitable for stable transformation of bacterial cells, for example to transcribe the nucleic acid molecule of the present invention. For example, the vector may be pUC18. In particular, WO2007/099133 discloses vectors expressing fusion proteins based on DCC, such as the vectors identified as 7800 and 7809 in Example 1 of WO2007/099133. The present invention thus provides for a vector encoding the fusion protein of SEQ ID NO: 2, 3 or 4 in WO2012025618, or for a vector the DNA sequence SEQ ID NO: 1 in WO2012025618, for the DCC fusion protein.

The present invention also provides for a vector such as pUC18 containing a nucleic acid molecule of the present invention coding for a fusion protein as described and provided herein. As far as it concerns UNC5, the present invention therefore relates to a vector such a pUC18 containing a nucleic acid molecule encoding the amino acids 20-217 and 220-446 of SEQ ID NO: 1 fused together, or the sequence SEQ ID NO:1; the amino acids 20-215 and 218-444 of SEQ ID NO: 2 fused together, or the sequence SEQ ID NO: 2; the amino acids 20-217 and 220-446 of SEQ ID NO: 3 fused together, or the sequence SEQ ID NO:3; or the amino acids 20-217 and 220-446 of SEQ ID NO: 4 fused together, or the sequence SEQ ID NO:4. Particularly, the present invention provides for a vector such as pUC18 containing a nucleic acid molecule comprising the nucleotide sequence 73-666 of SEQ ID NO : 5, or 73-660 of SEQ ID NO : 6, or 73-666 of SEQ ID NO : 7 or 73-666 of SEQ ID NO : 8. These vectors also comprise the nucleotide sequence of SEQ ID NO : 9, particularly nucleotides 7-

693. Generally, the vector may be capable of expressing said nucleic acid molecule in a eukaryotic host cell.

The vector as provided is an expression vector. Generally, expression vectors have been widely described in the literature. The expression vector may contain a selection marker gene and a replication-origin ensuring replication in the host. The expression vector may comprise a promoter. He may further comprise a termination signal for transcription. Between, the promoter and the termination signal there is preferably at least one restriction site or a polylinker which enables the insertion of a nucleic acid sequence/molecule desired to be expressed. In an embodiment, the vector is capable of expressing the protein *in vivo*, say the vector, once administered to a patient, is capable of expressing the protein *in situ*.

It is be understood that when the vector provided herein is generated by taking advantage of an expression vector known in the prior art that already comprises a promoter suitable to be employed in context of this invention, for example expression of a UNC5-fusion protein as described herein, the nucleic acid molecule is inserted into that vector in a manner that the resulting vector comprises preferably only one promoter suitable to be employed in context of this invention. The promoter may generally be heterologous or homologous. The vector described herein may also encompass more than one promoter, each respective promoter may be heterologous or homologous. The skilled person knows how such insertion can be put into practice. For example, the promoter can be excised either from the nucleic acid construct or from the expression vector prior to ligation.

The proteins according to the invention are preferably produced by recombinant means. Preferably, the protein expression is in eukaryotic cell with subsequent isolation of the polypeptide and usually purification to a pharmaceutically acceptable purity. For the protein expression, nucleic acids encoding the protein thereof are inserted into expression vectors by standard methods. Expression is performed in appropriate stable eukaryotic host cells like CHO cells, NS0 cells, SP2/0 cells, HEK293 cells, COS cells, and the protein is recovered from the cells (supernatant or cells after lysis). HEK293 cells appeared to be very suitable for this aim and forms a particular embodiment.

In an embodiment, the nucleic acid molecule of the present invention and/or the vector into which the polynucleotide described herein is cloned may be transduced, transformed or transfected or otherwise introduced into a host cell. For example, the host cell is a eukaryotic or a prokaryotic cell, preferably a eukaryotic cell. As a non-limiting example, the host cell is a mammalian cell. The host cell described herein is intended to be particularly

useful for generating the UNC5-fusion proteins described and provided in the present invention.

Generally, the host cell described hereinabove may be a prokaryotic or eukaryotic cell, preferably a eukaryotic cell, comprising a nucleic acid molecule provided in the present invention or the vector described herein or a cell derived from such a cell and containing the nucleic acid molecule or the vector described herein. In a preferred embodiment, the host cell comprises, *i.e.* is genetically modified with the nucleic acid molecule of the present invention or the vector described herein in such a way that it contains the nucleic acid molecule of the present invention integrated into the genome. For example, such host cell described herein may be a human, yeast, or fungus cell. In one particular aspect, the host cell is capable to transcribe the nucleic acid molecule of the present invention. An overview of examples of different corresponding expression systems to be used for generating the host cell described herein is for instance contained in Methods in Enzymology 153 (1987), 385-516, in Bitter (Methods in Enzymology 153 (1987), 516-544), in Sawers (Applied Microbiology and Biotechnology 46 (1996), Billman-Jacobe (Current Opinion in Biotechnology 7 (1996), 500-4), Hockney (Trends in Biotechnology 12 (1994), 456-463), and in Griffiths (Methods in Molecular Biology 75 (1997), 427-440). The transformation or genetically engineering of the host cell with a nucleic acid molecule of the present invention or vector described herein can be carried out by standard methods, as for instance described in Sambrook and Russell (2001), Molecular Cloning : A Laboratory Manual, CHS Press, Cold Spring Harbor, NY USA ; Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, 1990.

The host cell comprising the nucleic acid molecule provided herein or a vector described herein may be a HEK293 cell or a HEK293-Freestyle cell (Human embryonic kidney cell line 293, Invitrogen). The present invention thus provides for a method for producing the DCC and UNC5-fusion proteins as provided and described herein. This method comprises the steps of expressing a nucleic acid molecule as provided and described herein in a suitable host cell, especially as described herein, and recovering the DCC or UNC5-fusion protein from said cell or the cell culture supernatant.

