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(54) Title: STAT3 INACTIVATION BY INHIBITION OF THE FOLATE RECEPTOR PATHWAY

(57) Abstract: The invention provides a method for specific suppression of Stat3 by inhibition of at least one component of the folate receptor α pathway. The primary target is the folate receptor α , and the invention comprises compounds, including siRNA, antibodies and peptides, targeting the folate receptor as well as uses thereof in treatment of Stat3 related disorders, such as hyperproliferative diseases, including cancer. A method of treating, ameliorating or preventing a Stat3 related disorder by administration of a folate receptor α inhibitor is also provided. Moreover, a method of identifying suitable inhibitors of folate receptor α are provided.



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Stat3 inactivation by inhibition of the Folate receptor pathway

Field of invention

5 The present invention relates to inactivation of Stat3 by inhibition of the folate receptor α pathway. The invention comprises compounds for inhibition of the folate receptor α pathway as well as uses thereof in treatment of hyperproliferative disease, including cancer.

10 Background of invention

Hyperproliferative disorders include tumors or neoplasms, which are localized masses of cells that result from an aberrant, accelerated rate of growth (i.e., hyperproliferative cell growth). As long as the tumor cells remain confined to a single mass, the tumor is considered to be benign. A malignant tumor, also termed cancer, however, has the
15 ability to invade other tissues. Thus, cancer cells are characterized by two critical properties: the cells proliferate in defiance of normal restraints, and invade and colonize the territories of other cells.

The classification of cancers is based on the organ and cell tissue from which the
20 cancer originates. Cancer types include (i) carcinomas, which is the most common kind of cancer which originates from epithelial tissues, covering the body's surface or lining internal organs and various glands; (ii) leukemias, originating from the blood-forming tissues, including bone marrow, lymph nodes and the spleen; (iii) lymphomas from the cells of the lymph system; (iv) melanomas, originating from pigment cells located
25 among the epithelial cells of the skin; and (v) sarcomas, originates in the connective tissues of the body, such as bones, muscles and blood vessels. These broad cancer classifications comprise more than one hundred subclassifications, such as breast, lung, pancreatic, colon, and prostate cancer, to name a few.

30 In addition to cancer, several other hyperproliferative disorders exist. These hyperproliferative disorders are caused by non-cancerous (i.e. non-neoplastic) cells that overproduce, often in response to a particular growth factor. Examples of such hyperproliferative disorders include diabetic retinopathy, endometriosis, macular degenerative disorders and benign growth disorders such as prostate enlargement and

lipomas. Hyperproliferative disorders also comprise autoimmune diseases, such as a variety of skin disorders, including psoriasis.

5 The aggressive cell phenotype of hyperproliferative diseases is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signalling pathways. A commonality for hyperproliferative cells, including all cancer cells, is their failure to execute apoptosis. In fact, the lack of appropriate apoptosis due to defects in the normal apoptosis machinery is a hallmark of cancer. Consequently, most current cancer therapies, including chemotherapeutic agents, radiation, and immunotherapy, 10 work by inducing apoptosis in cancer cells. However, the defects in the normal apoptotic machinery in cancer cells are often associated with an increased resistance to chemotherapy, radiotherapy, or immunotherapy-induced apoptosis. Such primary or acquired resistance of human cancer of different origins to current treatment protocols due to apoptosis defects is a major problem in current cancer therapy. Accordingly, 15 current and future efforts towards designing and developing new molecular target-specific anticancer therapies to improve survival and quality of life of cancer patients must include strategies that specifically target cancer cell resistance to apoptosis. In this regard, targeting crucial negative regulators that play a central role in directly inhibiting apoptosis in cancer cells represents a highly promising therapeutic strategy 20 for new anticancer drug design.

Signal transducers and activators of transcription (STATs) are activated in response to cytokines and growth factors (Darnell et al., Science 264:1415 (1994)). The main domains of Stat3 protein include the tetramerization and leucine zipper at the N- 25 terminus, the DNA binding domain, and the SH2 transactivation domain at the carboxy-terminal end. The SH2 region is responsible for the binding of Stat3 to the tyrosinephosphorylated receptors and for the dimerization which is necessary for DNA binding and gene expression (Zhong et al., Science 264:95(1994)). Stat3 is activated by phosphorylation at Y-705, which leads to dimer formation, nuclear translocation, 30 followed by recognition of Stat3-specific DNA binding elements, and activation of target gene transcription (Darnell et al., Science 264:1415 (1994); Zhong et al., Science 264:95(1994)).

The constitutively activated form of Stat3 is frequently observed in breast carcinoma 35 cell lines but not in normal breast epithelial cells, and it has been reported that

approximately 60 percent of breast tumors contain persistently activated Stat3 (Dechow et al., Proc. Natl. Acad. Sci. USA 101:10602 (2004) Garcia et al., Cell, Growth. 8:1267 (1997); Bowman et al., Oncogene 19:2474 (2000)). Stat3 is classified as a proto-oncogene because activation of Stat3 can mediate oncogenic transformation and tumor formation (Bromberg et al., Cell 98:295 (1999)). Stat3 may participate in oncogenesis by stimulating cell proliferation, promoting angiogenesis, and conferring resistance to apoptosis induced by conventional therapies (Catlett-Falcone et al., Curr. Opin. Oncol. 11:1 (1999); Catlett-Falcone et al., Inznzunity 10:105 (1999); Alas et al., Clin. Cancer Res. 9:316 (2003); Wei et al., Oncogene 22: 15 17 (2003)).

Possible downstream targets through which Stat3 promotes oncogenesis include up-regulation of antiapoptotic factors (Bcl-2, survivin, Mcl-1, and Bcl-XL), cell-cycle regulators (cyclin D1, MEK5, and c-myc), and inducer of tumor angiogenesis (VEGF). Activated Stat3 signalling directly contributes to malignant progression of cancer. Stat3 oncogenic function acts through the pro-survival proteins such as survivin, Mcl-1, Bcl-2, and Bcl-XL and results in the prevention of apoptosis (Real et al., Oncogene 21:7611 (2002); Aoki et al., Blood 101:1535 (2003); Epling-Burnette et al., J. Clin. Invest. 102351 (2001); Nielsen et al., Lezckenzia 13:735 (1999)). Stat3 appears to be essential for the survival or growth of tumor cells, as blockage of Stat3 signaling inhibits cancer cell growth (Alas et al., Clin. Cancer Res. 9:316 (2003); Aoki et al., Blood 101:1535 (2003); Epling-Burnette et al., J. Clin. Invest. 102351 (2001); Burke et al., Oncogene 20:7925 (2001); Mora et al., Cancer Res. 62:6659 (2002); Ni et al., Cancer Res. 60:1225 (2000); Rahaman et al., Oncogene 21:8404 (2002)).

Since Stat3 is frequently activated in breast cancer (Dechow et al., Proc. Natl. Acad. Sci USA 101: 10602 (2004)), it is a putative target for cancer therapy with the potential of inhibiting the abnormal growth of breast cancer. Peptide-based Stat3 inhibitors, which mimic the Stat3 SH2 domain complementary binding structure, were reported to successfully block Stat3 function in vitro (Turkson et al., J. Biol. Chem. 276:45443 (2001)). Attempts have also been made to inhibit Stat3 upstream regulators such as Janus kinases, especially JAK2 (Blaskovich et al., Cancer Res. 63:1270 (2003)). However, there remains a pressing need for developing methods for suppression of Stat3 for use in cancer treatment.

The effectiveness of conventional antineoplastics, such as chemotherapy as well as radiation treatment is often limited by toxicity to other tissues in the body. These side

effects are caused by the fact that conventional anticancer therapies target proliferating cells in general, and therefore affect both cancer cells as well as normal (i.e. non-malignant) dividing cells. Thus, there is a need to obtain treatments that diminish the side effects of conventional cancer therapies, by more directly targeting malignant tumor cells.

Folate receptor α (FR α) is a mediator of folate uptake. Although the precise pathway has not been delineated, FR α has been associated with internalisation of folate via endocytosis (8, 9). Folate receptor α is highly expressed on the surface of a number of cancer cells including ovarian, endometrial, pancreas, breast and cervical cancers (3, 8). In general, more aggressive forms of cancer express higher amounts of FR α compared with their primary counterparts (3). Except from a few fast growing cell types like placenta and colorectal cells (8) most healthy tissues express non or negligible levels of FR α (3). Some results, however, indicate that transport of folate is not the primary function of FR α in ovarian cancer cells, since folate-uptake in these cells primarily is mediated by the reduced folate carrier in spite of highly up-regulated FR α (4). The primary function of FR α is, thus, still considered unknown, but it has been proposed that FR α might affect cell proliferation via cell signalling pathways (8).

Because FR α is either absent from normal tissues or localized to the apical surfaces of polarized epithelia, where it is inaccessible to circulating drugs, folate-linked drugs do not normally accumulate in healthy tissues. However, since the same receptor is fully accessible and highly upregulated on a number of cancer cell types, and also has a high affinity for folic acid, it has been suggested as a target for cancer cell specific delivery of chemotherapeutics by receptor-directed cellular uptake (10).

Summary of invention

The present invention offers a method of selectively suppressing Stat3 in hyperproliferative cells, by targeting the folate receptor α , thereby restoring the apoptotic ability of the hyperproliferative cells, while at the same time diminishing side effects of conventional antineoplastics.

Thus, the present invention relates to inactivation of Stat3 by inhibition of the folate receptor α pathway.

5 In one aspect, the present invention relates to a method for treating, ameliorating, or preventing a disorder comprising administration of a therapeutically effective amount of at least one folate receptor α inhibitor to an animal including a human being in need thereof.

10 In another aspect, the invention relates to a method of inducing apoptosis and/or cell cycle arrest in a cell, comprising contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.

15 In a third aspect, the invention relates to a method of rendering a cell sensitive to an inducer of apoptosis, comprising contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.

20 In a fourth aspect, the invention pertains to a method for identifying a compound suitable for folate receptor α inhibition, said method comprising the steps of bringing said compound in contact with a cell comprising the folate receptor α , and detecting the level of phosphorylated Stat3 in the presence and absence of said compound, wherein a decrease of phosphorylated Stat3 in the presence of said compound compared with the level of phosphorylated Stat3 in the absence of said compound is indicative of an inhibitory effect of said compound on the folate receptor α .

25 A fifth aspect of the invention relates to a method for identifying a peptide suitable for folate receptor α inhibition, said method comprising the steps of a. immobilizing folate receptor α or a fragment thereof on a solid support, b. expressing a peptide library in phages, which display said peptide on its surface, c. bringing said phages in contact with said immobilized folate receptor α or fragment thereof. d. removing unbound phage, e. eluting phages bound to said immobilized folate receptor α or fragment thereof, and f. analysing DNA comprised in said bound phages to obtain sequence of peptide suitable for folate receptor α inhibition.

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In a sixth aspect, the invention relates to a folate receptor α inhibitor for use as a medicament.

A seventh aspect relates to a folate receptor α inhibitor for treatment of a disorder as defined herein.

5 An eighth aspect relates to use of a folate receptor α inhibitor for the manufacture of a medicament for the treatment of a disorder as defined herein.

A ninth aspect of the present invention relates to a use of a folate receptor α inhibitor for the preparation of a medicament for the treatment of a hyperproliferative disease.

10 In a tenth aspect, the present invention relates to a nucleotide sequence encoding at least one peptide as defined in the present invention.

An eleventh aspect relates to a nucleotide sequence encoding at least one siRNA as defined herein.

15 A twelfth aspect relates to a recombinant vector comprising at least one nucleotide sequence as defined defined in the tenth aspect.

20 In a thirteenth aspect, the present invention relates to a cell comprising a nucleotide sequence as defined above integrated in said cells genome and/or carrying a recombinant vector according to the eleventh aspect within said cell.

A fourteenth aspect relates to a pharmaceutical composition comprising a pharmaceutically effective amount of folate receptor α inhibitor and a pharmaceutically acceptable carrier.

30 In a fifteenth aspect, the invention pertains to a kit comprising a pharmaceutically effective amount of folate receptor α inhibitor and instructions for administering said compound to an animal including a human being in need thereof.

In a sixteenth aspect, the invention relates to the use of a folate receptor α inhibitor for the preparation of a medicament for the treatment of a disease, as defined herein.

35 Embodiments of a folate receptor α inhibitor according to the present invention comprise antibodies, polypeptides, peptides, peptide fragments, peptide aptamers,

nucleic acid aptamers, small molecules, foline analogues, natural single domain antibodies, affibodies, affibody-antibody chimeras, and non-immunoglobulin folate receptor α inhibitors. In one embodiment, the folate receptor α is a peptide, and in a preferred embodiment, the peptide is selected from the group consisting of SEQ ID NO: 18 to SEQ ID NO: 35.

Embodiments of disorders, which may be treated, ameliorated and/or prevented by the methods, uses, kits, compositions and folate receptor α inhibitors of the present invention comprise hyperproliferative disorders, such as cancer, for example breast cancer, ovarian cancer, prostate cancer, lung cancer, renal cancer, colon cancer, gastric cancer, and cervical cancer. In a preferred embodiment said cancer is selected from the group consisting of breast cancer, ovarian cancer, lung cancer, and cervical cancer.

Description of Drawings

Figure 1: Stat3 activation in HeLa cells by folic acid and folinic acid. (a) Immunoblot analysis of Stat3 tyrosine phosphorylation (pStat3) in HeLa cells non-treated (control) or treated for 25 minutes with IL-6, folic acid or folinic acid. Immunoblotting with anti-Stat3 antibody verifies that the total amount of protein is equal in all samples. The presented blot is representative of three independent experiments. (b) Dose-response of folic acid on the phosphorylation of Stat3 in HeLa cells. The presented blot is representative of two independent experiments. (c) Binding of active pStat3-dimers to STAT consensus sequences in nuclear extracts from HeLa cells nontreated (control) or treated for 25 minutes with IL-6, folic acid or folinic acid. Data are represented as means $\pm$ s.d. of OD450-values from triplets. The results show one representative of at least three independent experiments carried out.

Figure 2: Expression of FR α on the surface of HeLa cells. (a) Immunostaining with anti-FR α antibody (Mov18/ZEL) showing the expression of FR α (red) on the surface of HeLa and not on HEK293. The nuclei (blue) are visualised with DAPI-staining. (b) Immunoblot analysis of Stat3 tyrosine phosphorylation (pStat3) in HEK293 cells non-treated (control) or treated for 25 minutes with IL-6, folic acid or folinic acid. Immunoblotting with anti-Stat3 antibody verifies that the total amount of protein is equal in all samples. The presented blot is representative of three independent experiments. (c) Flow Cytometry showing the expression of FR α on the surface of

HeLa and not on HEK293 cells. Cells were incubated with (purple) or without (green) anti-FR α antibody (Mov18/ZEL) followed by FITC-conjugated secondary antibody. The experiment was done three times with similar results.

- 5 Figure 3: FR α mediates the activation of Stat3 by folic acid and folinic acid. (a) Immunostaining with anti-FR α antibody (Mov18/ZEL) showing the expression of FR α (red) on the surface of HeLa cells transiently transfected with GFP-siRNA (negative control) or FR α -siRNA. The nuclei (blue) are visualised with DAPI-staining. The results show that FR α -siRNA effectively blocks the expression of FR α on the surface of HeLa
- 10 cells. (b) RT-PCR with primers for the FR α on mRNA isolated from HeLa cells transiently transfected with GFP-siRNA or FR α -siRNA. GAPDH-primers were used as controls. (c) Immunoblot analysis of Stat3 tyrosine phosphorylation (pStat3) in HeLa cells transiently transfected with GFP-siRNA or FR α -siRNA non-treated (control) or treated for 25 minutes with IL-6, folic acid and folinic acid. Immunoblotting with anti-
- 15 Stat3 antibody verifies that the total amount of protein is equal in all samples. The presented blot is representative of two independent experiments.

- Figure 4: Folic acid and folinic acid activate Stat3 through a JAK2-dependent mechanism. Immunoblot analysis of Y705-phosphorylated Stat3 (pStat3) in HeLa cells
- 20 treated with (+) or without (-) JAK2-inhibitor AG490 prior to induction with IL-6, folic acid or folinic acid. Immunoblotting with anti-Stat3 antibody verifies that the total amount of protein is equal in all samples. The experiment was repeated at least three times with similar outcome.