The present invention relates to compositions comprising a netrin-1 interfering drug. It relates in particular to compositions comprising a DCC and/or UNC5-fusion protein as provided herein or an *in vivo* expressing vector encoding a DCC and/or UNC5-fusion protein. It also relates in particular to compositions comprising an anti-netrin 1 antibody. These

compositions may further comprise a pharmaceutically acceptable carrier, excipient and/or diluent. These compositions may be used as a pharmaceutical co-ingredient or a pharmaceutical and form a pharmaceutical composition or a medicament, to be used in combination with the chemotherapeutic drug or treatment on the same patient.

In an embodiment, both the DCC and/or UNC5-fusion proteins as provided herein or an *in vivo* expressing vector encoding a DCC and/or UNC5-fusion protein and the chemotherapeutic drug are within the same composition with a pharmaceutically acceptable carrier, excipient and/or diluent.

In another embodiment, they are presented under separate pharmaceutical forms. This form a composition or kit of parts comprising a chemotherapeutic drug and a netrin-1 interfering drug, for a simultaneous, separate or sequential administration to a patient.

In an embodiment of the method of treatment, use and compositions for use, the administration is sequential. In a preferred embodiment, the chemotherapeutic drug is administered first, and the netrin-1 interfering drug after. The interval between both administrations may be at least 5, 10, 15, 20 or 24 hours, preferably between 24 and 96 hours, more preferably between 24 and 72 hours, especially between 24 and 48 hours, for example 24 hours. In an embodiment, the netrin-1 interfering drug is simply administered the day after the administration of the chemotherapeutic drug.

These different pharmaceutical forms may be used in the methods of treatment of the invention, in sufficient amounts.

Examples of suitable pharmaceutical carriers are well known in the art. They include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Pharmaceutical compositions comprising such carriers can be formulated by well known conventional methods.

These pharmaceutical compositions can be administered to a subject at a suitable dose, *i.e.* for the netrin-1 interfering drug at least 1 mg/kg body weight, e.g. about 10 mg/kg body weight to about 100 mg/kg weight of the subject in which cancer, is to be treated. The chemotherapeutic drug may be administered at the usual dose, or at a reduced dose with respect to the usual dose as far as the combination has a synergic efficacy. For example the dose of chemotherapeutic drug is reduced by 10, 20, 30, 40, 50%, or more. Administration of the composition may be effected or administered by different ways, *e.g.* orally, (e.g. pill, tablet, buccal, sublingual, disintegrating, capsule, thin film, liquid solution or suspension, powder, solid crystals or liquid), rectally (e.g. suppository, enema) via injection (e.g.

intravenously, subcutaneously, intramuscularly, intraperitoneally, intradermally) via inhalation (e.g., intrabronchially), topically, vaginally, epicutaneously or intranasally). The dosage regimen will be determined by the attending physician and clinical factors. As it well known in the medical arts, dosages for any one of patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex time and route of administration, general health, and other drugs being administered concurrently.

The compositions and pharmaceutical compositions of the invention may be administered locally or systemically. Administration will preferably be intravenously or subcutaneously. The compositions and pharmaceutical compositions may also be administered directly to the target site, *e.g.* by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based in Ringer's dextrose), and the like. Preservatives and others additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, also doses below or above of the exemplary ranges described hereinabove are envisioned, especially considering the aforementioned factors.

As already mentioned, the present invention relates to pharmaceutical compositions for use in treating a cancer overexpressing netrin-1.

Some embodiments of cancers include metastatic breast cancer, non-small cell lung cancer, aggressive neuroblastoma, pancreatic adenocarcinoma, primary melanoma (n=7), melanoma metastasis (n=6), ovarian cancers, glioblastoma, acute myeloid leukemia, chronic lymphocytic leukemia, aggressive B-cell lymphoma, sarcoma, renal adenocarcinoma, head and neck cancers, Testicular cancers (e.g. embryonal carcinoma, teratoma, yolk sac tumors), kidney cancers, stomach cancers, uterus cancers. Examples of cancers are listed *infra*.

Methods of determining whether a given cell expresses dependence receptors DCC

and/or UNC5 on the surface and/or shows significant up-regulation of netrin-1 gene expression are well known in the art and comprise, but are not limited to, IHC (Immunohistochemistry) of FACS (Fluorescence activated cell sorting), quantitative PCR (e.g. with hexamer primed cDNA) or alternatively Western Blot paired with chromogenic dye-based protein detection techniques (such as silver or coomassie blue staining) or fluorescence- and luminescence-based detection methods for proteins in solutions and on gels, blots and microarrays, such as immunostaining, as well as immunoprecipitation, ELISA, microarrays, and mass spectrometry. In the context of the present invention, examples for cancers to be treated are listed herein including refractory versions of any of the mentioned cancers.

The invention will now be described with further details, in a non limiting way, by reference to the drawing in which:

Figures 1-4: Netrin-1 and its dependence receptors are up-regulated upon Doxorubicin treatment.

Figure 1: Lung cancer cell lines A549 and H460 were treated with 2μM Doxorubicin for 24 hours and Netrin-1 gene expression, normalized with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and compared to not-treated cells, was evaluated by quantitative PCR. Doxorubicin treatment induced in both cell lines a strong induction of Netrin-1 gene expression. Results represent mean values of five independent experiments. Mann–Whitney tests were performed, and *P* value is indicated. DoxoR, Doxorubicin; Act.D, Actinomycin D; NT, not treated.

Figure 2: A549 cells were treated with 1μM and 2μM Doxorubicin for 48 hours and Netrin-1 protein levels were evaluated by western blotting. Netrin-1 protein, normalized to β-actin, was strongly accumulated following Doxorubicin treatment.

Netrin-1 up-regulation was confirmed by immunofluorescence staining, following treatment with 2μM Doxorubicin for 48 hours. Nuclei were counterstained with Hoescht staining (in blue). (Not shown)

Figure 3: Netrin-1 receptors gene expression was measured in A549 cells following Doxorubicin treatment. UNC5A, UNC5B and DCC gene expression was significantly up-regulated by Doxorubicin, while UNC5C and UNC5D showed non-significant variations. Mann–Whitney tests were performed, and *P* value is indicated. DoxoR, Doxorubicin; Act.D, Actinomycin D; NT, not treated.

Figure 4: Doxorubicin-induced Netrin-1 up-regulation is directly dependent by gene transcription. A549 cells were treated with 2μM Doxorubicin and with the potent RNA polymerases inhibitor Actinomycin D (100μg/ml) for 24 hours. Actinomycin D strongly inhibited Netrin-1 up-regulation following Doxorubicin treatment (Doxo..). Mann–Whitney tests were performed, and *P* value is indicated. DoxoR, Doxorubicin; Act.D, Actinomycin D; NT, not treated, Doxo., Doxorubicin + Actinomycin D.

Figures 5-9: Netrin-1 and its receptors expression is increased in several cancer cell lines and in ovarian tumors upon treatment with cytotoxic drugs.

Figures 5-8: Expression levels of Netrin-1 (NTN1), DCC, UNC5B and UNC5D were measured by quantitative RT-PCR. Breast cancer (HBL100), lung cancer (A549, H322, H358), colon cancer (HCT116, HCT8), pancreas cancer (MiaPacA-2, Panc-1), neuroblastoma (SH-Sy5y, IMR32), glioblastoma (SF767, U87MG) and ovary cancer (PA-1, TOV-112D, NIH-OVCAR3) cell lines were treated with classical chemotherapeutic drugs (Doxorubicin, Cisplatin, 5-Fluorouracil and Taxol), at different drugs concentration dependent on IC_{50} values calculated for each cell line and drug treatment for 24 hours. Netrin-1 and its receptors gene expression was compared to control, not-treated cells, and variations was scored as following: -, no changes or down-regulation; +, between 2 and 4 fold over control of gene expression; ++, between 4 and 100 fold over control; +++, more than 100 fold over control; n.d., not determined; n.e., not expressed. Positive cell lines were determined for gene expression variations more than 2 fold over control. Gray boxes represent resistant (i.e., more than 50% cell survival after treatment with maximal drugs concentrations) cancer cell lines.

Figure 9: Netrin-1 is over-expressed in ovarian tumor patients after chemotherapeutic treatment. Netrin-1 level, normalized with GAPDH, used as housekeeping gene, was analyzed in RNA extracted from ovarian biopsies of tumors from patients obtained before and after a chemotherapeutic cycle of carboplatin/taxol treatment. The median level of netrin-1 was calculated for each group.

Figures 10-15: Netrin-1 silencing sensitizes A549 cells to Doxorubicin and induces apoptotic cell death *via* UNC5B receptor.

Figure 10-12: Netrin-1 silencing sensitizes tumor cells to Doxorubicin. A549 cells were transfected with either a scramble siRNA (siCTRL, siRNA Universal Negative Control #1, Sigma-Aldrich) or with a specific siRNA targeting netrin-1 (siNet, sequence SEQ ID NO: 10: AAGCUGGACGCAGCAUGAUGC). 24 hours after transfection, cells were treated with

increasing concentrations of Doxorubicin. Cell death rate (Figure 10), measured by toxilight kit as described in materials and methods section, and cell survival (Figure 11), was evaluated 48 hours after treatment. Results were normalized to control, untreated cells. While scramble siRNA-transfected cells showed a general resistance to Doxorubicin treatment, netrin-1 silencing strongly induced cell death and decreased cell survival in presence of Doxorubicin. Evaluation of cell death percentage (Figure 12), measured by 4',6-diamidino-2-phenylindole (DAPI) exclusion as described in the materials and methods section, confirmed that Netrin-1 siRNA sensitized A549 cells to 0.5μM and 2 μM Doxorubicin treatment. *, $P<0.05$; **, $P<0.01$. DoxoR, Doxorubicin.

Figures 13-14: Netrin-1 silencing triggers apoptosis in combination with Doxorubicin treatment. A549 cells were transfected as in (Figures 10-12), and treated with the indicated Doxorubicin concentrations for 24 hours. Active caspase-3 (Figure 13), normalized to untreated cells, and DNA fragmentation (Figure 14) were evaluated as described in the materials and methods section. While Doxorubicin failed to induce apoptosis in A549 cells transfected with a scramble siRNA (siCTRL), cells silenced for netrin-1 showed a strong increase in the apoptotic rate. *, $P<0.05$; **, $P<0.01$. DoxoR, Doxorubicin.

Figure 15: Combination of netrin-1 silencing and Doxorubicin treatment induces cell death through netrin-1 receptor UNC5B. A549 cells were transfected with scramble siRNA (siCTRL), netrin-1-specific siRNA (siNet), UNC5B-specific siRNA (siUnc5B) and with a combination of netrin-1 and UNC5B-targeting siRNA. 24 hours after transfection, cells were treated with the indicated Doxorubicin concentrations for 48 hours, and cell death rate was measured by toxilight and normalized to control, untreated cells. While netrin-1 silencing (siNet) sensitized A549 cells to Doxorubicin treatment, as compared to siCTRL-transfected cells, the simultaneously silencing of netrin-1 and UNC5B (siNet+siUnc5B) rescued cell death induction by siNet and Doxorubicin treatment. *, $P<0.05$; **, $P<0.01$. DoxoR, Doxorubicin.

Figures 16-21: Interference to netrin-1 and its receptors interaction sensitizes tumor cells to cytotoxic drugs.