- 25 Figure 5: STAT3 and not STAT1 is activated by folic acid and folinic acid in HeLa cells. A. Immunoblot analysis of STAT3 tyrosine Y705-phosphorylation (pSTAT3) in HeLa cells non-treated (control) or treated for 25 minutes with IL-6, folic acid or folinic acid. Immunoblotting with anti-STAT3 antibody verifies that the total amount of protein is equal in all samples. The presented blot is a representative of three independent
- 30 experiments. B. Immunoblot analysis of STAT1 tyrosine phosphorylation (pSTAT1) in HeLa cells non-treated (control) or treated for 25 minutes with IFN α , folic acid or folinic acid. Immunoblotting with anti-STAT1 antibody verifies that the total amount of protein is equal in all samples. The presented blot is a representative of two independent experiments. C. Binding of active pSTAT3-dimers to STAT consensus sequences in
- 35 nuclear extracts from HeLa cells non-treated (control) or treated for 25 minutes with IL-

6, folic acid or folinic acid. Data are represented as means $\pm$ s.d. of OD450-values from triplets. The results show one representative of at least three independent experiments carried out. D. Dose-response of folic acid on the phosphorylation of STAT3 in HeLa cells. Cells were treated with the indicated amounts of folic acid. The presented blot is a representative of two independent experiments.

Figure 6: Folic acid and folinic acid activate STAT3 differently upon gp130 antibody binding. Immunoblot analysis of Y705-phosphorylated STAT3 (pSTAT3) in HeLa cells treated with (+) or without (-) A. the EGF receptor inhibitor AG1478 or B. the anti-gp130 antibody prior to induction with IL-6, folic acid or folinic acid. Immunoblotting with anti-STAT3 antibody verifies that the total amount of protein is equal in all samples. The experiments were repeated twice.

Figure 7: Folic acid stimulates cell proliferation of HeLa but not HEK293 cells. Cell proliferation assay with HeLa and HEK293 cells non-treated or treated for 48 hours with the indicated amounts of folic acid. Proliferation was measured by 5-Bromo-2'-deoxyuridine (BrdU) incorporation, and depicted as relative to non-treated cells. The experiment was repeated at least twice.

Figure 8: Co-receptors for folic and folinic acid induced STAT3 activation. Immunoblot analysis of Y705-phosphorylated STAT3 (pSTAT3) in HeLa cells treated with (+) or without (-) A. and B. the EGF receptor inhibitor AG1478, C. the anti-gp130 antibody, or D. the anti-FR α antibody Mov18/ZEL prior to induction with EGF, IL-6, folic acid or folinic acid. Immunoblotting with anti-STAT3 antibody verifies that the total amount of protein is equal in all samples. The experiments were repeated twice. The arrows indicate the position of the EGF induced autophosphorylated EGF receptor of 170 kDa recognized by the pSTAT3 antibody.

Detailed description of the invention

The present invention relates to suppression of Stat3 activity by inhibition of folate receptor α . Suppression of stat3 leads to inhibition of cell growth in cells with increased Stat3 activity. Inhibition of folate receptor α also sensitizes cells to inducers of apoptosis and/or cell cycle arrest, and may even it self induce apoptosis and/or cell cycle arrest. Therefore, the invention relates to methods of inhibiting cell growth,

methods of sensitizing cells to inducers of apoptosis and/or cell cycle arrest and methods of inducing apoptosis and/or cell cycle arrest in cells, comprising inhibiting folate receptor α either alone or in combination with an inducer of apoptosis. The invention further relates to methods of treating, ameliorating, or preventing disorders that are associated with elevated Stat3 activity or responsive to induction of apoptosis comprising administering to said animal an inhibitor of folate receptor α and optionally an inducer of apoptosis. Such disorders include those characterized by a dysregulation of apoptosis and those characterized by the proliferation of cells having elevated Stat3 activity.

The terms "disease" and "disorder" are used interchangeably.

Folate and folate receptor α

Folate is a form of the water-soluble vitamin B9. The term "folate" and "folate derivative or analogue thereof", as used herein refers to folate as well as all possible derivatives and analogues thereof. In particular, the term "folate" comprises any analogue or derivative of folate. Thus, folate comprises folic acid, as well as reduced and non-reduced forms of folate, such as for example folinic acid (formyl tetrahydrofolate), dihydrofolate, tetrahydrofolate, methylene tetrahydrofolate, N⁵-methyltetrahydrofolate.

Folate acts as single-carbon donor and is essential for nucleotide synthesis as well as synthesis of some amino acids, such as methionine and serine. Therefore, folate is essential for cellular proliferation and tissue regeneration, which is especially important during periods of rapid cell division and growth such as infancy and pregnancy.

As mammalian cells cannot synthesize folates *de novo*, folate must be taken up from the cellular environment. Folates, however, pass very inefficiently through biological membranes, and therefore, cellular uptake is tightly regulated to sustain sufficient levels of intracellular folate to support biosynthesis of purines, pyrimidines, and some amino acids (serine, methionine). Uptake of folates from food can be mediated by the reduced-folate carrier (RFC), which is one of the major proteins mediating folate transport and is present on all cell surfaces. RFC is a typical transport protein with 12 membrane-spanning domains. RFC preferentially transports reduced folates.

In contrast to RFC, folate receptor α (FR α) has a significantly higher affinity for the synthetic nonreduced form of folate, folic acid, than for reduced folates (8). The presence of highly upregulated FR α on a number of cancer cell types combined with the high affinity for folic acid has made FR α a possible target for chemo- and immuno
5 cancer therapy as well as for folate-targeted imaging (10).

The term "folate receptor α " or "FR α " as used herein, is meant to comprise any analog, fragment or derivative thereof, including folate receptor β (beta) and folate receptor γ (gamma) as defined in SEQ ID NOs.: 12-14 and 15-17, respectively, and fragments
10 thereof.

The present invention relates to a novel function of FR α in activation of the oncogene Stat3. It has been found that folic acid activates Stat3 through FR α , which may therefore prove to be a target for inactivation of Stat3, and thereby serve as treatment
15 of diseases that are associated with elevated Stat3 activity or responsive to induction of apoptosis, such as hyperproliferative disorders, including a number of cancer forms and psoriasis.

Inhibitors of folate receptor α

Inhibitors of folate receptor α according to the present invention can be provided as pharmaceutically acceptable salts. The term "pharmaceutically acceptable salt" as used herein, refers to any salt (e.g. obtained by reaction with an acid or a base) of a compound of the present invention that is physiologically tolerated in the target animal (e.g., a mammal). Salts of the compounds of the present invention may be derived from
25 inorganic or organic acids and bases. Examples of acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, sulfonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in
30 themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.

Examples of bases include, but are not limited to, alkali metal (e.g., sodium) hydroxides, alkaline earth metal (e.g., magnesium) hydroxides, ammonia, and compounds of formula NW_4^+ , wherein W is C₁₋₄ alkyl, and the like.

- 5 Examples of salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, chloride, bromide, iodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, 10 pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples' of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na⁺, NH₄⁺, and NW_4^+ (wherein W is a C₁₋₄ alkyl group), and the like. For 15 therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.
- 20 Inhibition of folate receptor α can occur both by direct and/or by indirect interaction with folate receptor α . Indirect interaction with folate receptor α comprises depletion of the cellular environment of folate receptor α agonists. It is further understood that the term "inhibition of folate receptor α " as used herein comprises inhibition of the entire pathway of folate receptor α activation and the resulting stimulation of Stat3 activity.
- 25 Therefore, inhibition of folate receptor α can occur by abolishing the activation of folate receptor α and/or disrupting the upstream and/or downstream pathway that leads to the activation of Stat3. Furthermore, the inhibition of folate receptor α can occur both on the transcriptional and translational level. Moreover, folate receptor α inhibition may be achieved by targeting co-receptors for folate receptor α , for example the co-receptor 30 gp130.

It is, therefore, also understood that a folate receptor α inhibitor comprise both compounds, which bind directly and/or indirectly to the folate receptor α . A folate receptor α inhibitor also comprise compounds, which binds agonists to folate receptor

α , thereby preventing the activation of said folate receptor α . Thus, inhibition of folate receptor α comprises inhibition of the entire pathway of folate receptor α .

5 Inhibitors of folate receptor α and/or the folate receptor α pathway as identified in the present invention also include derivatives, analogs, prodrugs, or pharmaceutically acceptable salts thereof.

10 The term "prodrug," as used herein, refers to a pharmacologically inactive derivative of a parent "drug" molecule that requires biotransformation (e.g., either spontaneous or enzymatic) within the target physiological system to release, or to convert (e.g., enzymatically, mechanically, electromagnetically) the prodrug into the active drug. Prodrugs are designed to overcome problems associated with stability, toxicity, lack of specificity, or limited bioavailability. Exemplary prodrugs comprise an active drug molecule itself and a chemical masking group (e.g., a group that reversibly suppresses the activity of the drug). Some preferred prodrugs are variations or derivatives of compounds that have groups cleavable under metabolic conditions. Exemplary prodrugs become pharmaceutically active in vivo or in vitro when they undergo solvolysis under physiological conditions or undergo enzymatic degradation or other biochemical transformation (e.g., phosphorylation, hydrogenation, dehydrogenation, glycosylation). Prodrugs often offer advantages of solubility, tissue compatibility, or delayed release in the mammalian organism. Common prodrugs include acid derivatives such as esters prepared by reaction of parent acids with a suitable alcohol (e.g., a lower alkanol), amides prepared by reaction of the parent acid compound with an amine, or basic groups reacted to form an acylated base derivative (e.g., a lower alkylamide).

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In one embodiment of the present invention, the folate receptor α inhibitor is selected from the group consisting of antibodies, polypeptides, peptides, peptide fragments, peptide aptamers, nucleic acid aptamers, small molecules, foline/folate analogues, natural single domain antibodies, affibodies, affibody-antibody chimeras, and non-immunoglobulin folate receptor α inhibitors.

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In one preferred embodiment of the present invention, the folate receptor α inhibitor is a peptide selected from the group consisting of SEQ ID NO: 18 to 35. In one embodiment, the peptide is selected from SEQ ID NO: 18, 19, 20, 21, 22, 23, 24, 25,

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26, 27, 28, 29, 30, 31, 32, 33, 34, or 35. Each of the peptide defined by SEQ ID NO: 18-35 are intended as a single embodiment, and can accordingly be claimed individually. In a specifically preferred embodiment, the peptide is SEQ ID NO: 18. In another specifically preferred embodiment, the peptide is SEQ ID NO: 19.

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The six amino acid peptides as defined in SEQ ID NO: 18 to 35 are also comprised in a number of larger polypeptides, which are also claimed as folate receptor α inhibitors according to the present invention. Examples of such polypeptides, which comprise a hexameric peptide selected from SEQ ID NO: 18 to 35 are SEQ ID NO: 36 to 46. Thus, 10 in one embodiment, the folate receptor α inhibitor of the present invention is a peptide comprising a consecutive amino acid sequence selected from a peptide selected from the group consisting of SEQ ID NO: 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46. In a specific embodiment, the folate receptor α of the present invention is a peptide comprising a consecutive amino acid sequence selected from a peptide selected from 15 SEQ ID NO: 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, or 46. Each peptide comprising such a consecutive amino acid sequence is intended as a separate embodiment, and is accordingly claimed individually. The consecutive amino acid sequence comprise at least 3 amino acids, such as at least 5 amino acids, for example at least 10 amino acids, such as 15 amino acids, such as at least 20 amino acids, for example at least 30 20 amino acids, such as 40 amino acids, such as at least 50 amino acids, for example at least 60 amino acids, such as 70 amino acids, such as at least 80 amino acids, for example at least 90 amino acids, such as 100 amino acids, such as at least 120 amino acids, for example at least 140 amino acids, such as 160 amino acids, such as at least 180 amino acids, for example at least 180 amino acids, such as 200 amino acids, such 25 as at least 300 amino acids, for example at least 400 amino acids, such as 500 amino acids, such as at least 600 amino acids, for example at least 700 amino acids, such as 800 amino acids, such as at least 900 amino acids, for example at least 1000 amino acids. Preferably, the consecutive amino acid sequence comprise between 3 and 50 amino acids, such as more preferably between 5 and 20 amino acids.

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In one embodiment of the present invention, the inhibitors of folate receptor α are compounds that deplete the cellular pool of folate receptor α activators. Activators of folate receptor α include without restriction agonists of folate receptor α . Agonists of folate receptor α include non-limiting examples of folic acid, folate, foline, reduced 35 and/or non-reduced folic acid. Compounds, which deplete the cellular environment of

folate receptor α agonists include but are not restricted to folate receptor α or a fragment thereof, soluble fragments of folate receptor α and/or fragments of the Reduced Folate Carrier. In one such embodiment, the soluble folate receptor α is folate receptor α , in which the GPI-anchor has been cleaved off. In another embodiment, the soluble folate receptor α corresponds to the soluble folate receptor α as found in leukaemia, cow milk, and umbilical cord serum. These receptors are known to bind folic acid. In a preferred embodiment, the folate receptor α inhibitor or the present invention is folate receptor α or a fragment thereof.

10 In one embodiment, the soluble FR α peptide is the bovine folate receptor α variant. In one such embodiment, the bovine FR α peptide corresponds to SEQ ID NO.: 4, or a fragment thereof.

15 In another embodiment, a compound which depletes the cellular pool of folate receptor α activators is an antibody. Antibodies according to the present invention are described below.

In another embodiment of the present invention, the at least one inhibitor of folate receptor α is a protease. In one such embodiment, said protease is capable of cleaving a membrane-attached folate receptor α , thus releasing said folate receptor α from the cellular membrane. In one such embodiment, the folate receptor α is soluble upon cleavage and capable of binding free folate receptor α agonists, thereby serving to deplete cellular levels of said folate receptor α agonists. In a particular embodiment, said protease is a phospholipase. In another embodiment, said protease is a metalloprotease.

20 A phospholipase is an enzyme that converts phospholipids into fatty acids and other lipophilic substances. There are four major classes, termed A, B, C and D distinguished by the type of reaction catalyzed. Phospholipase A comprises phospholipase A1, which cleaves the SN-1 acyl chain, and phospholipase A2, which cleaves the SN-2 acyl chain. Phospholipase B is also known as a lysophospholipase and cleaves both SN-1 and SN-2 acyl chains. Phospholipase C cleaves before the phosphate, releasing diacylglycerol and a phosphate-containing head group. Phospholipase Cs play a central role in signal transduction, releasing the second messenger Inositol triphosphate. Phospholipase D cleaves after the phosphate, releasing phosphatidic acid and an alcohol. Phospholipases C and D are considered phosphodiesterases.

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A metalloproteases or metalloproteinases constitute a family of enzymes, classified by the nature of the most prominent functional group in their active site. There are two subgroups of metalloproteinases: Exopeptidases (metalloexopeptidases) (EC number: 3.4.17), and endopeptidases (metalloendopeptidases) (EC number: 3.4.24). Well
5 known metalloendopeptidases include ADAM proteins and matrix metalloproteinases.

In another embodiment, the inhibitors interact directly with folate receptor α . In one such embodiment, the inhibitors block the site for agonist interaction with folate receptor α , thereby inhibiting activation of said folate receptor α . In one embodiment,
10 the inhibitor is an antibody. In one embodiment, the inhibitor is a blocking antibody. In one embodiment, the inhibitor is a human antibody. In another embodiment, the inhibitor is an antibody developed from rabbit or mouse by immunization of said rabbit or mouse with folate receptor α or fragments thereof. In a specific embodiment, the anti-FR α antibody is Mov18/ZEL. In another specific embodiment, the anti-FR α
15 antibody is Mov19/ZEL.

In yet another embodiment of the present invention, the inhibitor of folate receptor α is siRNA. In a preferred mechanism, siRNA negatively affects the amount of in the cell and/or the level of transcriptional product encoding folate receptor α in the cell. In one
20 embodiment, the at least one siRNA inhibitor is selected from the group of siRNAs consisting of SEQ ID NOs: 9-11. In one embodiment, the at least one inhibitor is a combination of siRNAs as defined by SEQ ID NOs: 9 and 10. In one embodiment, the at least one inhibitor is a combination of siRNAs as defined by SEQ ID NOs: 10 and 11. In one embodiment, the at least one inhibitor is a combination of siRNAs as defined by
25 SEQ ID NOs: 9 and 11. In one embodiment, the at least one inhibitor is the siRNAs defined by SEQ ID NO: 9. In one embodiment, the at least one inhibitor is the siRNAs defined by SEQ ID NO: 10. In one embodiment, the at least one inhibitor is the siRNAs defined by SEQ ID NO: 11.