Figures 16-17: A549 cells were treated for 48 hours with the indicated Doxorubicin concentrations in presence or not of 2μg/mL TRAP-netrin^{DCC} (Figure 16) and TRAP-netrin^{Unc5A} (Figure 17). The co-treatment with Doxorubicin and the two recombinant fusion proteins, increased cell death rate, measured by toxilight, as compared to Doxorubicin- and

PBS-treated cells. Results were normalized to untreated cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. DoxoR, Doxorubicin.

Figures 18-19: A549 cells were treated with PBS or 2µg/mL TRAP-netrin^{Unc5A}, in presence of the indicated concentrations of 5-Fluorouracil (5-FU, Figure 18) or Cisplatin (Figure 19). 48 hours after co-treatment, cell survival was measured by MTS and normalized to untreated cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. DoxoR, Doxorubicin.

Figures 20-21: MiaPacA cells were treated with PBS or 2µg/mL TRAP-netrin^{Unc5A}, in presence of the indicated concentrations of 5-FU (Figure 20) or Doxorubicin (Figure 21). 48 hours after co-treatment, cell survival was measured by MTS and normalized to untreated cells. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. DoxoR, Doxorubicin.

Figure 22: Netrin-1 interfering potentiates Doxorubicin anti-cancer effect in a preclinical animal model. A549 cells were engrafted in seven-weeks old female athymic nude mice. Once tumors reached a 100mm^3-volume, mice were treated intraperitoneally with TRAP-netrin^{UNC5A} (20mg/kg), Doxorubicin (2mg/kg) or with a combination of both drugs, twice a week for two weeks. As a control, mice were injected with PBS. Histogram represents tumor volume growth for each group as a function of days post-xenografts. While both drug alone was not able to reduce tumor growth, combination of TRAP-netrin^{UNC5A} and Doxorubicin treatment significantly reduced tumor growth.

Figures 23-27- Netrin-1 receptors gene expression following cytotoxic drugs treatment.

Figures 23-24: Cancer cells were treated as described in Fig.5, and UNC5A and UNC5C gene expression was evaluated after drugs treatment. Scoring system is the same used in Fig.5. Both receptors showed poor expression levels changes after treatment, as compared to untreated cells.

Figures 25-27: Netrin-1 receptors expression levels in ovarian biopsies of tumors from patients before and after carboplatin/taxol treatment. The median values were calculated from each group. UNC5B (Figure 25), UNC5D (C=Figure 26) and DCC (Figure 27) gene expression showed a similar up-regulation after chemo-therapeutic treatment. Gene expression levels were normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as housekeeping gene.

Figure 28: Cell sensitivity to cytotoxic drugs. The inhibitory concentration (IC) IC_{10}, IC_{30} and IC_{50} in response to Cisplatin, 5-Fluoruracil (5FU), Doxorubicin, and paclitaxel (Taxol) was determined for the indicated cell lines by MTS assays. IC_{50} values were calculated by

linear regression of double reciprocal plots. For resistant cancer cell lines (i.e., more than 50% cell survival after treatment with maximal drugs concentrations IC_{MAX}), represented by gray boxes, IC_{MAX} and fractions were calculated.

Figure 29: Map of expression plasmid NP-X.

Sequence listing:

SEQ ID NO:	Amino acid sequence	Nucleic acid sequence
1	UNC5A-TRAP with peptide signal	
2	UNC5B-TRAP with peptide signal	
3	UNC5C-TRAP with peptide signal	
4	UNC5D-TRAP with peptide signal	
5		UNC5A
6		UNC5B
7		UNC5C
8		UNC5D
9		Human IgG1 Fc
10		siRNA strand (sense)
11		primer
12		primer
13	Netrin-1	
14		Netrin-1

I. Materials and Methods:

1. Quantitative RT-PCR allowing to assess netrin-1 expression or overexpression:

Total RNA was extracted using NucleoSpin® RNA II Kit (Macherey Nagel, Düren, Germany) according to manufacturer's protocol. RT-PCR reactions were performed with iScript® cDNA Synthesis Kit (Biorad). One µg total RNA was reverse-transcribed using the following program: 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. For expression studies, the target transcripts were amplified in LightCycler® 2.0 apparatus (Roche Applied Science), using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche Applied Science). Expression of target genes was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) genes, used as housekeeping genes. The amount of target transcripts, normalized to the housekeeping gene, was calculated using the comparative C_T method. A validation experiment was

performed, in order to demonstrate that efficiencies of target and housekeeping genes were approximately equal. The sequences of the primers are as follows:

Forward primer: aaaagtactgcaagaaggactatgc SEQ ID NO:11.

Reverse primer: ccctgcttatacacggagatg SEQ ID NO:12.

2. Netrin-1 protein quantification in human cancer cells:

For immunoblot analysis, cells were lysed by sonication in modified RIPA buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitor cocktail and 5mM DTT) and incubated 1h at 4°C. Cellular debris were pelletted by centrifugation (10.000g 15' at 4°C) and protein extracts (200 µg per lane) were loaded onto 10% SDS-polyacrylamide gels and blotted onto PVDF sheets (Millipore Corporation, Billerica, MA, U.S.A.). Filters were blocked with 10% non-fat dried milk and 5% BSA in PBS/0.1% Tween 20 (PBS-T) over-night and then incubated for 2h with rabbit polyclonal α-netrin-1 (dilution 1:500, clone H104, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal β-actin (Santa Cruz Biotechnologies) antibodies. After three washes with PBS-T, filters were incubated with the appropriate HRP-conjugated secondary antibody (1:10000, Jackson ImmunoResearch, Suffolk, UK) for 1h. Detection was performed using West Dura Chemiluminescence System (Pierce, Rockford, IL, U.S.A.).