30 In another embodiment, the at least one inhibitor of folate receptor α is an aptamer. The term aptamer as used herein refers to an antagonist, which has been identified by a systematic evolution of ligands by exponential enrichment (SELEX) procedure. The SELEX procedure is well known to persons of skill within the art. The aptamer can be a nucleic acid, such as DNA or RNA and modified versions thereof, as well as a peptide
35 and modified versions thereof.

In one embodiment of the present invention, the at least one inhibitor of folate receptor α is hormone. In one such embodiment, the at least one inhibitor of folate receptor α is estrogen.

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In a further embodiment of the present invention, the at least one inhibitor of folate receptor α is a combination of folate receptor α inhibitors.

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The term "inhibitor of folate receptor α " or folate receptor α inhibitor, as used herein, refers to any chemical form of inhibitors of the folate receptor α -Stat3 pathway and which is based on the basic structure of the specific inhibitors as specified elsewhere herein. Certain of the compounds of the present invention may exist as stereoisomers including optical isomers. The invention includes all stereoisomers and both the racemic mixtures of such stereoisomers as well as the individual enantiomers that may

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In a preferred embodiment, the folate receptor α of the present invention, which are capable of binding folate receptor α , do not activate the pathway leading to Stat3 activation. Thus, the folate receptor α inhibitors of the present invention preferably do not function as receptor agonists.

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Antibodies

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The term "antibody" as referred to herein includes whole antibodies and/or any antigen binding fragment (i.e., "antigen-binding portion") or single chain thereof. The antibodies of the present invention include human antibodies, recombinant human antibodies, heterologous antibody, isolated antibody,

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An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region (abbreviated herein as CH). Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region (abbreviated herein as CL). The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved,

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termed framework regions. Each VH and VL is composed of three CDRs and four framework sequences, arranged from amino-terminus to carboxy-terminus in the following order: framework sequence1, CDR1, framework sequence2, CDR2, framework sequence3, CDR3, framework sequence4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

10 The term "antigen-binding portion" of an antibody, as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; (vi) an isolated complementarity determining region (CDR), and (vii) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. A further example is binding-domain immunoglobulin fusion proteins comprising (i) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH₂ constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH₃ constant region fused to the CH₂ constant region. The binding domain polypeptide can be a heavy chain variable region or a light chain variable region. Such binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939. These antibody fragments are

obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

5 The term "epitope" means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

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The term "discontinuous epitope", as used herein, means a conformational epitope on a protein antigen which is formed from at least two separate regions in the primary sequence of the protein.

15 The term "bispecific molecule" is intended to include any agent, e.g., a protein, peptide, or protein or peptide complex, which has two different binding specificities. For example, the molecule may bind to, or interact with, (a) a cell surface antigen and (b) an Fc receptor on the surface of an effector cell. The term "multispecific molecule" or "heterospecific molecule" is intended to include any agent, e.g., a protein, peptide, or
20 protein or peptide complex, which has more than two different binding specificities. For example, the molecule may bind to, or interact with, (a) a cell surface antigen, (b) an Fc receptor on the surface of an effector cell, and (c) at least one other component. Accordingly, the invention includes, but is not limited to, bispecific, trispecific, tetraspecific, and other multispecific molecules which are directed to folate receptor α ,
25 and to other cell surface antigens or targets, such as Fc receptors on effector cells.

As used herein, a human antibody is "derived from" a particular germline sequence if the antibody is obtained from a system using human immunoglobulin sequences, e.g., by immunizing a transgenic mouse carrying human immunoglobulin genes or by
30 screening a human immunoglobulin gene library, and wherein the selected human antibody is at least 90%, more preferably at least 95%, even more preferably at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, a human antibody derived from a particular human germline sequence will display no more than 10 amino acid
35 differences, more preferably, no more than 5, or even more preferably, no more than 4,

3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene.

5 The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). However, the term "human antibody", as used herein, is not intended to include
10 antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a)
15 antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom (described further in Section I, below), (b) antibodies isolated from a host cell transformed to express the antibody, e.g., from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial human antibody library, and (d) antibodies prepared,
20 expressed, created or isolated by any other means that involve splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to in vitro mutagenesis (or, when an animal
25 transgenic for human Ig sequences is used, in vivo somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire in vivo.

30 As used herein, a "heterologous antibody" is defined in relation to the transgenic non-human organism producing such an antibody. This term refers to an antibody having an amino acid sequence or an encoding nucleic acid sequence corresponding to that found in an organism not consisting of the transgenic non-human animal, and generally from a species other than that of the transgenic non-human animal.

An "isolated antibody", as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (e.g., an isolated antibody that specifically binds to folate receptor α is substantially free of antibodies that specifically bind antigens other than folate receptor α). An isolated antibody that specifically binds to an epitope, isoform or variant of human folate receptor α may, however, have cross-reactivity to other related antigens, e.g., from other species (e.g., folate receptor α species homologs). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals. In one embodiment of the invention, a combination of "isolated" monoclonal antibodies having different specificities are combined in a well defined composition.

As used herein, "specific binding" refers to antibody binding to a predetermined antigen. Typically, the antibody binds with an affinity corresponding to a KD of about 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or even less, when measured as apparent affinities based on IC50 values in FACS, and binds to the predetermined antigen with an affinity corresponding to a KD that is at least ten-fold lower, such as at least 100-fold lower than its affinity for binding to a non-specific antigen (e.g., BSA, casein) other than the predetermined antigen or a closely-related antigen.

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Affinity: the strength of binding between receptors and their ligands, for example between an antibody and its antigen.

Avidity: The functional combining strength of an antibody with its antigen which is related to both the affinity of the reaction between the epitopes and paratopes, and the valencies of the antibody and antigen

Antibody Classes: Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five (5) major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1 and IgA-2. The heavy chains constant domains that correspond to the different classes of immunoglobulins are called alpha (α), delta (δ), epsilon (ϵ), gamma (γ) and mu (μ), respectively. The light chains of antibodies can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino

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sequences of their constant domain. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

5 Antibody Combining Site: An antibody combining site is that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen. The term immunoreact in its various forms means specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof. Alternatively, an antibody combining site
10 is known as an antigen binding site.

Chimeric antibody: An antibody in which the variable regions are from one species of animal and the constant regions are from another species of animal. For example, a chimeric antibody can be an antibody having variable regions which derive from a
15 mouse monoclonal antibody and constant regions which are human.

Complementarity determining region or CDR: Regions in the V-domains of an antibody that together form the antibody recognizing and binding domain.

20 Constant Region or constant domain or C-domain: Constant regions are those structural portions of an antibody molecule comprising amino acid residue sequences within a given isotype which may contain conservative substitutions therein. Exemplary heavy chain immunoglobulin constant regions are those portions of an immunoglobulin molecule known in the art as CH1, CH2, CH3, CH4 and CH5. An exemplary light chain
25 immunoglobulin constant region is that portion of an immunoglobulin molecule known in the art as CL.

Diabodies: This term refers to a small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (VH) connected to a
30 light chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al., Proc. Natl. Acad Sci. USA 90: 6444-
35 6448 (1993).

Fv: dual chain antibody fragment containing both a VH and a VL.

5 Human antibody framework: A molecule having an antigen binding site and essentially all remaining immunoglobulin-derived parts of the molecule derived from a human immunoglobulin.

10 Humanised antibody framework: A molecule having an antigen binding site derived from an immunoglobulin from a non-human species, whereas some or all of the remaining immunoglobulin-derived parts of the molecule is derived from a human immunoglobulin. The antigen binding site may comprise: either a complete variable domain from the non-human immunoglobulin fused onto one or more human constant domains; or one or more of the complementarity determining regions (CDRs) grafted onto appropriate human framework regions in the variable domain. In a humanized antibody, the CDRs can be from a mouse monoclonal antibody and the other regions of the antibody are human.

Immunoglobulin: The serum antibodies, including IgG, IgM, IgA, IgE and IgD.

20 Immunoglobulin isotypes: The names given to the Ig which have different H chains, the names are IgG (IgG1,2,3,4), IgM, IgA (IgA1,2), sIgA, IgE, IgD.

25 Immunologically distinct: The phrase immunologically distinct refers to the ability to distinguish between two polypeptides on the ability of an antibody to specifically bind one of the polypeptides and not specifically bind the other polypeptide.

30 Monoclonal Antibody: The phrase monoclonal antibody in its various grammatical forms refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen, e.g., a bispecific monoclonal antibody.

Polyclonal antibody: Polyclonal antibodies are a mixture of antibody molecules recognising a specific given antigen, hence polyclonal antibodies may recognise different epitopes within said antigen.

- 5 Single Chain Antibody or scFv: The phrase single chain antibody refers to a single polypeptide comprising one or more antigen binding sites. Furthermore, although the H and L chains of an Fv fragment are encoded by separate genes, they may be linked either directly or via a peptide, for example a synthetic linker can be made that enables them to be made as a single protein chain (known as single chain antibody, sAb; Bird et al. 1988 Science 242:423-426; and Huston et al. 1988 PNAS 85:5879-5883) by recombinant methods. Such single chain antibodies are also encompassed within the term "antibody", and may be utilized as binding determinants in the design and engineering of a multispecific binding molecule.
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- 15 Valency: The term valency refers to the number of potential antigen binding sites, i.e. binding domains, in a polypeptide. A polypeptide may be monovalent and contain one antigen binding site or a polypeptide may be bivalent and contain two antigen binding sites. Additionally, a polypeptide may be tetravalent and contain four antigen binding sites. Each antigen binding site specifically binds one antigen. When a polypeptide comprises more than one antigen binding site, each antigen binding site may specifically bind the same or different antigens. Thus, a polypeptide may contain a plurality of antigen binding sites and therefore be multivalent and a polypeptide may specifically bind the same or different antigens.
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- 25 V-domain: Variable domain are those structural portions of an antibody molecule comprising amino acid residue sequences forming the antigen binding sites. An exemplary light chain immunoglobulin variable region is that portion of an immunoglobulin molecule known in the art as VL.
- 30 VL: Variable domain of the light chain.

VH: Variable domain of the heavy chain.

- It is one aspect of the present invention to provide antibodies or functional equivalents thereof specifically recognising and binding at least one epitope of folate receptor α
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and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof, or a functional homologue of such epitopes. The epitope may be any of the epitopes mentioned herein below.

5 The antibody or functional equivalent thereof may be any antibody known in the art, for example a polyclonal or a monoclonal antibody derived from a mammal or a synthetic antibody, such as a single chain antibody or hybrids comprising antibody fragments. Furthermore, the antibody may be mixtures of monoclonal antibodies or artificial polyclonal antibodies. In addition functional equivalents of antibodies may be antibody
10 fragments, in particular epitope binding fragments. Furthermore, antibodies or functional equivalent thereof may be small molecule mimic, mimicking an antibody. Naturally occurring antibodies are immunoglobulin molecules consisting of heavy and light chains. In preferred embodiments of the invention, the antibody is a monoclonal antibody.

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Monoclonal antibodies (Mab's) are antibodies, wherein every antibody molecule are similar and thus recognises the same epitope. Monoclonal antibodies are in general produced by a hybridoma cell line. Methods of making monoclonal antibodies and antibody-synthesizing hybridoma cells are well known to those skilled in the art.

20 Antibody producing hybridomas may for example be prepared by fusion of an antibody producing B lymphocyte with an immortalized B-lymphocyte cell line. Monoclonal antibodies according to the present invention may for example be prepared as described in Antibodies: A Laboratory Manual, By Ed Harlow and David Lane, Cold Spring Harbor Laboratory Press, 1988. Said monoclonal antibodies may be derived
25 from any suitable mammalian species, however frequently the monoclonal antibodies will be rodent antibodies for example murine or rat monoclonal antibodies. It is preferred that the antibodies according to the present invention are monoclonal antibodies or derived from monoclonal antibodies.

30 Polyclonal antibodies is a mixture of antibody molecules recognising a specific given antigen, hence polyclonal antibodies may recognise different epitopes within said antigen. In general polyclonal antibodies are purified from serum of a mammal, which previously has been immunized with the antigen. Polyclonal antibodies may for example be prepared by any of the methods described in Antibodies: A Laboratory
35 Manual, By Ed Harlow and David Lane, Cold Spring Harbor Laboratory Press, 1988.

Polyclonal antibodies may be derived from any suitable mammalian species, for example from mice, rats, rabbits, donkeys, goats, sheeps, cows or camels. The antibody is preferably not derived from a non-mammalian species, i.e. the antibody is for example preferably not a chicken antibody. The antibody may also for example be
5 an artificial polyclonal antibody as for example described in US 5,789,208 or US 6,335,163, both patent specifications are hereby incorporated by reference into the application in their entirety.

The antibodies according to the present invention may also be recombinant antibodies.
10 Recombinant antibodies are antibodies or fragments thereof or functional equivalents thereof produced using recombinant technology. For example recombinant antibodies may be produced using a synthetic library or by phage display. Recombinant antibodies may be produced according to any conventional method for example the methods outlined in "Recombinant Antibodies", Frank Breitling, Stefan Dübel, Jossey-Bass, September 1999.
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The antibodies according to the present invention may also be bispecific antibodies, i.e. antibodies specifically recognising two different epitopes. Bispecific antibodies may in general be prepared starting from monoclonal antibodies, or from recombinant
20 antibodies, for example by fusing two hybridoma's in order to combine their specificity, by Chemical crosslinking or using recombinant technologies. Antibodies according to the present invention may also be tri-specific antibodies.

Functional equivalents of antibodies may in one preferred embodiment be a fragment
25 of an antibody, preferably an antigen binding fragment or a variable region. Examples of antibody fragments useful with the present invention include Fab, Fab', F(ab')₂ and Fv fragments. Papain digestion of antibodies produces two identical antigen binding fragments, called the Fab fragment, each with a single antigen binding site, and a residual "Fc" fragment, so-called for its ability to crystallize readily. Pepsin treatment
30 yields an F(ab')₂ fragment that has two antigen binding fragments which are capable of cross-linking antigen, and a residual other fragment (which is termed pFc'). Additional fragments can include diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. As used herein, "functional fragment" with respect to antibodies, refers to Fv, F(ab) and F(ab')₂
35 fragments.

Preferred antibody fragments retain some or essential all the ability of an antibody to selectively binding with its antigen or receptor. Some preferred fragments are defined as follows:

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(1) Fab is the fragment that contains a monovalent antigen-binding fragment of an antibody molecule. A Fab fragment can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain.

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(2) Fab' is the fragment of an antibody molecule and can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain. Two Fab' fragments are obtained per antibody molecule. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region.

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(3) (Fab')₂ is the fragment of an antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction. F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds.

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(4) Fv is the minimum antibody fragment that contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in a tight, non-covalent association (VH -V L dimer). It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the VH -V L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

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In one embodiment of the present invention the antibody is a single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Such single chain antibodies are also referred to as "single-chain Fv" or "scFv" antibody fragments. Generally, the Fv

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polypeptide further comprises a polypeptide linker between the VH and VL domains that enables the scFv to form the desired structure for antigen binding.

5 The antibody may also be selected for useful properties, for example it may be desirable to control serum half life of the antibody. In general, complete antibody molecules have a very long serum persistence, whereas fragments (<60-80 kDa) are filtered very rapidly through the kidney. Glycosylation on complete antibodies in general, prolongs serum persistence. Hence, if long term action of the antibody is desirable, the antibody is preferably a complete antibody, whereas if shorter action of
10 the MASP-2 antibody is desirable, an antibody fragment might be preferred.

In another embodiment of the present invention the functional equivalent of an antibody is a small molecule mimic, mimicking an antibody.

15 In one embodiment of the present invention the antibody or functional equivalent thereof comprises specific hypervariable regions, designated CDR. Preferably, the CDRs are CDRs according to the Kabat CDR definition. CDRs or hypervariable regions may for example be identified by sequence alignment to other antibodies.

20 Human Antibodies

Human monoclonal antibodies of the invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology, e.g., the standard somatic cell hybridization technique of Kohler and Milstein, Nature 256:495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other
25 techniques for producing monoclonal antibody can be employed, e.g., viral or oncogenic transformation of B-lymphocytes or phage display techniques using libraries of human antibody genes.

In a preferred embodiment, human monoclonal antibodies directed against epitopes of
30 folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof, can be generated using transgenic or transchromosomal mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomal mice include mice referred to herein as HuMAb mice and KM mice, respectively, and are collectively referred to
35 herein as "transgenic mice."

The HuMAb mouse contains a human immunoglobulin gene miniloci that encodes unarranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (Lonberg, N. et al. (1994) *Nature* 368 (6474):856-859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ and in response to immunization, the introduced human heavy and light chain transgenes, undergo class switching and somatic mutation to generate high affinity human IgG, κ monoclonal antibodies (Lonberg, N. et al. (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13:65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764:536-546). The preparation of HuMAb mice is described in detail in Taylor, L. et al. (1992) *Nucleic Acids Research* 20:6287-6295; Chen, J. et al. (1993) *International Immunology* 5:647-656; Tuailon et al. (1994) *J. Immunol.* 152:2912-2920; Lonberg et al., (1994) *Nature* 368(6474):856-859; Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Taylor, L. et al. (1994) *International Immunology* 6:579-591; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* Vol. 13:65-93; Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci* 764:536-546; Fishwild, D. et al. (1996) *Nature Biotechnology* 14:845-851. See further, US Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay, as well as US 5,545,807 to Surani et al.; WO 98/24884, WO 94/25585, WO 93/1227, WO 92/22645, WO 92/03918 and WO 01/09187.

The KM mouse contains a human heavy chain transchromosome and a human kappa light chain transgene. The endogenous mouse heavy and light chain genes also have been disrupted in the KM mice such that immunization of the mice leads to production of human immunoglobulins rather than mouse immunoglobulins. Construction of KM mice and their use to raise human immunoglobulins is described in detail in WO 02/43478.

Immunizations

To generate fully human monoclonal antibodies to folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof, transgenic or transchromosomal mice containing human

immunoglobulin genes (e.g., HCo12, HCo7 or KM mice) can be immunized with an enriched preparation of antigen and/or cells expressing folate receptor α , as described, for example, by Lonberg et al. (1994), supra; Fishwild et al. (1996), supra, and WO 98/24884. Alternatively, mice can be immunized with DNA encoding human folate receptor α . Preferably, the mice will be 6-16 weeks of age upon the first infusion. For example, an enriched preparation (5-50 μ g) of the folate receptor α antigen can be used to immunize the HuMAb mice intraperitoneally. In the event that immunizations using a purified or enriched preparation of the folate receptor α antigen do not result in antibodies, mice can also be immunized with cells expressing folate receptor α , e.g., a cell line, to promote immune responses.

Cumulative experience with various antigens has shown that the HuMAb transgenic mice respond best when initially immunized intraperitoneally (i.p.) or subcutaneously (s.c.) with folate receptor α expressing cells in complete Freund's adjuvant, followed by every other week i.p. immunizations (up to a total of 10) with cells expressing cells expressing folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof in PBS. The immune response can be monitored over the course of the immunization protocol with plasma samples being obtained by retroorbital bleeds. The plasma can be screened by FACS analysis, and mice with sufficient titers of anti-folate receptor α human immunoglobulin can be used for fusions. Mice can be boosted intravenously with folate receptor α expressing cells for example 4 and 3 days before sacrifice and removal of the spleen.

Generation of Hybridomas Producing Human Monoclonal Antibodies to folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof:

To generate hybridomas producing human monoclonal antibodies to human folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof, splenocytes and lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can then be screened for the production of antigen-specific antibodies. For example, single cell suspensions of splenic lymphocytes from immunized mice can be fused to SP2/0 nonsecreting mouse myeloma cells (ATCC, CRL 1581) with 50% PEG (w/v). Cells can be plated at approximately 1×10^5 per well in flat bottom microtiter plate, followed by a two week

incubation in selective medium containing besides usual reagents 10% fetal Clone Serum, 5-10% origin hybridoma cloning factor (IGEN) and 1X HAT (Sigma). After approximately two weeks, cells can be cultured in medium in which the HAT is replaced with HT. Individual wells can then be screened by ELISA for human kappa-
5 light chain containing antibodies and by FACS analysis using folate receptor α expressing cells for folate receptor α specificity. Once extensive hybridoma growth occurs, medium can be observed usually after 10-14 days. The antibody secreting hybridomas can be replated, screened again, and if still positive for human IgG, anti-folate receptor α monoclonal antibodies can be subcloned at least twice by limiting
10 dilution. The stable subclones can then be cultured in vitro to generate antibody in tissue culture medium for characterization.

Generation of Transfectomas Producing Human Monoclonal Antibodies to folate receptor α and/or fragments thereof, as well as its activators, including agonists of
15 folate receptor α , and/or fragments thereof:

Human antibodies of the invention also can be produced in a host cell transfectoma using, for example, a combination of recombinant DNA techniques and gene transfection methods as is well known in the art, see e.g. Morrison, S. (1985) Science
20 229:1202.

For example, to express the antibodies, or antibody fragments thereof, DNAs encoding partial or full-length light and heavy chains, can be obtained by standard molecular biology techniques (e.g., PCR amplification, site directed mutagenesis) and can be
25 inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene.
30 The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vectors or, more typically, both genes are inserted into the same expression vector. The antibody genes are inserted into the expression vector by standard methods (e.g., ligation of complementary restriction sites
35 on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are

present). The light and heavy chain variable regions of the antibodies described herein can be used to create full-length antibody genes of any antibody isotype by inserting them into expression vectors already encoding heavy chain constant and light chain constant regions of the desired isotype such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively, the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (i.e., a signal peptide from a non-immunoglobulin protein).

In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology. Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). It will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as promoters and/or enhancers derived from cytomegalovirus (CMV), Simian Virus 40 (SV40), adenovirus, (e.g., the adenovirus major late promoter (AdMLP)) and polyoma. Alternatively, nonviral regulatory sequences may be used, such as the ubiquitin promoter or β -globin promoter.

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see e.g., US 4,399,216, US 4,634,665 and US 5,179,017, all by Axel et al.). For example, typically

the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in dhfr-host cells with methotrexate selection/amplification) and the neo gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection, lipofectin transfection and the like.

In one embodiment the antibodies are expressed in eukaryotic cells, such as mammalian host cells. Preferred mammalian host cells for expressing the recombinant antibodies of the invention include CHO cells (including dhfr-CHO cells, described in Urlaub and Chasin, (1980) Proc. Natl. Acad. Sci. USA 77:4216-4220, used with a DHFR selectable marker, e.g., as described in R. J. Kaufman and P. A. Sharp (1982) Mol. Biol. 159:601-621), NS/0 myeloma cells, COS cells, HEK293 cells and SP2.0 cells. In particular for use with NS/0 myeloma cells, another preferred expression system is the GS (glutamine synthetase) gene expression system disclosed in WO 87/04462, WO 89/01036 and EP 338 841. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Further Recombinant Means for Producing Human Monoclonal Antibodies to folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof:

Alternatively the cloned antibody genes can be expressed in other expression systems, including prokaryotic cells, such as microorganisms, e.g. E. coli for the production of scFv antibodies, algi, as well as insect cells. Furthermore, the antibodies can be

produced in transgenic non-human animals, such as in milk from sheep and rabbits or eggs from hens, or in transgenic plants. See e.g. Verma, R., et al. (1998) "Antibody engineering: Comparison of bacterial, yeast, insect and mammalian expression systems", J.Immunol.Meth. 216:165-181; Pollock, et al. (1999) "Transgenic milk as a method for the production of recombinant antibodies", J.Immunol.Meth. 231:147-157; and Fischer, R., et al. (1999) "Molecular farming of recombinant antibodies in plants", Biol.Chem. 380:825-839.

Use of Partial Antibody Sequences to Express Intact Antibodies

Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, e.g., Riechmann, L. et al. (1998) Nature 332:323-327; Jones, P. et al. (1986) Nature 321:522-525; and Queen, C. et al. (1989) Proc. Natl. Acad. Sci. USA 86:10029-10033). Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences. These germline sequences will differ from mature antibody gene sequences because they will not include completely assembled variable genes, which are formed by V(D)J joining during B cell maturation. Germline gene sequences will also differ from the sequences of a high affinity secondary repertoire antibody which contains mutations throughout the variable gene but typically clustered in the CDRs. For example, somatic mutations are relatively infrequent in the amino terminal portion of framework region 1 and in the carboxy-terminal portion of framework region 4. For this reason, it is not necessary to obtain the entire DNA sequence of a particular antibody in order to recreate an intact recombinant antibody having binding properties similar to those of the original antibody (see WO 99/45962). Partial heavy and light chain sequence spanning the CDR regions is typically sufficient for this purpose. The partial sequence is used to determine which germline variable and joining gene segments contributed to the recombined antibody variable genes. The germline sequence is then used to fill in

missing portions of the variable regions. Heavy and light chain leader sequences are cleaved during protein maturation and do not contribute to the properties of the final antibody. To add missing sequences, cloned cDNA sequences can be combined with synthetic oligonucleotides by ligation or PCR amplification. Alternatively, the entire
5 variable region can be synthesized as a set of short, overlapping, oligonucleotides and combined by PCR amplification to create an entirely synthetic variable region clone. This process has certain advantages such as elimination or inclusion or particular restriction sites, or optimization of particular codons.

10 The nucleotide sequences of heavy and light chain transcripts from hybridomas are used to design an overlapping set of synthetic oligonucleotides to create synthetic V sequences with identical amino acid coding capacities as the natural sequences. The synthetic heavy and kappa chain sequences can differ from the natural sequences in three ways: strings of repeated nucleotide bases are interrupted to facilitate
15 oligonucleotide synthesis and PCR amplification; optimal translation initiation sites are incorporated according to Kozak's rules (Kozak, 1991, J. Biol. Chem. 266:19867-19870); and HindIII sites are engineered upstream of the translation initiation sites.

For both the heavy and light chain variable regions, the optimized coding and
20 corresponding non-coding, strand sequences are broken down into 30 - 50 nucleotides approximately at the midpoint of the corresponding non-coding oligonucleotide. Thus, for each chain, the oligonucleotides can be assembled into overlapping double stranded sets that span segments of 150 - 400 nucleotides. The pools are then used as templates to produce PCR amplification products of 150 - 400 nucleotides. Typically, a
25 single variable region oligonucleotide set will be broken down into two pools which are separately amplified to generate two overlapping PCR products. These overlapping products are then combined by PCR amplification to form the complete variable region. It may also be desirable to include an overlapping fragment of the heavy or light chain constant region (including the BbsI site of the kappa light chain, or the AgeI site of the
30 gamma heavy chain) in the PCR amplification to generate fragments that can easily be cloned into the expression vector constructs.

The reconstructed heavy and light chain variable regions are then combined with cloned promoter, leader, translation initiation, constant region, 3' untranslated,
35 polyadenylation, and transcription termination, sequences to form expression vector

constructs. The heavy and light chain expression constructs can be combined into a single vector, co-transfected, serially transfected, or separately transfected into host cells which are then fused to form a host cell expressing both chains.

- 5 In another aspect of the invention, the structural features of the human anti-folate receptor α antibodies of the invention are used to create structurally related human anti-folate receptor α antibodies that retain at least one functional property of the antibodies of the invention, such as binding to folate receptor α . More specifically, one or more CDR regions can be combined recombinantly with known human framework regions and CDRs to create additional, recombinantly-engineered, human anti-folate receptor
10 antibodies of the invention.

Monovalent antibodies:

- The monospecific binding member, which bind folate receptor α , may be monovalent,
15 i.e. having only one binding domain.

- For a monovalent antibody, the immunoglobulin constant domain amino acid residue sequences comprise the structural portions of an antibody molecule known in the art as CH1, CH2, CH3 and CH4. Preferred are those binding members which are known in
20 the art as CL. Preferred CL polypeptides are selected from the group consisting of Ckappa and Clambda.

- Furthermore, insofar as the constant domain can be either a heavy or light chain constant domain (CH or CL, respectively), a variety of monovalent binding member
25 compositions are contemplated by the present invention. For example, light chain constant domains are capable of disulfide bridging to either another light chain constant domain, or to a heavy chain constant domain. In contrast, a heavy chain constant domain can form two independent disulfide bridges, allowing for the possibility of bridging to both another heavy chain and to a light chain, or to form polymers of heavy
30 chains.

- Thus, in another embodiment, the invention contemplates a composition comprising a monovalent polypeptide wherein the constant chain domain C has a cysteine residue capable of forming at least one disulfide bridge, and where the composition comprises
35 at least two monovalent polypeptides covalently linked by said disulfide bridge.

In preferred embodiments, the constant chain domain C can be either CL or CH.

Where C is CL, the CL polypeptide is preferably selected from the group consisting of Ckappa and Clambda.

5

In another embodiment, the invention contemplates a binding member composition comprising a monovalent polypeptide as above except where C is CL having a cysteine residue capable of forming a disulfide bridge, such that the composition contains two monovalent polypeptides covalently linked by said disulfide bridge.

10

Multispecificity, including bispecificity

In a preferred embodiment the present invention relates to multispecific binding members, which have affinity for and are capable of binding at least two different entities. Multispecific binding members can include bispecific binding members.

15

In one embodiment the multispecific molecule is a bispecific antibody (BsAb), which carries at least two different binding domains, at least one of which is of antibody origin.

20

A bispecific molecule of the invention can also be a single chain bispecific molecule, such as a single chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding domain, or a single chain bispecific molecule comprising two binding domains. Multispecific molecules can also be single chain molecules or may comprise at least two single chain molecules.

25

The multispecific, including bispecific, antibodies may be produced by any suitable manner known to the person skilled in the art.

30

The traditional approach to generate bispecific whole antibodies was to fuse two hybridoma cell lines each producing an antibody having the desired specificity.

Because of the random association of immunoglobulin heavy and light chains, these hybrid hybridomas produce a mixture of up to 10 different heavy and light chain combinations, only one of which is the bispecific antibody. Therefore, these bispecific antibodies have to be purified with cumbersome procedures, which considerably decrease the yield of the desired product.

35

Alternative approaches include in vitro linking of two antigen specificities by chemical cross-linking of cysteine residues either in the hinge or via a genetically introduced C-terminal Cys as described above. An improvement of such in vitro assembly was
5 achieved by using recombinant fusions of Fab's with peptides that promote formation of heterodimers. However, the yield of bispecific product in these methods is far less than 100%.

A more efficient approach to produce bivalent or bispecific antibody fragments, not
10 involving in vitro chemical assembly steps, was described by Holliger et al. (1993). This approach takes advantage of the observation that scFv's secreted from bacteria are often present as both monomers and dimers. This observation suggested that the VH and VL of different chains could pair, thus forming dimers and larger complexes. The dimeric antibody fragments, also named "diabodies" by Holliger et al., are in fact small
15 bivalent antibody fragments that assembled in vivo. By linking the VH and VL of two different antibodies 1 and 2, to form "cross-over" chains VH 1VL 2 and VH 2-VL 1, the dimerisation process was shown to reassemble both antigen-binding sites. The affinity of the two binding sites was shown to be equal to the starting scFv's, or even to be 10-fold increased when the polypeptide linker covalently linking VH and VL was removed,
20 thus generating two proteins each consisting of a VH directly and covalently linked to a VL not pairing with the VH. This strategy of producing bispecific antibody fragments was also described in several patent applications. Patent application WO 94/09131 (SCOTGEN LTD; priority date Oct. 15, 1992) relates to a bispecific binding protein in which the binding domains are derived from both a VH and a VL region either present
25 at two chains or linked in an scFv, whereas other fused antibody domains, e.g. C-terminal constant domains, are used to stabilise the dimeric constructs. Patent application WO 94/13804 (CAMBRIDGE ANTIBODY TECHNOLOGY/MEDICAL RESEARCH COUNCIL; first priority date Dec. 4, 1992) relates to a polypeptide containing a VH and a VL which are incapable of associating with each other, whereby
30 the V-domains can be connected with or without a linker.

Mallender and Voss, 1994 (also described in patent application WO 94/13806; DOW
CHEMICAL CO; priority date Dec. 11, 1992) reported the in vivo production of a single-chain bispecific antibody fragment in E. coli. The bispecificity of the bivalent protein
35 was based on two previously produced monovalent scFv molecules possessing distinct

specificities, being linked together at the genetic level by a flexible polypeptide linker. Traditionally, whenever single-chain antibody fragments are referred to, a single molecule consisting of one heavy chain linked to one (corresponding) light chain in the presence or absence of a polypeptide linker is implicated. When making bivalent or bispecific antibody fragments through the "diabody" approach (Holliger et al., (1993) and patent application WO 94/09131) or by the "double scFv" approach (Mallender and Voss, 1994 and patent application WO 94/13806), again the VH is linked to a (the corresponding) VL.