For immunofluorescence study, cells were detached, centrifuged on cover slips with a cytospiner (Shandon Cytospin 3, Thermo Scientific) and fixed for 30 minutes with 4% (v/v) paraformaldehyde. Cells were then permeabilized for 30 minutes in 0.2% Triton X-100/PBS and blocked in PBS containing 2% BSA and 2% normal donkey serum. Endogenous netrin-1 was stained using rat monoclonal α-netrin-1 antibody (R&D systems) and Alexa-488 Donkey anti-rat IgG (Molecular probes). Nuclei were counterstained using Hoescht staining (Sigma).

3. Cell death assay and conventional drugs treatment:

Cell death was evaluated by means of different methods. For total cell death assays, $5*10^3$ cells per well were grown in 96-well plate in serum-poor medium and treated with Doxorubicin. 48 hours later, cell death was evaluated using the bioluminescent cytotoxicity assay ToxiLight (Lonza, Basel, Switzerland), according to manufacturer's instruction. Alternatively, cell death percentage was measured by acridine orange and DAPI staining, using the NucleoCounter NC-3000 system (ChemoMetec A/S, Allerød, Denmark). Briefly, $5*10^4$ cells were plated in 12-well plate and treated with Doxorubicin. 48 hours after treatment, floating and adherent cells were collected, suspended in PBS and mixed with two different dyes, acridine orange, staining the entire population of cells, and 4',6-diamidino-2-

phenylindole (DAPI), staining the non-viable cells. Cell death rate, measured as DAPI-positive cells in total cell population, was then determined by NucleoCounter NC-3000, following the manufacture's application note. Cell survival was measured by MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) in 96-well plates. MTS assay was performed according to the manufacturer's procedures on $3*10^3$ cells grown in serum-poor medium for 16 hours and then treated for 48 hours with the indicated Doxorubicin concentrations in serum-free medium. Caspase-3 activity assay was performed as previously described (21) using the Caspase 3/CPP32 Fluorimetric Assay Kit (Gentaur Biovision, Brussel, Belgium), according to the manufacturer's instructions. Caspase activity (activity / min / microgram of protein) was calculated from a 1h kinetic cycle reading on a spectrofluorimeter (405nm / 510nm, Infinite F500, Tecan, Männedorf, Switzerland).

4. Candidate drugs:

TRAP-netrin^{DCC} and TRAP-netrin^{UNC5A} are respectively the fifth fibronectin domain of DCC ectodomain and the two immunoglobin (Ig1-Ig2) domains of the UNC5A ectodomain, fused to IgG1 Fc portion. These two recombinant proteins were produced respectively in 293-free-style and CHO-free-style.

TRAP-netrin^{DCC} has been produced according to examples 1 – 4 of WO2012025618 using plasmid 7800. A similar fusion protein may be produced using vector 7809 also disclosed in these examples of WO2012025618.

TRAP-netrin^{UNC5A} has been produced using the method described under 5.

5. Production of TRAP-netrin^{UNC5A} (UNC5A-Fc), TRAP-netrin^{UNC5B} (UNC5B-Fc)and TRAP-netrin^{UNC5C} (UNC5C-Fc)

Plasmid construction

Standard methods were used to manipulate DNA as described in Sambrook, J. *et al.,* Molecular Cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions. Desired gene segments were prepared by gene synthesis. The synthesized gene fragments were cloned into a specified expression vector. The DNA sequence of the subcloned gene fragments were confirmed by DNA sequencing.

Expression plasmid is represented on Figure 11 and noted PS-UNC5-Fc-NP-X (PS for peptide signal, and X may be V-01 for UNC5A, V-02 for UNC5B and V-03 for UNC5C.

This vector is an expression plasmid e.g. for transient expression of an artificial Ig Fc fusion protein in which the Ig-like domains of the human UNC5A, B, or C receptor is fused to

the hinge region of human IgG 1 antibody (Fc constant region; Hinge-CH2-CH3) with the introduction of a 2 amino acid artificial linker sequence.

Chemical gene synthesis was used to prepare the DNA segments of 672 (SEQ ID NO: 5, 7) or 676 bps (SEQ ID NO: 6) flanked by a unique HindIII and KpnI restriction endonuclease at the 5'- and the 3'-end, respectively. Similarly, was prepared the DNA segment of 699 bps (SEQ ID N0: 9) flanked by a unique KpnI and XbaI restriction endonuclease at the 5'- and the 3'-end, respectively. A DNA segment coding for the open reading frame (ORF) of the desired UNC5-fusion protein (UNC5-Fc fusion protein) (SEQ ID NO: 1, 2, 3) with the Kappa 2 signal peptide at the N-terminal position was obtained by ligation of the two DNA segments cited above. The gene was introduced in an expression vector (NP-V) to the immediate promoter of CMV-IE and enhancer hE1 and the bovine growth hormone (bGH) polyadenylation site.

The UNC5-fusion protein (UNC5-Fcusion protein) is composed of a murine signal sequence (amino acids 1 to 19 of SEQ ID NO: 1, 2 or 3), the two immunoglobulin-like domains of the human UNC5 receptor (UNC5A : amino acids 20 to 217 of SEQ ID NO: 1; correspond to amino acids 34 to 240 of UNC5A of amino acid sequence UniProt ID: Q6ZN44; UNC5B : amino acids 20 to 215 of SEQ ID NO: 2; correspond to amino acids 49 to 244 of UNC5B of amino acid sequence UniProt ID: Q8IZJ1; UNC5C : amino acids 20 to 217 of SEQ ID NO: 3; correspond to amino acids 61 to 258 of UNC5C of amino acid sequence UniProt ID: O95185; two amino acid linker (from cloning site; amino acids 199 to 200 of SEQ ID NO: 3) and the human IgGI antibody Fc constant region (amino acids 220 to 446 of SEQ ID NO: 1 or 3; amino acids 218 to 444 of SEQ ID NO: 2). The mature UNC5-Fc fusion proteins lack the signal peptide.