10 The multispecific molecules described above can be made by a number of methods. For example, all specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the multi-specific molecule is a mAb X mAb, mAb X Fab, Fab X F(ab')₂ or ligand X Fab fusion protein. Various other methods for preparing bi- or multivalent antibodies are
15 described for example described in U.S. Pat. Nos. 5,260,203; 5,455,030; 4,881,175; 5,132,405; 5,091,513; 5,476,786; 5,013,653; 5,258,498; and 5,482,858.

By using a bispecific or multispecific binding member according to the invention the invention offers several advantages as compared to monospecific/monovalent binding
20 members.

A bispecific/multispecific binding member has a first binding domain capable of specifically recognising and binding a Streptococcus protein, in particular Pneumolysin, whereas the other binding domain(s) may be used for other purposes:

25 In one embodiment at least one other binding domain is used for binding to folate receptor α , such as binding to another epitope on the same folate receptor α as compared to the first binding domain. Thereby specificity for the folate receptor α species may be increased as well as increase of avidity of the binding member.

30 In another embodiment the at least one other binding domain may be used for specifically binding a mammalian cell, such as a human cell. It is preferred that the at least other binding domain is capable of binding an hyperproliferative cell, such as a cancer cell or a cell involved in psoriasis. This may be accomplished by establishing
35 that the at least one other binding domain is capable of specifically binding a

mammalian protein, such as a human protein, such as a protein specific for cancer cells and/or cells involved in psoriasis, for example epidermal cells.

5 Accordingly, the present invention includes bispecific and multispecific molecules comprising at least one first binding specificity for folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof, and a second binding specificity for a second target epitope. Therefore, the invention includes bispecific and multispecific molecules capable of binding both to specific target cells, such as cancer cells and/or cells involved in
10 psoriasis, and cells expressing folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof. These bispecific and multispecific molecules target cells expressing folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof, to effector cells and, like the human monoclonal antibodies of
15 the invention, trigger Fc receptor-mediated effector cell activities, such as phagocytosis of folate receptor α and/or fragments thereof, as well as its activators, including agonists of folate receptor α , and/or fragments thereof expressing cells, antibody dependent cellular cytotoxicity (ADCC), cytokine release, or generation of superoxide anion.

20 Bispecific and multispecific molecules of the invention can further include a third binding specificity. In one embodiment, the third binding specificity is an anti-enhancement factor (EF) portion, e.g., a molecule which binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against the target cell. The "anti-enhancement factor portion" can be an antibody, functional
25 antibody fragment or a ligand that binds to a given molecule, e.g., an antigen or a receptor, and thereby results in an enhancement of the effect of the binding determinants for the folate receptor α or target cell antigen. The "anti-enhancement factor portion" can bind a target cell antigen. Alternatively, the anti-enhancement factor
30 portion can bind to an entity that is different from the entity to which the first and second binding specificities bind. For example, the anti-enhancement factor portion can bind a cytotoxic T cell (e.g., via CD2, CD3, CD8, CD28, CD4, CD40, ICAM-1 or other immune cell that results in an increased immune response against the target cell).

In one embodiment, the bispecific and multispecific molecules of the invention comprise as a binding specificity at least one further antibody, including, e.g., an Fab, Fab', F(ab')₂, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner et al. in US 4,946,778. The antibody may also be a binding-
5 domain immunoglobulin fusion protein as disclosed in US 2003/0118592 and US 2003/0133939.

In one embodiment, the binding specificity for an Fc receptor is provided by a human
10 monoclonal antibody, the binding of which is not blocked by human immunoglobulin G (IgG). As used herein, the term "IgG receptor" refers to any of the eight γ -chain genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fc* receptor classes: Fc γ RI (CD64), Fc γ RII (CD32), and Fc γ RIII (CD16). In one preferred embodiment, the Fc γ
15 receptor is a human high affinity Fc γ RI.

The production and characterization of these preferred monoclonal antibodies are described by Fanger et al. in WO 88/00052 and in US 4,954,617. These antibodies bind to an epitope of Fc γ RI, Fc γ RII or Fc γ RIII at a site which is distinct from the Fc γ
20 binding site of the receptor and, thus, their binding is not blocked substantially by physiological levels of IgG. Specific anti-Fc γ RI antibodies useful in this invention are mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. In other embodiments, the anti-Fc γ receptor antibody is a humanized form of mAb 22 (H22). The production and characterization of the H22 antibody is described in Graziano, R.F. et al. (1995) J.
25 Immunol. 155 (10):4996-5002 and WO 94/10332. The H22 antibody producing cell line was deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the accession No. CRL 11177.

Fc α RI, Fc γ RI, Fc γ RII and Fc γ RIII, especially Fc γ RII and Fc γ RIII, are preferred trigger
30 receptors for use in the invention because they (1) are expressed primarily on immune effector cells, e.g., monocytes, PMNs, macrophages and dendritic cells; (2) are expressed at high levels (e.g., 5,000-100,000 per cell); (3) are mediators of cytotoxic activities (e.g., ADCC, phagocytosis); and (4) mediate enhanced antigen presentation of antigens, including self-antigens, targeted to them.

35

While human monoclonal antibodies are preferred, other antibodies which can be employed in the bispecific or multispecific molecules of the invention are murine, chimeric and humanized monoclonal antibodies. Such murine, chimeric and humanized monoclonal antibodies can be prepared by methods known in the art.

5

Bispecific and multispecific molecules of the present invention can be made using chemical techniques (see e.g., D. M. Kranz et al. (1981) Proc. Natl. Acad. Sci. USA 78:5807), "polydoma" techniques (see US 4,474,893), or recombinant DNA techniques.

- 10 In particular, bispecific and multispecific molecules of the present invention can be prepared by conjugating the constituent binding specificities, e.g., the anti-FcR and anti-folate receptor α binding specificities, using methods known in the art. For example, each binding specificity of the bispecific and multispecific molecule can be generated separately and then conjugated to one another. When the binding
- 15 specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-
- 20 maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) see e.g., Karpovsky et al. (1984) J. Exp. Med. 160:1686; Liu, M. A., et al. (1985) Proc. Natl. Acad. Sci. USA 82:8648. Other methods include those described by Paulus (Behring Ins. Mitt. (1985) No. 78, 118-132); Brennan et al. (1985) Science 229:81-83, and Glennie et al. (1987) J. Immunol. 139:2367-2375. Preferred conjugating agents are SATA and sulfo-SMCC,
- 25 both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies, they can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of

30 sulfhydryl residues, preferably one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific and multispecific molecule is a mAb x mAb, mAb x Fab, Fab x

35 F(ab')₂ or ligand x Fab fusion protein. A bispecific and multispecific molecule of the

invention, e.g., a bispecific molecule can be a single chain molecule, such as a single chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific and multispecific molecules can also
5 be single chain molecules or may comprise at least two single chain molecules.

Methods for preparing bi- and multispecific molecules are described for example in US 5,260,203; US 5,455,030; US 4,881,175; US 5,132,405; US 5,091,513; US 5,476,786; US 5,013,653; US 5,258,498; and US 5,482,858.

10 Binding of the bispecific and multispecific molecules to their specific targets can be confirmed by enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), FACS analysis, a bioassay (e.g., growth inhibition), or a Western Blot Assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (e.g., an antibody) specific for the
15 complex of interest. For example, the FcR-antibody complexes can be detected using e.g., an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example,
20 Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

25 Humanised antibody framework

It is not always desirable to use non-human antibodies for human therapy, since the non-human "foreign" epitopes may elicit immune response in the individual to be treated. To eliminate or minimize the problems associated with non-human antibodies, it is desirable to engineer chimeric antibody derivatives, i.e., "humanized" antibody
30 molecules that combine the non-human Fab variable region binding determinants with a human constant region (Fc). Such antibodies are characterized by equivalent antigen specificity and affinity of the monoclonal and polyclonal antibodies described above, and are less immunogenic when administered to humans, and therefore more likely to be tolerated by the individual to be treated.

Accordingly, in one embodiment the binding member, which target folate receptor α , has a binding domain carried on a humanised antibody framework, also called a humanised antibody.

- 5 Humanised antibodies are in general chimeric antibodies comprising regions derived from a human antibody and regions derived from a non-human antibody, such as a rodent antibody. Humanisation (also called Reshaping or CDR-grafting) is a well-established technique for reducing the immunogenicity of monoclonal antibodies (mAbs) from xenogeneic sources (commonly rodent), increasing the homology to a
- 10 human immunoglobulin, and for improving their activation of the human immune system. Thus, humanized antibodies are typically human antibodies in which some CDR residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies.
- 15 It is further important that humanized antibodies retain high affinity for the antigen and other favourable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin
- 20 models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of certain residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence
- 25 the ability of the candidate immunoglobulin to bind its antigen. In this way, framework sequence residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is maximized, although it is the CDR residues that directly and most substantially influence antigen binding.
- 30 One method for humanising MAbs related to production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody are fused to constant domains derived from a second antibody, preferably a human antibody. Methods for carrying out such chimerisation procedures are for example
- 35 described in EP-A-0 120 694 (Celltech Limited), EP-A-0 125 023 (Genentech Inc.), EP-

A-0 171 496 (Res. Dev. Corp. Japan), EP-A-0173494 (Stanford University) and EP-A-0 194 276 (Celltech Limited). A more complex form of humanisation of an antibody involves the re-design of the variable region domain so that the amino acids constituting the non-human antibody binding site are integrated into the framework of a human antibody variable region (Jones et al., 1986).

The humanized antibody of the present invention may be made by any method capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on Mar. 26, 1987), the contents of which are expressly incorporated by reference. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed mutagenesis as described in the examples below.

As an example the humanized antibody of the present invention may be made as described in the brief explanation below. The humanized antibodies of the present invention may be produced by the following process:

(a) constructing, by conventional techniques, an expression vector containing an operon with a DNA sequence encoding an antibody heavy chain in which the CDRs and such minimal portions of the variable domain framework region that are required to retain antibody binding specificity are derived from a non-human immunoglobulin, and the remaining parts of the antibody chain are derived from a human immunoglobulin, thereby producing the vector of the invention;

(b) constructing, by conventional techniques, an expression vector containing an operon with a DNA sequence encoding a complementary antibody light chain in which the CDRs and such minimal portions of the variable domain framework region that are required to retain donor antibody binding specificity are derived from a non-human immunoglobulin, and the remaining parts of the antibody chain are derived from a human immunoglobulin, thereby producing the vector of the invention;

(c) transfecting the expression vectors into a host cell by conventional techniques to produce the transfected host cell of the invention; and

(d) culturing the transfected cell by conventional techniques to produce the humanised antibody of the invention.

5 The host cell may be cotransfected with the two vectors of the invention, the first vector containing an operon encoding a light chain derived polypeptide and the second vector containing an operon encoding a heavy chain derived polypeptide. The two vectors contain different selectable markers, but otherwise, apart from the antibody heavy and light chain coding sequences, are preferably identical, to ensure, as far as possible, equal expression of the heavy and light chain polypeptides. Alternatively, a single
10 vector may be used, the vector including the sequences encoding both the light and the heavy chain polypeptides. The coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both.

15 The host cell used to express the altered antibody of the invention may be either a bacterial cell such as *Escherichia coli*, or a eukaryotic cell. In particular a mammalian cell of a well defined type for this purpose, such as a myeloma cell or a Chinese hamster ovary cell may be used.

20 The general methods by which the vectors of the invention may be constructed, transfection methods required to produce the host cell of the invention and culture methods required to produce the antibody of the invention from such host cells are all conventional techniques. Likewise, once produced, the humanized antibodies of the invention may be purified according to standard procedures as described below.

25 Human antibody framework

In a more preferred embodiment the invention relates to a binding member, wherein the binding domain is carried by a human antibody framework, i.e. wherein the antibodies have a greater degree of human peptide sequences than do humanised antibodies.

30

Human mAb antibodies directed against human proteins can be generated using transgenic mice carrying the complete human immune system rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for
35 epitopes from a human protein (see, e.g., Wood et al. International Application WO

- 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. International Application WO 92/03918; Kay et al. International Application 92/03917; Lonberg, N. et al. 1994 Nature 368:856-859; Green, L. L. et al. 1994 Nature Genet. 7:13-21; Morrison, S. L. et al. 1994 Proc. Natl. Acad. Sci. USA 81:6851-6855;
- 5 Bruggeman et al. 1993 Year Immunol 7:33-40; Tuailon et al. 1993 PNAS 90:3720-3724; Bruggeman et al. 1991 Eur J Immunol 21:1323-1326).

Such transgenic mice are available from Abgenix, Inc., Fremont, Calif., and Medarex, Inc., Annandale, N.J. It has been described that the homozygous deletion of the

10 antibody heavy-chain joining region (IH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA 90:2551 (1993); Jakobovits et al., Nature 362:255-258

15 (1993); Bruggermann et al., Year in Immunol. 7:33 (1993); and Duchosal et al. Nature 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., J. Mol. Biol. 227: 381 (1991); Marks et al., J. Mol. Biol. 222:581-597 (1991); Vaughan, et al., Nature Biotech 14:309 (1996)).

20 Alternative binding members:

Natural single domain antibodies

Heavy-chain antibodies (HCAbs) are naturally produced by camelids (camels, dromedaries and llamas). HCAbs are homodimers of heavy chains only, devoid of light chains and the first constant domain (Hamers-Casterman et al., 1993). The possibility

25 to immunize these animals allows for the cloning, selection and production of an antigen binding unit consisting of a single-domain only. Furthermore these minimal-sized antigen binding fragments are well expressed in bacteria, interact with the antigen with high affinity and are very stable.

30 New or Nurse Shark Antigen Receptor (NAR) protein exists as a dimer of two heavy chains with no associated light chains. Each chain is composed of one variable (V) and five constant domains. The NAR proteins constitute a single immunoglobulin variable-like domain (Greenberg, A. S., Avila, D., Hughes, M., Hughes, A., McKinney, E. C. & Flajnik, M. F. (1995) Nature (London) 374, 168-173.) which is much lighter than an

35 antibody molecule.

Non-immunoglobulin folate receptor α inhibitors

In one preferred embodiment, the present invention relates to folate receptor α inhibitors derived from a naturally occurring protein or polypeptide; said protein or polypeptide may for example be designed de novo, or may be selected from a library. The binding member may be a single moiety, e.g., a polypeptide or protein domain, or it may include two or more moieties, e.g., a pair of polypeptides such as a pair of polypeptides. The folate receptor α inhibitors may for example, but exclusively, be a lipocalin, a single chain MHC molecule, an Anticalin™ (Pieris), an Affibody™, or a Trinectin™ (Phylos), Nanobodies (Ablynx). The folate receptor α inhibitors may be selected or designed by recombinant methods known by people well known in the art.

Affibody

Affibody: A recombinant immunologically active molecule, selected from a library constructed by combinatorial variegation of the Fc binding surface of of a protein that is not an antibody, preferably the 58 residue staphylococcal protein A (SPA).

Affibodies are produced recombinantly by methods well known to those skilled in the art of recombinant DNA technology. Phage display techniques may be used to identify affibodies capable of specifically recognising a particular folate receptor α or part thereof. Affibodies can be produced in any suitable host, as for example, but not exclusively E. coli or S. cerevisiae (see below) (Hansson M et al., "An in vitro selected binding protein (affibody) shows conformation-dependent recognition of the respiratory syncytial virus (RSV) G protein", Immunotechnology. 1999 Mar; 4(3-4): 237-52.)

Affibody-antibody chimeras

In another embodiment of the present invention, the folate receptor α inhibitor is an affibody-antibody chimera (Ronnmark J et al, Construction and characterization of affibody-Fc chimeras produced in Escherichia coli. J Immunol Methods. 2002 Mar 1; 261(1-2): 199-211). According to the invention affibody-antibody chimeras can be constructed by several methods, for example by fusion of nucleotide sequences or fusion of polypeptide sequences. The nucleic acid sequence of an affibody may be fused to a nucleic acid sequence of an antibody by DNA recombinant technology for the production of the binding member in a suitable host. The affibody nucleotide sequences may for example be fused to an antibody light chain nucleotide sequence or

an antibody heavy chain nucleic acid sequence. In an embodiment of the invention the affibody sequence may be fused with a fragment of an antibody sequences. The affibody sequence may for example, but not exclusively, be fused with an Fc fragment of an antibody, thus potentially allowing dimers to form by homo-dimerisation. The affibody antibody chimeras may contain multiple affibody sequences, such as at least two, three, four of at least six affibody sequences. In an embodiment of the invention a fusion of two affibodies may be fused with an Fc fragment resulting in a tetravalent binding member upon dimerisation.