Transient transfection, expression and purification

Recombinant proteins were obtained by transient transfection of Freestyle HEK 293 cells (Invitrogen) growing in suspension in 293 Freestyle culture medium (Invitrogen) with 8 % CO_2 at 37°C. For transfection 293fectin® reagent (Invitrogen) was used according to manufacturer's instructions. Three days after transfection, supernatants were harvested and clarified by centrifugation (10min at 200g). The Fc-fusion proteins were purified using Protein G Sepharose 4 FF according to the manufacturer's instructions. Elutions were performed in 0.1M Glycin pH 2.8. Eluates were neutralized in 1M Tris-Hcl pH 9.0 and dialyzed over night against PBS. Final analytics were performed using polyacrylamide gel electrophoresis in denaturing and non denaturing conditions followed by coomassie blue staining or by western

blot analysis after nitrocellulose transfer (using an anti-human IgG (Fc specific)-HRP antibody, Sigma).

Recombinant UNC5A-Fcas also obtained by transient transfection of Freestyle CHO-S cells (Chine Hamster Ovary, Invitrogen) growing in suspension in a chemically defined, animal-component free, serum-free media with 8 % CO_2 at 31°C. For transfection FreeStyleTM MAX Reagent (Invitrogen) was used according to manufacturer's instructions. The UNC5A-Fcusion protein (SEQ ID NO: 1) could be secreted with high efficiency at a rate of at least 300 mg/L at transient expression in Freestyle CHO-S cells. Supernatant was harvested by centrifugation and sterile filtered (0.2 µm). The concentration of UNC5A-Fc in the supernatant was determined using the BioRad Experion system. The Fc-fusion protein was subsequently purified via cation exchange chromatography followed by Protein A affinity chromatography (PALL Protein A Ceramic Hyper D) according to manufacturer's instructions with the exception of elution in 0.1M Glycine HCl pH 3.0. The eluate was neutralized with 1M Tris-HCl pH 9 and dialyzed overnight against 20 mM Citrate, 134 mM NaCl, pH 6.2. Final analytics were performed with the Bio-Rad Experion system for quantification, size verification and presence of contaminants.

Table : Results of expression

Plasmid #	Characteristic	Sequence	MW kDa	Expression yield µg/mL (Freestyle HEK 293 supernatant day 3)	Expression yield µg/mL (Freestyle CHO-S supernatant day 8)
NP-V-01	UNC5A-Fc	SEQ ID NO. 1	48,17	3.5	300
NP-V-02	UNC5B-Fc	SEQ ID NO. 2	48,31	25	-
NP-V-03	UNC5C-Fc	SEQ ID NO. 3	48,55	5	-

In the following experiment, UNC5A-Fc produced in CHO-S cells has been used.

6. Animal model:

Seven-week-old (20-22 g body weight) female athymic nu/nu mice were obtained from Charles River animal facility. The mice were housed in sterilized filter-topped cages and maintained in a pathogen-free animal facility. A549 cells were implanted by s.c. injection of 10^7 cells in 200µL of PBS into the right flank of the mice. Once tumors were established

(V≈100 mm^3), mice were treated with netrin-1 interfering drugs and/or cytotoxic drugs for two weeks. Tumor sizes were measured with a caliper. The tumor volume was calculated with the formula v = 0.5*(length*$width^2$). At the end of the treatment, tumors were harvested, weighted and were embedded in 7.5% gelatin – 0.12M sucrose and sectioned into 20 µm slices.

7. Statistical analysis:

The data reported are the mean ± S.D. of at least three independent determinations, each performed in triplicate. Statistical analysis was performed by the nonparametric Mann-Whitney *U* test unless indicated.

II. Results and Discussion

1. Netrin-1 and its receptors are up-regulated in tumor cells upon conventional chemotherapies.

We first analyzed by quantitative RT-PCR the level of netrin-1 in two lung cancer cell lines A549 and H460 in response to Doxorubicin. As shown in Fig.1, netrin-1 mRNA level was massively increased in both cell lines (respectively by 430 and 300 fold) upon treatment with 2 µM Doxorubicin. This increase of mRNA was associated with a robust increase of netrin-1 protein expression (Fig.2 and immunofluorescence (not shown).

Next, we analyzed the level of the netrin-1 receptors DCC and UNC5H –UNC5A, UNC5B, UNC5C and UNC5D - in response to Doxorubicin. As shown in Fig.3, levels of DCC, UNC5A, UNC5B and UNC5D increase concomitantly to netrin-1 levels, in A549 cells treated with Doxorubicin. This increase reached 44 folds for the UNC5B receptor. To monitor whether this up-regulation of netrin-1 and its receptors is related to increased gene transcription, A549 cells were treated with Doxorubicin in the presence of the RNA polymerase inhibitor Actinomycin D. As shown in Fig.4, Actinomycin D fully prevents Doxorubicin-mediated netrin-1 up-regulation, thus supporting the view that conventional therapeutic drugs triggers increase of netrin-1 and its receptors via enhanced gene transcription.

To investigate whether netrin-1 and its receptors up-regulation was restricted to Doxorubicin or was a general response to chemotherapeutic agents, netrin-1 levels were analyzed by quantitative RT-PCR in a panel of 15 cancer cell lines in response to various conventional chemotherapeutic drugs, such as Doxorubicin, 5-Fluoruracil (5FU), paclitaxel (Taxol) and Cisplatin. Analysis of netrin-1 level was performed upon treatment with 3 concentrations corresponding to the determined IC_{10}, IC_{30} or IC_{50} of each drug for each cell

lines (Fig. 28). In cell lines which appeared to be resistant to specific drugs (Fig. 28), a concentration corresponding to maximal effective concentration (IC_{MAX}) was used to monitor netrin-1 level. As shown in Fig.5-8, Doxorubicin and 5FU both trigger a significant (i.e.,> 2 fold over control) increase of netrin-1 in respectively 60% and 36% of cancer cell lines. Treatment with Taxol and Cisplatin was associated with netrin-1 up-regulation in only 20% and 21% of cell lines respectively. Netrin-1 up-regulation upon chemotherapeutic drugs treatment is not tumor type specific as netrin-1 up-regulation was seen in at least one cell line of breast, lung, pancreatic and ovarian cancers and as well as in neuroblastoma and glioblastoma cell lines. We could not detect any correlation between netrin-1 up-regulation and chemoresistance, as netrin-1 up-regulation was detected in both resistant and sensitive cell lines, and as some resistant cell lines did not show netrin-1 up-regulation (Fig.5-8).

The expression of netrin-1 dependence receptors in response to these cytotoxic agents was also investigated in the 15 cancer cell lines (Fig.5-8). Similarly to netrin-1 response, Doxorubicin seemed to have the largest effect, as it is associated with the up-regulation of DCC, UNC5B and UNC5D in, respectively, 87%, 80% and 67% of the cell lines screened. DCC, which displays an overall low expression in the screened cancer cell lines, is the netrin-1 receptor showing the largest spectrum of up-regulation as DCC expression was strongly increased in 36%, 43%, and 53% of cell lines in response to respectively Cisplatin, 5FU and Taxol. Levels of the netrin-1 receptors UNC5A and UNC5C remained largely unaffected by treatment with cytotoxic drugs in most of the cell lines that were screened (Fig. 23-24). Together, these data support the view that netrin-1 and its receptors up-regulation frequently occurs in response to conventional drugs treatment.

We finally analysed netrin-1 and receptors level in ovarian cancer specimens from patients before and after treatment with carboplatin/taxol. As shown in Fig.9, netrin-1 mRNA was up-regulated after chemotherapy. Moreover, DCC, UNC5B and UNC5D level was also affected by carboplatin/taxol treatment (Fig.25-27).

2. Netrin-1 interference potentiates cytotoxic drugs induced cell death.

The fact that both netrin-1 and its receptors are up-regulated upon conventional drugs treatment suggests that the dependence for survival on netrin-1 is amplified in chemo-treated cancer cells. We thus first analysed Doxorubicin-induced cell death upon silencing of netrin-1 by a siRNA strategy. A549 cells were then transfected with a netrin-1 siRNA and treated with increasing concentration of Doxorubicin. Silencing of netrin-1 was associated with a marked potentiation of Doxorubicin-induced cell death as shown by measurement of

loss of cell permeability (Fig.10), cell survival (Fig.11), DAPI exclusion (Fig.12), caspase activation (Fig.13) or DNA fragmentation (Fig.14). To determine whether this increased sensitivity was due to the pro-apoptotic engagement of unbound netrin-1 dependence receptors, a similar experiment was performed in settings of silencing of UNC5B, the main netrin-1 receptor expressed upon Doxorubicin treatment in A549 cells. As shown in Fig.15, silencing of UNC5B is associated with the inhibition of the potentiation of cell death induced by netrin-1 silencing and Doxorubicin treatment.

We thus looked at a possible similar potentiation effect using a more therapeutically relevant way for netrin-1 interference. Two drug candidates, TRAP-netrin^{DCC} and TRAP-netrin^{UNC5A}, which are Fc-stabilized ectodomains of respectively DCC or UNC5A, have been shown to trigger death of netrin-1 expressing tumor cells *in vitro* and tumor growth inhibition in engrafted mice models (not shown). As shown in Fig.16-17, these two candidate drugs strongly potentiate Doxorubicin-induced cell death in A549 cells. As netrin-1 and receptors were also up-regulated upon 5FU and Cisplatin treatment (Fig.5-8), we performed similar combination of TRAP-netrin^{UNC5A} with 5FU and Cisplatin. Comparable potentiating effect on cell death was observed upon co-treatment with 5FU or Cisplatin and TRAP-netrin^{UNC5A} (Fig.18-19). Similarly, in pancreatic cancer cell line MiaPacA where 5FU and Doxorubicin have been shown to up-regulate netrin-1 and its receptors, co-treatment of 5FU or Doxorubicin and TRAP-netrin^{UNC5A} potentiated cell death (Fig20-21).

3. Netrin-1 interference potentiates cytotoxic drugs anti-cancer effect in a preclinical animal model of cancer.

We then assessed whether the *in vitro* effect seen above could be translated *in vivo* in a therapeutic perspective. A549 cells were engrafted in *nude* mice and animals with palpable tumors were treated twice a week by i.p. injection of vehicule or TRAP-netrin^{UNC5A} at 20mg/kg alone or in combination with 2 mg/kg of Doxorubicin. Single agent –TRAP-netrin^{UNC5A} or Doxorubicin- treatment used upon these administration schemes and doses were associated with detectable but weak tumor growth inhibiting effect (Fig.22). However, co-treatment of Doxorubicin and TRAP-netrin^{UNC5A} was associated with a strong and prolonged inhibition of tumor growth. Taken together, these data support the view that combining netrin-1 interference based treatment with a conventional chemotherapy is associated with synergic anti-cancer effect.