Alternatively the chimeras may be obtained by linking of the two protein/polypeptide molecules together by methods known to people skilled in the art.

Stat3

The Signal Transducers and Activator of Transcription (STAT) proteins are involved in the regulation of several aspects of cell growth, survival and differentiation. The transcription factors of this family are activated by members of the Janus Kinase family (JAK) and dysregulation of this pathway is frequently observed in primary tumors and leads to increased angiogenesis and enhanced survival of tumors.

Activation of Stat3 is tightly regulated. Interleukin-6 (IL-6) binding to its receptor induces the homodimerisation of the gp130 IL-6 transducer, which then leads to phosphorylation of the JAKs. The JAKs induces phosphorylation of STAT3 tyrosine-705, which leads to its dimerisation. Stat3 subsequently translocates to the nucleus, where it binds to SIE/GAS elements in the promoters of target genes, thus, activating transcription. The proteins encoded by these Stat3 activated genes lead to the carcinogenic effects observed in cancer cells (7).

Stat3 contributes to tumorigenesis in several ways, including through stimulation of cell division, angiogenesis, and metastasis as well as inhibition of apoptosis (5).

Furthermore, Stat3 has a direct inhibitory effect on the transcription of one of the most important defences against cancer, p53 (6).

There have been several reports that anti-apoptotic genes are at least partly regulated Stat3, including Bcl-2, Bcl-xL, and Mcl-1 (13,14). Interestingly, knock-down of Bcl-2 with an antisense oligonucleotide can lead to enhanced sensitivity of the cells to

dexamethasone-induced apoptosis, suggesting that inactivation of Stat3 may also sensitize cells to exposure to apoptosis-inducing drugs (15). The importance of the JAK/Stat pathway in the survival and proliferation of myeloma is also apparent by the number of ways that this pathway is altered. It has been shown that approximately 48% of multiple myeloma patients have a constitutively active form of Stat3 (16).

Thus, Stat3 is an oncogene and is constitutively active in several cancer types, including ovarian, breast, prostate, lung, renal, colon, gastric and cervical cancers (1). Since Stat3 is considered an important contributor to the oncogenic transformation in a number of cancer types, Stat3 is a putative molecular target in cancer therapy (1, 5-7).

The term "Stat3", as used herein, refers to any form of Stat3 known to those of skill in the art, including, but not limited to, Stat3 α and Stat3 β , as well as modified Stat3 forms, such as phosphorylated and dephosphorylated Stat3.

The term "cells having elevated Stat3 activity" as used herein, refers to cells in which Stat3 is constitutively activated (e.g. phosphorylated) or cells in which Stat3 is activated for a greater percentage of time or at a higher level than is found in normal (i.e. non-diseased) cells. In particular, elevated Stat3 activity comprises an increase in the activity of Stat3 as compared with Stat3 activity in normal (i.e. non-diseased) cells of 5-10000%. More preferably the elevated Stat3 activity exceed the Stat3 activity in normal cells by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 550%, 600%, 650%, 700%, 750%, 800%, 850%, 900%, 950%, 1000%, 1500%, 2000%, 2500%, 3000%, 3500%, 4000%, 4500%, 5000%, 5500%, 6000%, 6500%, 7000%, 7500%, 8000%, 8500%, 9000%, 9500% or 10000%.

The activity of Stat3 can be detected by methods known to those of skill within the art. Examples of such detection methods are immunoblotting and Stat3 transcription factor assay, as described elsewhere herein. Furthermore, Stat3 activity may be determined by measurement of the expression levels of Stat3 dependent genes by techniques, which are known to people skilled within the art, and includes DNA array technologies, northern blotting, western blotting, southern blotting, immunostaining, PCR techniques, including real-time PCR and quantitative PCR and so forth.

Treatment of disorders

The present invention relates to a method for treating, ameliorating, or preventing a disorder by administration of a therapeutically effective amount of at least one inhibitor of folate receptor α to an animal including a human being. In a preferred embodiment, said disorder is a hyperproliferative disorder.

In one embodiment, the methods, compounds, folate receptor α inhibitors, compositions, uses and kits comprise inhibiting Stat3 activity in a cell by contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.

The terms "treating" or "treatment", as used herein, refers to treatment, amelioration and prevention of a specific disease as defined herein. Thus, treatment also comprises prophylactic treatment (i.e. prevention) of a cell or an animal, including a human being, in which the pathologic and/or physical phenotype is not yet apparent.

The terms "prevent," "preventing," and "prevention", as used herein, refer to a decrease in the occurrence of pathological cells (e.g., hyperproliferative or neoplastic cells) in an animal. The prevention may be complete, e.g., the total absence of pathological cells in a subject. The prevention may also be partial, such that the occurrence of pathological cells in a subject is less than that which would have occurred without the present invention. Prevention also refers to reduced susceptibility to a clinical condition.

The term "therapeutically effective amount," as used herein, refers to the amount of therapeutic agent sufficient to result in amelioration of one or more symptoms of a disorder, or prevent advancement of a disorder, or cause regression of the disorder. Regarding to the treatment of cancer, for example, a therapeutically effective amount preferably refers to the amount of a therapeutic agent that decreases the rate of tumor growth, decreases tumor mass, decreases the number of metastases, increases time to tumor progression, or increases survival time by at least 5%, preferably at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 100%.

The term "hyperproliferative disorder," as used herein, refers to any condition in which a localized population of proliferating cells in an animal is not regulated by the usual limitations of normal growth. Thus, hyperproliferative disorders are derived from cells, which proliferate at a higher rate than normal. The higher proliferation rate may result in a disease phenotype; however, the disease phenotype does not need to be manifested physically for an animal to be in need of treatment according to the present invention.

Examples of hyperproliferative disorders include tumors, neoplasms, and lymphomas. A neoplasm is said to be benign if it does not undergo invasion or metastasis, while an invasive and metastase forming neoplasm is said to be malignant. A metastatic cell is a cell that can invade and destroy neighboring body structures. Hyperplasia is a form of cell proliferation involving an increase in cell number in a tissue or organ without significant alteration in structure or function. Metaplasia is a form of controlled cell growth in which one type of fully differentiated cell substitutes for another type of differentiated cell.

In addition to cancer, several other hyperproliferative disorders exist. These hyperproliferative disorders are caused by non-cancerous (i.e. non-neoplastic) cells that overproduce in response to a particular growth factor. Examples of such hyperproliferative disorders include diabetic retinopathy, endometriosis, macular degenerative disorders and benign growth disorders such as prostate enlargement and lipomas. Hyperproliferative disorders also comprise autoimmune diseases, such as a variety of skin disorders, including psoriasis.

Many cancer cells display increased levels of folate receptor α , and thus, one embodiment of the present invention relates to the treatment of FR α positive cells, including FR α positive cancer cells by inhibition of FR α . The term "folate receptor α positive cell", as used herein designates a cell in which the level of functional FR α is increased compared with a normal (i.e. non-diseased) cell. In particular, the term "increased FR α level" comprises an increase in the level of FR α as compared with FR α levels in normal (i.e. non-diseased) cells of at least between 5-10000%. Most preferably, the increased FR α level exceed the FR α level in normal cells by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 550%, 600%, 650%, 700%, 750%, 800%, 850%, 900%,

950%, 1000%, 1500%, 2000%, 2500%, 3000%, 3500%, 4000%, 4500%, 5000%, 5500%, 6000%, 6500%, 7000%, 7500%, 8000%, 8500%, 9000%, 9500% or 10000%.

The level of FR α can be detected by methods known to those of skill within the art.

5 Examples of such detection methods include techniques for detecting the transcriptional and translational expression of FR α , said techniques including immunostaining, FACS, fluorescent in situ hybridization, DNA array technologies, northern blotting, western blotting, southern blotting, PCR techniques, including real-time PCR and quantitative PCR and so forth.

10

The methods, compounds, folate receptor α inhibitors, compositions, uses and kits are particularly suitable for disorders that are associated with an increase in STAT3 activity. In one embodiment, the STAT3 activity is increased by at least 10 %, such as at least 20 %, for example at least 30 %, such as at least 40 %, for example at least 50
15 %, such as at least 60 %, for example at least 70 %, such as at least 80 %, for example at least 90 %, such as at least 100 %, such as at least 200 %, for example at least 300 %, such as at least 400 %, for example at least 500 %, such as at least 600 %, for example at least 700 %, such as at least 800 %, for example at least 900 %, such as at least 1000 %. In one embodiment, the disorder is associated with 50-100 % increase in
20 Stat3 activity.

20

STAT3 activation may be observed by analysing STAT3 phosphorylation. Thus, in one embodiment, the methods, compounds, folate receptor α inhibitors, compositions, uses and kits of the present invention are suitable for disorders that are associated with an
25 increase in STAT3 phosphorylation. In one embodiment, the STAT3 phosphorylation is increased by at least 10 %, such as at least 20 %, for example at least 30 %, such as at least 40 %, for example at least 50 %, such as at least 60 %, for example at least 70 %, such as at least 80 %, for example at least 90 %, such as at least 100 %, such as at least 200 %, for example at least 300 %, such as at least 400 %, for example at least
30 500 %, such as at least 600 %, for example at least 700 %, such as at least 800 %, for example at least 900 %, such as at least 1000 %. In one embodiment, the disorder is associated with a 50-100 % increase in phosphorylated Stat3.

30

Hyperproliferative disorders which can be treated by the methods, compounds, folate
35 receptor α inhibitors, compositions, uses and kits of the present invention include

35

hyperproliferative diseases and/or disorders, such as any number of cancers.

Generally, such cancers include, but is not limited to, cancers of the bladder, brain, breast, cervix, colon, endometrium, esophagus, head and neck, kidney, larynx, liver, lung, oral cavity, ovaries, pancreas, prostate, skin, stomach, and testis. Certain of

5 these cancers may be more specifically referred to as acute and chronic lymphocytic leukemia, acute granulocytic leukemia, adrenal cortex carcinoma, bladder carcinoma, breast carcinoma, cervical carcinoma, cervical hyperplasia, choriocarcinoma, chronic granulocytic leukemia, chronic lymphocytic leukemia, colon carcinoma, endometrial carcinoma, esophageal carcinoma, essential thymocyto-
10 hairy cell leukemia, head and neck carcinoma, Hodgkin's disease, Kaposi's sarcoma, lung carcinoma, lymphoma, malignant carcinoid carcinoma, malignant hypercalcemia, malignant melanoma, malignant pancreatic insulinoma, medullary thyroid carcinoma, melanoma, multiple myeloma, mycosis fungoides, myeloid and lymphocytic leukemia, neuroblastoma, non-Hodgkin's lymphoma, osteogenic sarcoma, ovarian carcinoma,
15 pancreatic carcinoma, polycythemia Vera, primary brain carcinoma, primary macroglobulinemia, prostatic carcinoma, renal cell carcinoma, rhabdomyosarcoma, skin cancer, small-cell lung carcinoma, soft-tissue sarcoma, squamous cell carcinoma, stomach carcinoma, testicular carcinoma, thyroid carcinoma, and Wilms' tumor. In one embodiment, the cancer is a solid tumor. In another embodiment, the cancer is
20 selected from the group consisting of colon cancer, brain cancer, glioma, multiple myeloma, head and neck cancer (except for esophageal cancer), hepatocellular cancer, melanoma, ovarian cancer, cervical cancer, renal cancer, and non-small cell lung cancer.

25 In a preferred embodiment, the disorders which can be treated by methods, compounds, folate receptor α inhibitors, compositions, uses and kits of the present invention are selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, lung cancer, renal cancer, colon cancer, gastric cancer, and cervical cancer. In another embodiment the disorders which can be treated with the compounds
30 of the present invention can be selected from the group consisting of breast cancer, ovarian cancer, lung cancer, and cervical cancer. In a specifically preferred embodiment the disorder is breast cancer. In another preferred embodiment the disorder is prostate cancer.

In another embodiment the hyperproliferative disorder which can be treated by the methods, compounds, folate receptor α inhibitors, compositions, uses and kits of the present invention is psoriasis. In yet a further embodiment the hyperproliferative disorder is head and neck squamous cell carcinoma (HNSCC).

5

The present invention also relates to a method, a compound, a folate receptor α inhibitor, a composition, a use and/or a kit for inducing apoptosis and/or cell cycle arrest in a cell, comprising contacting the cell with a therapeutically effective amount of folate receptor α inhibitor. Moreover, the invention relates to a method, a compound, a
10 folate receptor α inhibitor, a composition, a use and/or a kit for rendering a cell sensitive to an inducer of apoptosis, comprising contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.

In some embodiments, the methods, compounds, compositions, uses, and kits of the
15 present invention are used to treat diseased cells, tissues, organs, or pathological conditions and/or disease states in an animal (e.g. a mammalian subject including, but not limited to, humans and veterinary animals). In this regard, various diseases and pathologies are amenable to treatment or prophylaxis using the present methods and compositions. A non-limiting exemplary list of these diseases and conditions includes
20 without restriction, breast cancer, prostate cancer, lymphoma, skin cancer, pancreatic cancer, colon cancer,
melanoma, malignant melanoma, ovarian cancer, brain cancer, primary brain carcinoma, head-neck cancer, glioma, glioblastoma, liver cancer, bladder cancer, non-small cell lung cancer, head or neck carcinoma, breast carcinoma, ovarian carcinoma,
25 lung carcinoma, small-cell lung carcinoma, Wilms' tumor, cervical carcinoma, testicular carcinoma, bladder carcinoma, pancreatic carcinoma, stomach carcinoma, colon carcinoma, prostatic carcinoma, genitourinary carcinoma, thyroid carcinoma, esophageal carcinoma, myeloma, multiple myeloma, adrenal carcinoma, renal cell carcinoma, endometrial carcinoma, adrenal cortex carcinoma, malignant pancreatic
30 insulinoma, malignant carcinoid carcinoma, choriocarcinoma, mycosis fungoides, malignant hypercalcemia, cervical hyperplasia, leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, chronic granulocytic leukemia, acute granulocytic leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, polycythemia Vera,
35 essential thrombocytosis, Hodgkin's disease, non-Hodgkin's lymphoma, soft-tissue

sarcoma, osteogenic sarcoma, primary macroglobulinemia, and retinoblastoma, and the like, T and B cell mediated autoimmune diseases; inflammatory diseases; infections; hyperproliferative diseases; AIDS; degenerative conditions, vascular diseases, and the like. In one embodiment, the cancer is breast cancer or ovarian cancer. In some embodiments, the cancer cells being treated are metastatic. In other
5 embodiments, the cancer cells being treated are resistant to anticancer agents.

The terms "anticancer agent" and "anticancer drug" as used herein, refer to any therapeutic agents (e.g., chemotherapeutic compounds and/or molecular therapeutic
10 compounds), antisense therapies, radiation therapies, or surgical interventions, used in the treatment of hyperproliferative diseases including cancer.

In some embodiments, infections suitable for treatment by a method, a compound, a folate receptor α inhibitor, a composition, a use and/or a kit of the present invention
15 include, but are not limited to, infections caused by viruses, bacteria, fungi, mycoplasma, prions, and the like.

The present invention also relates to folate receptor α inhibitor for use as a medicament. Thus, the folate receptor α inhibitors of the present invention or folate
20 receptor α inhibitors identified by the methods of the present invention are also claimed for use as a medicament. In a particular embodiment, said use is intended for the manufacture of a medicament for the treatment of any disorder as defined elsewhere herein, however, preferably a hyperproliferative disorder such as cancer.

25 *Combination with other therapies*

The methods, compounds, folate receptor α inhibitors, compositions, uses or kits may in some cases be combined with additional therapies. In one such embodiment, the methods, compounds, folate receptor α inhibitors, compositions, uses or kits according to the present invention further comprise one or more other therapies against cancer.
30 Such therapies include but are not limited to surgery, chemotherapy, radiotherapy, gene therapy, therapy with cytokines and immunotherapy. In a specific embodiment, the methods, compounds, folate receptor α inhibitors, compositions, uses or kits of the present invention further comprises contacting the cell with an inducer of apoptosis. Examples of an inducer of apoptosis are without restriction chemotherapeutic agents
35 and/or radiation.