4. To combine netrin-1 interference and cytotoxic drugs is a promising therapeutic approach.

We show here that, cancer cell lines up-regulate expression of netrin-1 in response to treatment with cytotoxic drugs, The cytotoxic drugs tested here, which include Doxorubicin, Cisplatin, 5FU, and paclitaxel (Taxol) are commonly used in the management of patients with non-small cell lung cancer, breast, colorectal and ovarian cancers both in the adjuvant and advanced setting. Moreover we have shown, using a so far restricted panel of human samples, that primary ovarian tumors from patients treated with Carboplatin/Taxol, display an increase in netrin-1 level compared to the same tumors before treatment. Even though in cell culture this netrin-1 up-regulation differs in kinetics and amplitude depending on the drug used and the cancer cell type (Fig.1), the fact that these drugs are known to affect different cellular mechanisms support the view that netrin-1 up-regulation is rather a general survival stress response than a specific alteration of a specific pathway affected by a specific chemotherapeutic drug. It is then interesting to speculate that this netrin-1 up-regulation may be a survival mechanism employed by cancer cell in response to these drugs.

Although the mechanisms for this up-regulation of netrin-1 remain to be determined, it may have significant therapeutic consequences. Indeed netrin-1 interfering drugs are currently under preclinical development; combination of these compounds with conventional cytotoxic agents may prove synergistic. We have shown that netrin-1 expression is up-regulated in samples from breast, ovarian, pancreatic and non-small cell lung cancer patients and that interfering with the Netrin-1 autocrine/paracrine loop triggers apoptosis of cancer cells in several models. Furthermore, the data presented here suggests that an even larger subset of patient may benefit from Netrin-1 targeting agents, either alone or in combination with cytotoxic agents. Based on our in vivo observation on tumor bearing mice, the combination does not appear to increase toxicity compared to cytotoxic agents alone. The pre-clinical data showed here support the view that combining conventional drugs plus netrin-1 interference may lead to an increased efficacy with reduced concentration of conventional drugs. Together these data support the rationale of testing netrin-1 interference based therapy in early clinical trials in combination with conventional chemotherapies.

5. Example of cancers over-expressing netrin-1 and expressing DCC and/or UNC5A and/or B and/or C and/or D.

The percentage of netrin-1 overexpressing cases is given for each type of cancers for which expression of netrin-1 and its receptors have been quantified.

- 60 % of metastatic breast cancer (Fitamant et al., PNAS 2008),
- 47 % of non-small cell lung cancer (Delloye-Bourgeois et al., JNCI 2009),

- 38 % of aggressive neuroblastoma (Delloye-Bourgeois et al., J. Exp. Med. 2009),
- 61 % of pancreatic adenocarcinoma (Link et al., Annals of Chir. Onco. 2007; Dumartin et al., Gastro 2010),
- 100 % of primary melanoma (n=7), melanoma metastasis (n=6) (Kaufmann et al., Cellular Oncology 2009),
- 76 % of ovarian cancers (Panastasiou et al., Oncotarget 2011),
- 65% of glioblastoma,
- > 60 % of acute myeloid leukemia and chronic lymphocytic leukemia
- > 50 % of aggressive B-cell lymphoma,
- 30 % of sarcoma,
- 40 % of renal adenocarcinoma,
- 22 % of head and neck cancers,
- Testicular cancers (36 % of embryonal carcinoma, 50 % of teratoma, 100 % of yolk sac tumors)
- 50 % of kidney cancers,
- 26 % of stomach cancers,
- 19 % of uterus cancers.

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CLAIMS

1. A pharmaceutical composition comprising a chemotherapeutic drug and a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*, in a pharmaceutically acceptable carrier or vehicle, for use as a medicament for treating a cancer expressing or over-expressing netrin-1.

2. The composition for the use according to claim 1, wherein the netrin-1 interfering drug promotes netrin-1 receptors-induced apoptosis.

3. The composition for the use according to claim 2, wherein the netrin-1 interfering drug is an antibody binding to netrin-1 or to a netrin-1 receptor.

4. The composition for the use according to claim 2, wherein the netrin-1 interfering drug is a compound comprising an extracellular domain of a netrin-1 receptor or a fragment of said extracellular domain.

5. The composition for the use according to claim 2, wherein the netrin-1 interfering drug is a compound comprising an extracellular domain of a netrin-1 receptor DCC, UNC5A, UNC5B, UNC5C or UNC5D or a fragment of said extracellular domain.

6. The composition for the use according to claim 4 or 5, wherein the netrin-1 interfering drug is a fusion protein comprising an extracellular domain of a netrin-1 receptor or fragment thereof and an antibody Fc part.

7. The composition for the use according to claim 2, wherein the netrin-1 interfering drug is a compound comprising a peptidic moiety, which is able to bind to a netrin-1 receptor, this binding being able to prevent netrin-1 ability to block apoptosis induction by a netrin-1 receptor.

8. The composition for the use according to claim 2, wherein the netrin-1 interfering drug is a siRNA which reduces expression of the gene coding for netrin-1.

9. The composition for the use according to any one of the preceding claims, wherein the chemotherapeutic drug is a drug which is able to induce an over-expression of netrin-1 in cancer cells.

10. The composition for the use according to claim 9, wherein the chemotherapeutic drug is doxorubicin, 5-fluorouracil (5FU), paclitaxel, or cisplatin.

11. A pharmaceutical composition comprising a chemotherapeutic drug which is able to induce an over-expression of netrin-1 in cancer cells, for use as an anti-cancer medicament to be used in a patient in combination with a netrin-1 interfering drug or a vector

capable of expressing a netrin-1 interfering drug *in vivo*, to treat a cancer expressing or over-expressing netrin-1.

12. The composition for the use according to any one of the preceding claims, comprising a chemotherapeutic drug and a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*, for a simultaneous, separate or sequential administration to a patient.

13. A method for treating a cancer expressing or overexpressing netrin-1, wherein an efficient amount of a chemotherapeutic drug and of a netrin-1 interfering drug or a vector capable of expressing a netrin-1 interfering drug *in vivo*, in a pharmaceutically acceptable carrier or vehicle, is administered to a patient in need thereof.