Thus, an additional aspect of the present invention is a method for treating, ameliorating, or preventing hyperproliferative disorders in an animal comprising administering to the animal a therapeutically effective amount of a folate receptor α inhibitor in combination with one or more active agents or treatments, e.g. chemotherapeutic agents or treatments.

Therefore, some embodiments of the present invention provide methods, compounds, folate receptor α inhibitors, compositions, uses or kits for administering an effective amount of a compound of the present invention and at least one additional therapeutic agent (including, but not limited to, chemotherapeutic antineoplastics, apoptosis modulating agents, antimicrobials, antivirals, antifungals, and anti-inflammatory agents) and/or therapeutic technique (e.g., surgical intervention, and/or radiotherapies).

In preferred embodiments of the invention, the at least one additional therapeutic agent can be any chemotherapeutic agent which is used, has been used, or is known to be useful for the treatment of hyperproliferative disorders.

A number of suitable anticancer agents can be used in the methods of the present invention. These anticancer agents include, but is not limited to, agents that induce apoptosis; polynucleotides (e.g., anti-sense, ribozymes, siRNA); polypeptides (e.g., enzymes and antibodies); biological mimetics (e.g., gossypol or BH3 mimetics); agents that bind (e.g., oligomerize or complex) with a Bcl-2 family protein such as Bax; alkaloids; alkylating agents; antitumor antibiotics; antimetabolites; hormones; platinum compounds; monoclonal or polyclonal antibodies (e.g., antibodies conjugated with anticancer drugs, toxins, defensins), toxins; radionuclides; biological response modifiers (e.g., interferons (e.g., IFN- α) and interleukins (e.g., IL-2)); adoptive immunotherapy agents; hematopoietic growth factors; agents that induce tumor cell differentiation (e.g., all-trans-retinoic acid); gene therapy reagents (e.g., antisense therapy reagents and nucleotides); tumor vaccines; angiogenesis inhibitors; proteasome inhibitors; NF- κ B modulators; anti-CDK compounds; HDAC inhibitors; and the like. Numerous other examples of chemotherapeutic compounds and anticancer therapies suitable for coadministration with the disclosed compounds are known to those skilled in the art.

In preferred embodiments, anticancer agents comprise agents that induce or stimulate apoptosis. Agents that induce apoptosis include, but are not limited to, radiation (e.g., X-rays, gamma rays, UV); tumor necrosis factor (TNF)-related factors (e.g., TNF family receptor proteins, TNF family ligands, TRAIL, antibodies to TRAILRI or TRAILR2);

5 kinase inhibitors (e.g., epidermal growth factor receptor (EGFR) kinase inhibitor, vascular growth factor receptor (VGFR) kinase inhibitor, fibroblast growth factor receptor (FGFR) kinase inhibitor, platelet-derived growth factor receptor (PDGFR) kinase inhibitor, and Bcr-Abl kinase inhibitors (such as GLEEVEC)); antisense molecules; antibodies (e.g., HERCEPTIN, RITUXAN, ZEVALIN, and AVASTIN); anti-

10 estrogens (e.g., raloxifene and tamoxifen); antiandrogens (e.g., flutamide, bicalutamide, finasteride, aminoglutethamide, ketoconazole, and corticosteroids); cyclooxygenase 2 (COX-2) inhibitors (e.g., celecoxib, meloxicam, NS-398, and non-steroidal anti-inflammatory drugs (NSAIDs)); anti-inflammatory drugs (e.g., butazolidin, DECADRON, DELTASONE, dexamethasone, dexamethasone intensol, DEXONE,

15 HEXADROL, hydroxychloroquine, METICORTEN, ORADEXON, ORASONE, oxyphenbutazone, PEDIAPRED, phenylbutazone, PLAQUENIL, prednisolone, prednisone, PRELONE, and TANDEARIL); and cancer chemotherapeutic drugs (e.g., irinotecan (CAMPTOSAR), CPT-11, fludarabine (FLUDARA), dacarbazine (DTIC), dexamethasone, mitoxantrone, MYLOTARG, VP-16, cisplatin, carboplatin, oxaliplatin,

20 5-FU, doxorubicin, gemcitabine, bortezomib, gefitinib, bevacizumab, TAXOTERE or TAXOL); cellular signaling molecules; ceramides and cytokines; staurosporine, and the like.

In still other embodiments, the methods of the present invention provide at least one

25 inhibitor of folate receptor α and at least one antihyperproliferative or antineoplastic agent selected from alkylating agents, antimetabolites, and natural products (e.g., herbs and other plant and/or animal derived compounds).

Alkylating agents suitable for use in the present invention include, but are not limited to:

30 1) nitrogen mustards (e.g., mechlorethamine, cyclophosphamide, ifosfarnide, melphalan (L-sarcolysin); and chlorambucil); 2) ethylenimines and methylmelamines (e.g., hexamethylmelamine and thiotepa); 3) alkyl sulfonates (e.g., busulfan); 4) nitrosoureas (e.g., carmustine (BCNU); lomustine (CCNU); semustine (methyl-CCNU); and streptozocin (streptozotocin)); and 5) triazenes (e.g., dacarbazine

35 (DTIC; dimethyltriazenoimidazolecarboxamide).

In some embodiments, antimetabolites suitable for use in the present compositions and methods include, but are not limited to: 1) folic acid analogs (e.g., methotrexate (amethopterin)); 2) pyrimidine analogs (e.g., fluorouracil (5-fluorouracil; 5-FU),
5 floxuridine (fluorode-oxyuridine; FudR), and cytarabine (cytosine arabinoside)); and 3) purine analogs (e.g., mercaptopurine (6-mercaptopurine; 6-MP), thioguanine (6-thioguanine; TG), and pentostatin (2'-deoxycoformycin)).

In still further embodiments, chemotherapeutic agents suitable for use in the present
10 invention include, but are not limited to: 1) vinca alkaloids (e.g., vinblastine (VLB), vincristine); 2) epipodophyllotoxins (e.g., etoposide and teniposide); 3) antibiotics (e.g., dactinomycin (actinomycin D), daunorubicin (daunomycin; rubidomycin), doxorubicin, bleomycin, plicamycin (mithramycin), and mitomycin (mitomycin C)); 4) enzymes (e.g., L-asparaginase); 5) biological response modifiers (e.g., interferon-alfa); 6) platinum
15 coordinating complexes (e.g., cisplatin (cis-DDP) and carboplatin); 7) anthracenediones (e.g., mitoxantrone); 8) substituted ureas (e.g., hydroxyurea); 9) methylhydrazine derivatives (e.g., procarbazine (N-methylhydrazine; MM)); 10) adrenocortical suppressants (e.g., mitotane (o,p'-DDD) and aminoglutethimide); 11) adrenocorticosteroids (e.g., prednisone); 12) progestins (e.g., hydroxyprogesterone
20 caproate, medroxyprogesterone acetate, and megestrol acetate); 13) estrogens (e.g., diethylstilbestrol and ethinyl estradiol); 14) antiestrogens (e.g., tamoxifen); 15) androgens (e.g., testosterone propionate and fluoxymesterone); 16) antiandrogens (e.g., flutamide); and 17) gonadotropin-releasing hormone analogs (e.g., leuprolide).

25 In a preferred embodiment, the present invention relates to a method of rendering a cell sensitive to an inducer of apoptosis, comprising contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.

The terms "rendering sensitive", "sensitize" and "sensitizing", as used herein, refer to
30 by administering a first agent increasing the susceptibility and/or responsiveness of an animal or a cell within an animal towards the biological effects, such as promotion or retardation of an aspect of cellular function including, but not limited to, cell growth, proliferation, invasion, angiogenesis, or apoptosis, of another agent. The sensitizing effect of a first agent on a target cell can be measured as the difference in the intended
35 biological effect (e.g., promotion or retardation of an aspect of cellular function

including, but not limited to, cell growth, proliferation, invasion, angiogenesis, or apoptosis) observed upon the administration of a second agent with and without administration of the first agent. The response of the sensitized cell can be increased by at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, at least 150%, at least 200%, at least 350%, at least 300%, at least 350%, at least 400%, at least 450%, or at least 500% over the response in the absence of the first agent.

Therefore, an important feature of the present invention is that inhibitors of folate receptor α can inhibit cell growth, at least in part by inducing cell cycle arrest and/or apoptosis, and can also reinforce the induction of cell cycle arrest and/or apoptosis in response to apoptosis induction signals. It is contemplated that these inhibitors of folate receptor α sensitize cells to inducers of apoptosis, including cells that have acquired resistance to such inducers. The folate receptor α inhibitors of the present invention can be used to induce apoptosis in any disorder that can be treated, ameliorated, or prevented by the induction of apoptosis. In one embodiment, the inhibitors can be used to induce apoptosis in cells having elevated Stat3 activity.

The term "apoptosis" define the cellular process of programmed cell death. Programmed cell death as signalled by the nuclei in normally functioning human and animal cells occur when age or state of cell health and condition dictates. Orderly cell death is characterized by slow dissolving and reuse of cell parts by neighbouring tissue. Some chemotherapy drugs induce apoptosis, while others cause cell lysis or bursting.

In one embodiment, the invention pertains to modulating an apoptosis associated state which is associated with one or more apoptosis modulating agents, including apoptosis inducing agents. Examples of apoptosis modulating agents include, but are not limited to, FasCD95, TRAMP, TNF RI, DR1, DR2, DR3, DR4, DR5, DR6, FADD, RIP, TNFa, Fas ligand, TRAIL, antibodies to TRAILRI or TRAILR2, Bcl-2, p53, BAX, BAD, Akt, CAD, PI3 kinase, PP1, and caspase proteins. Other agents involved in the initiation, decision and degradation phase of apoptosis are also included. Examples of apoptosis modulating agents include agents, the activity, presence, or change in concentration of which can modulate apoptosis in a subject. Preferred apoptosis modulating agents are inducers of

apoptosis, such as TNF or a TNF-related ligand, particularly a TRAMP ligand, a Fas/CD95 ligand, a TNFR-1 ligand, or TRAIL.

5 The term "apoptosis modulating agents," as used herein, refers to agents which are involved in modulating (e.g., inhibiting, decreasing, increasing, promoting) apoptosis. Examples of apoptosis modulating agents include proteins which comprise a death domain such as, but not limited to, Fas/CD95, TRAMP, TNF RI, DR1, DR2, DR3, DR4, DR5, DR6, FADD, and RIP. Other examples of apoptotic modulating agents include, but are not limited to, TNF α , Fas ligand, antibodies to Fas/CD95 and other TNF family
10 receptors, TRAIL, antibodies to TRAILRI or TRAILR2, Bcl-2, p53, BAX, BAD, Akt, CAD, PI3 kinase, PP1, and caspase proteins. Modulating agents broadly include agonists and antagonists of TNF family receptors and TNF family ligands. Apoptosis modulating agents may be soluble or membrane bound (e.g. ligand or receptor). Preferred apoptosis modulating agents are inducers of apoptosis, such as TNF or a TNF-related
15 ligand, particularly a TRAMP ligand, a Fas/CD95 ligand, a TNFR-1 ligand, or TRAIL.

One embodiment of the present invention pertains to the administration of an effective amount of folate receptor α inhibitor in combination with one or more radiotherapeutic agents or treatments. In preferred embodiments of the invention, the one or more
20 radiotherapeutic agents or treatments can be external-beam radiation therapy, brachytherapy, therrnotherapy, radiosurgery, charged-particle radiotherapy, neutron radiotherapy, photodynamic therapy, or radionuclide therapy.

The present invention provides methods, compounds, folate receptor α inhibitors, compositions, uses and kits for administering a compound of the invention with
25 radiation therapy. The invention is not limited by the types, amounts, or delivery and administration systems used to deliver the therapeutic dose of radiation to an animal. For example, the animal may receive photon radiotherapy, particle beam radiation therapy, other types of radiotherapies, and combinations thereof. In some
30 embodiments, the radiation is delivered to the animal using a linear accelerator. In still other embodiments, the radiation is delivered using a gamma knife.

The source of radiation can be external or internal to the animal. External radiation therapy is most common and involves directing a beam of high-energy radiation to a
35 tumor site through the skin using, for instance, a linear accelerator. While the beam of

radiation is localized to the tumor site, it is nearly impossible to avoid exposure of normal, healthy tissue. However, external radiation is usually well tolerated by patients. Internal radiation therapy involves implanting a radiation-emitting source, such as beads, wires, pellets, capsules, particles, and the like, inside the body at or near the tumor site including the use of delivery systems that specifically target cancer cells (e.g., using particles attached to cancer cell binding ligands). Such implants can be removed following treatment, or left in the body inactive. Types of internal radiation therapy include, but are not limited to, brachytherapy, interstitial irradiation, intracavity irradiation, radioimmunotherapy, and the like.

The animal may optionally receive radiosensitizers (e.g., metronidazole, misonidazole, intra-arterial Budr, intravenous iododeoxyuridine (IudR), nitroimidazole, 5-substituted-4-nitroimidazoles, 2Hisoindolediones, [[(2-bromoethyl)-amino]methyl]-nitro- 1H-imidazole- 1- ethanol, nitroaniline derivatives, DNA-affinic hypoxia selective cytotoxins, halogenated DNA ligand, 1,2,4 benzotriazine oxides, 2-nitroimidazole derivatives, fluorine-containing nitroazole derivatives, benzamide, nicotinamide, acridine-intercalator, 5-thiotetrazole derivative, 3-nitro- 1,2,4-triazole, 4,5-dinitroimidazole derivative, hydroxylated texaphrins, cisplatin, mitomycin, tiripazamine, nitrosourea, mercaptopurine, methotrexate, fluorouracil, bleomycin, vincristine, carboplatin, epirubicin, doxorubicin, cyclophosphamide, vindesine, etoposide, paclitaxel, heat (hyperthermia), and the like), radioprotectors (e.g., cysteamine, aminoalkyl dihydrogen phosphorothioates, amifostine (WR 2721), IL-1, IL-6, and the like). Radiosensitizers enhance the killing of tumor cells. Radioprotectors protect healthy tissue from the harmful effects of radiation.

Any type of radiation can be administered to a patient, so long as the dose of radiation is tolerated by the patient without unacceptable negative side-effects. Suitable types of radiotherapy include, for example, ionizing (electromagnetic) radiotherapy (e.g., X-rays or gamma rays) or particle beam radiation therapy (e.g., high linear energy radiation). Ionizing radiation is defined as radiation comprising particles or photons that have sufficient energy to produce ionization, i.e., gain or loss of electrons (as described in, for example, U.S. 5,770,581 incorporated herein by reference in its entirety). The effects of radiation can be at least partially controlled by the clinician. The dose of radiation is preferably fractionated for maximal target cell exposure and reduced toxicity.

The total dose of radiation administered to an animal preferably is about .01 Gray (Gy) to about 100 Gy. More preferably, about 10 Gy to about 65 Gy (e.g., about 15 Gy, 20 Gy, 25 Gy, 30 Gy, 35 Gy, 40 Gy, 45 Gy, 50 Gy, 55 Gy, or 60 Gy) are administered over the course of treatment. While in some embodiments a complete dose of radiation can be administered over the course of one day, the total dose is ideally fractionated and administered over several days. Desirably, radiotherapy is administered over the course of at least about 3 days, e.g., at least 5, 7, 10, 14, 17, 21, 25, 28, 32, 35, 38, 42, 46, 52, or 56 days (about 1-8 weeks). Accordingly, a daily dose of radiation will comprise approximately 1-5 Gy (e.g., about 1 Gy, 1.5 Gy, 1.8 Gy, 2 Gy, 2.5 Gy, 2.8 Gy, 3 Gy, 3.2 Gy, 3.5 Gy, 3.8 Gy, 4 Gy, 4.2 Gy, or 4.5 Gy), preferably 1-2 Gy (e.g., 1.5-2 Gy). The daily dose of radiation should be sufficient to induce destruction of the targeted cells. If stretched over a period, radiation preferably is not administered every day, thereby allowing the animal to rest and the effects of the therapy to be realized. For example, radiation desirably is administered on 5 consecutive days, and not administered on 2 days, for each week of treatment, thereby allowing 2 days of rest per week. However, radiation can be administered 1 day/week, 2 days/week, 3 days/week, 4 days/week, 5 days/week, 6 days/week, or all 7 days/week, depending on the animal's responsiveness and any potential side effects. Radiation therapy can be initiated at any time in the therapeutic period. Preferably, radiation is initiated in week 1 or week 2, and is administered for the remaining duration of the therapeutic period. For example, radiation is administered in weeks 1-6 or in weeks 2-6 of a therapeutic period comprising 6 weeks for treating, for instance, a solid tumor. Alternatively, radiation is administered in weeks 1-5 or weeks 2-5 of a therapeutic period comprising 5 weeks. These exemplary radiotherapy administration schedules are not intended, however, to limit the present invention.

Antimicrobial therapeutic agents may also be used as therapeutic agents in the present invention. Any agent that can kill, inhibit, or otherwise attenuate the function of microbial organisms may be used, as well as any agent contemplated to have such activities. Antimicrobial agents include, but are not limited to, natural and synthetic antibiotics, antibodies, inhibitory proteins (e.g., defensins), antisense nucleic acids, membrane disruptive agents and the like, used alone or in combination. Indeed, any type of antibiotic may be used including, but not limited to, antibacterial agents, antiviral agents, antifungal agents, and the like.

In some embodiments of the present invention, the folate receptor α inhibitor or a derivative, analog, prodrug, or pharmaceutically acceptable salt thereof and one or more therapeutic agents or anticancer agents are administered to an animal under one or more of the following conditions: at different periodicities, at different durations, at different concentrations, by different administration routes, etc. In some embodiments, the inhibitor is administered prior to the therapeutic or anticancer agent, e.g., 0.5, 1, 2, 3, 4, 5, 10, 12, or 18 hours, 1, 2, 3, 4, 5, or 6 days, or 1, 2, 3, or 4 weeks prior to the administration of the therapeutic or anticancer agent. In some embodiments, the inhibitor is administered after the therapeutic or anticancer agent, e.g., 0.5, 1, 2, 3, 4, 5, 10, 12, or 18 hours, 1, 2, 3, 4, 5, or 6 days, or 1, 2, 3, or 4 weeks after the administration of the anticancer agent. In some embodiments, the inhibitor and the therapeutic or anticancer agent are administered concurrently but on different schedules, e.g., the inhibitor is administered daily while the therapeutic or anticancer agent is administered once a week, once every two weeks, once every three weeks, or once every four weeks. In other embodiments, the compound is administered once a week while the therapeutic or anticancer agent is administered daily, once a week, once every two weeks, once every three weeks, or once every four weeks.

20 *Compositions and administration*

Also within the scope of the present invention are pharmaceutical compositions comprising a pharmaceutically effective amount of folate receptor α inhibitor and a pharmaceutically acceptable carrier. In one embodiment, said folate receptor α inhibitor is capable of binding to a polypeptide comprising at least one region of selected from SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 or part thereof, thereby inhibiting binding of folate receptor α agonist. In one example, said agonist is selected from the group consisting of foline/folate, folic acid and derivatives thereof.

The invention also provide the pharmaceutical composition as defined herein for treatment of a disorder as defined elsewhere herein, preferably hyperproliferative disorders such as cancer. Preferably, the folate receptor α inhibitor of the pharmaceutical composition is as defined elsewhere herein, or identified by a method as defined elsewhere herein.

Thus, compositions within the scope of this invention include all compositions, wherein the compounds, such as a folate receptor α inhibitor, of the present invention are contained in an effective amount. Although the individual needs are variable, the optimal ranges of effective amounts of each component can be determined by a person skilled within the art. Typically, the compounds may be administered to mammals, e.g. humans, orally at a dose of 0.0025 to 50 mg/kg, or an equivalent amount of the pharmaceutically acceptable salt thereof, per day of the body weight of the mammal being treated for disorders responsive to induction of apoptosis. Preferably, about 0.01 to about 10 mg/kg is orally administered to treat, ameliorate, or prevent such disorders. For intramuscular injection, the dose is generally about one-half of the oral dose. For example, a suitable intramuscular dose would be about 0.0025 to about 25 mg/kg, and most preferably, from about 0.01 to about 5 mg/kg.

An oral dose may comprise from about 0.01 to about 1000 mg, preferably about 0.1 to about 100 mg of the compound, such as a folate receptor α inhibitor. Such a unit dose may be administered one or more times daily as one or more tablets or capsules each containing from about 0.1 to about 10 mg, conveniently about 0.25 to 50 mg of the compound or its solvates.

In a topical formulation, the compound may be present at a concentration of about 0.01 to 100 mg per gram of carrier. In a preferred embodiment, the compound is present at a concentration of about 0.07-1.0 mg/ml, more preferably, about 0.1-0.5 mg/ml, most preferably, about 0.4 mg/ml.

The compounds, such as a folate receptor α inhibitor, of the present invention may be administered either as a raw chemical or as part of a pharmaceutical preparation containing suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally or topically and which can be used for the preferred type of administration, such as tablets, dragees, slow release lozenges and capsules, mouth rinses and mouth washes, gels, liquid suspensions, hair rinses, hair gels, shampoos and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection, topically or

orally, contain from about 0.01 to 99 percent, preferably from about 0.25 to 75 percent of active compound(s), together with the excipient.

5 The pharmaceutical compositions of the invention may be administered to any animal in need thereof. Such animals are preferably mammals, e.g., humans, although the invention is not intended to be so limited. Other animals include veterinary animals, such as cows, sheep, pigs, horses, dogs, cats and the like. The compounds and pharmaceutical compositions thereof may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral,
10 subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, buccal, intrathecal, intracranial, intranasal or topical routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

15 The pharmaceutical composition, folate receptor α inhibitors and/or compounds of the present invention may be manufactured in a manner which is known to those of skill in the art. Accordingly, the pharmaceutical preparations may be manufactured by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing
20 processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

25 Suitable excipients are, in particular, fillers such as saccharides, for example lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, as well as binders such as starch paste, using, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium
30 carboxymethylcellulose, and/or polyvinyl pyrrolidone. If desired, disintegrating agents may be added such as the above-mentioned starches and also carboxymethyl-starch, crosslinked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate. Auxiliaries are, above all, flow-regulating agents and lubricants, for example, silica, talc, stearic acid or salts thereof, such as magnesium stearate or calcium
35 stearate, and/or polyethylene glycol. Dragee cores are provided with suitable coatings

which, if desired, are resistant to gastric juices. For this purpose, concentrated saccharide solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropylmethyl-cellulose phthalate, are used. Dye stuffs or pigments may be added to the tablets or dragee coatings, for example, for identification or in order to characterize combinations of active compound doses.

Other pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer such as glycerol or sorbitol. The push-fit capsules can contain the active compounds in the form of granules which may be mixed with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds are preferably dissolved or suspended in suitable liquids, such as fatty oils, or liquid paraffin. In addition, stabilizers may be added.

Possible pharmaceutical preparations which can be used rectally include, for example, suppositories, which consist of a combination of one or more of the active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, watersoluble salts and alkaline solutions. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides or polyethylene glycol-400. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The topical compositions of this invention are formulated preferably as oils, creams, lotions, ointments and the like by choice of appropriate carriers. Suitable carriers include vegetable or mineral oils, white petrolatum (white soft paraffin), branched chain
5 fats or oils, animal fats and high molecular weight alcohol (greater than C12). The preferred carriers are those in which the active ingredient is soluble. Emulsifiers, stabilizers, humectants and antioxidants may also be included as well as agents imparting color or fragrance, if desired. Additionally, transdermal penetration enhancers can be employed in these topical formulations. Examples of such enhancers can be
10 found in U.S. Pat. Nos. 3,989,816 and 4,444,762.

Creams are preferably formulated from a mixture of mineral oil, selfemulsifying beeswax and water in which mixture the active ingredient, dissolved in a small amount of an oil such as almond oil, is admixed. A typical example of such a cream is one
15 which includes about 40 parts water, about 20 parts beeswax, about 40 parts mineral oil and about 1 part almond oil.

Ointments may be formulated by mixing a solution of the active ingredient in a vegetable oil such as almond oil with warm soft paraffin and allowing the mixture to
20 cool. A typical example of such an ointment is one which includes about 30% almond oil and about 70% white soft paraffin by weight.

Lotions may be conveniently prepared by dissolving the active ingredient, in a suitable high molecular weight alcohol such as propylene glycol or polyethylene glycol.
25

The following examples are illustrative, but not limiting, of the method and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in clinical therapy and which are obvious to those skilled in the art are within the spirit and scope of the invention.
30

Kits

The present invention also comprise kits comprising a pharmaceutically effective amount of folate receptor α inhibitor and instructions for administering said compound to an animal including a human being in need thereof.
35

In one embodiment, the kit comprise a folate receptor α inhibitor as defined elsewhere herein, of a folate receptor α inhibitor identified by a method as defined herein.

5 In addition to at least on folate receptor α inhibitor, the kit may also comprise an additional therapeutic agent. Examples of such additional therapeutic agent are without restriction agents provided in chemotherapy, radiotherapy, gene therapy, therapy with cytokines and immunotherapy. In a preferred embodiment, the kit further comprises an inducer of apoptosis, for example a chemotherapeutic agent.

10 The kit may may also be provided with instructions for administering said folate receptor α inhibitor and/or additional agent to an animal having a disease as defined elsewhere herein, preferably a hyperproliferative disorder, such as cancer. Such instructions preferably relates to administration schemes, administration regimes, administration methods, and/or dose recommendations.

15

Methods of identifying folate receptor α inhibitors

Apart from the folate receptor α inhibitors disclosed in the present invention, additional inhibitors may be identified. In one embodiment, such inhibitors are identified from a suitable library on the basis of their ability to bind folate receptor α or a fragment thereof.

20

In a specific aspect, the present invention relates to a method for identifying a compound suitable for folate receptor α inhibition, said method comprising the steps of bringing said compound in contact with a cell comprising the folate receptor α , and detecting the level of phosphorylated Stat3 in the presence and/or absence of said compound, wherein a decrease of phosphorylated Stat3 in the presence of said compound compared with the level of phosphorylated Stat3 in the absence of said compound is indicative of an inhibitory effect of said compound on the folate receptor α .

25
30

Compounds, which may be tested for their ability to bind folate receptor α and/or affects Stat3 activity include antibodies, polypeptides, peptides, peptide fragments, peptide aptamers, nucleic acid aptamers, small molecules, foline analogues, natural single domain antibodies, affibodies, affibody-antibody chimeras, and non-

immunoglobulin folate receptor α inhibitors. In particular, the compounds are the folate receptor α inhibitors as defined elsewhere herein.

- Another method for identifying peptides with affinity for folate receptor α is the phage display technique. Thus, in one aspect, the invention provides a method for identifying a peptide suitable for folate receptor α inhibition, said method comprising the steps of
- a. immobilizing folate receptor α or a fragment thereof on a solid support,
 - b. expressing a peptide library in phages, which display said peptide on its surface,
 - 10 c. bringing said phages in contact with said immobilized folate receptor α or fragment thereof.
 - d. removing unbound phage,
 - e. eluting phages bound to said immobilized folate receptor α or fragment thereof, and
 - 15 f. analysing DNA comprised in said bound phages to obtain sequence of peptide suitable for folate receptor α inhibition.

In a preferred embodiment, the folate receptor α or fragment thereof is a soluble folate receptor α fragment. One soluble fragment is the C-terminal domain of folate receptor α , e.g. amino acids 1-234, wherein the GPI anchor has been cleaved of.

Functional homologues

Functional homologues of peptides/polypeptides according to the present invention are meant to comprise any polypeptide sequence which is capable of inhibiting folate receptor α . In particular, the peptides, compounds and folate receptor α inhibitors of the present invention also comprise any functional homologue thereof.

Functional homologues according to the present invention comprise polypeptides with an amino acid sequence, which are sharing at least some homology with the predetermined polypeptide sequences as outlined herein above. For example such polypeptides are at least about 40 percent, such as at least about 50 percent homologous, for example at least about 60 percent homologous, such as at least about 70 percent homologous, for example at least about 75 percent homologous, such as at least about 80 percent homologous, for example at least about 85 percent

homologous, such as at least about 90 percent homologous, for example at least 92 percent homologous, such as at least 94 percent homologous, for example at least 95 percent homologous, such as at least 96 percent homologous, for example at least 97 percent homologous, such as at least 98 percent homologous, for example at least 99 percent homologous with the predetermined polypeptide sequences as outlined herein above.

The homology between amino acid sequences may be calculated using well known algorithms such as for example any one of BLOSUM 30, BLOSUM 40, BLOSUM 45, BLOSUM 50, BLOSUM 55, BLOSUM 60, BLOSUM 62, BLOSUM 65, BLOSUM 70, BLOSUM 75, BLOSUM 80, BLOSUM 85, and BLOSUM 90.

Functional homologues may comprise an amino acid sequence that comprises at least one substitution of one amino acid for any other amino acid. For example such a substitution may be a conservative amino acid substitution or it may be a non-conservative substitution.

A conservative amino acid substitution is a substitution of one amino acid within a predetermined group of amino acids for another amino acid within the same group, wherein the amino acids within predetermined groups exhibit similar or substantially similar characteristics. Within the meaning of the term "conservative amino acid substitution" as applied herein, one amino acid may be substituted for another within groups of amino acids characterised by having

- i) polar side chains (Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, Tyr, and Cys,)
- ii) non-polar side chains (Gly, Ala, Val, Leu, Ile, Phe, Trp, Pro, and Met)
- iii) aliphatic side chains (Gly, Ala Val, Leu, Ile)
- iv) cyclic side chains (Phe, Tyr, Trp, His, Pro)
- v) aromatic side chains (Phe, Tyr, Trp)
- vi) acidic side chains (Asp, Glu)

- vii) basic side chains (Lys, Arg, His)
- viii) amide side chains (Asn, Gln)
- 5 ix) hydroxy side chains (Ser, Thr)
- x) sulphur-containing side chains (Cys, Met), and
- 10 xi) amino acids being monoamino-dicarboxylic acids or monoamino-monocarboxylic-monoamidocarboxylic acids (Asp, Glu, Asn, Gln).

Non-conservative substitutions are any other substitutions. A non-conservative substitution leading to the formation of a functional homologue would for example i)

15 differ substantially in hydrophobicity, for example a hydrophobic residue (Val, Ile, Leu, Phe or Met) substituted for a hydrophilic residue such as Arg, Lys, Trp or Asn, or a hydrophilic residue such as Thr, Ser, His, Gln, Asn, Lys, Asp, Glu or Trp substituted for a hydrophobic residue; and/or ii) differ substantially in its effect on polypeptide backbone orientation such as substitution of or for Pro or Gly by another residue;

20 and/or iii) differ substantially in electric charge, for example substitution of a negatively charged residue such as Glu or Asp for a positively charged residue such as Lys, His or Arg (and vice versa); and/or iv) differ substantially in steric bulk, for example substitution of a bulky residue such as His, Trp, Phe or Tyr for one having a minor side chain, e.g. Ala, Gly or Ser (and vice versa).

25 Functional homologues according to the present invention may comprise more than one such substitution, such as e.g. two amino acid substitutions, for example three or four amino acid substitutions, such as five or six amino acid substitutions, for example seven or eight amino acid substitutions, such as from 10 to 15 amino acid substitutions,

30 for example from 15 to 25 amino acid substitution, such as from 25 to 30 amino acid substitutions, for example from 30 to 40 amino acid substitution, such as from 40 to 50 amino acid substitutions, for example from 50 to 75 amino acid substitution, such as from 75 to 100 amino acid substitutions, for example more than 100 amino acid substitutions.

The addition or deletion of an amino acid may be an addition or deletion of from 2 to 5 amino acids, such as from 5 to 10 amino acids, for example from 10 to 20 amino acids, such as from 20 to 50 amino acids. However, additions or deletions of more than 50 amino acids, such as additions from 50 to 200 amino acids, are also comprised within the present invention.

The polypeptides according to the present invention, including any variants and functional homologues thereof, may in one embodiment comprise more than 5 amino acid residues, such as more than 10 amino acid residues, for example more than 20 amino acid residues, such as more than 25 amino acid residues, for example more than 50 amino acid residues, such as more than 75 amino acid residues, for example more than 100 amino acid residues, such as more than 150 amino acid residues, for example more than 200 amino acid residues.

Additional factors may be taken into consideration when determining functional homologues according to the meaning used herein. For example functional homologues may be capable of associating with antisera which are specific for the polypeptides according to the present invention.

In a further embodiment the present invention relates to functional equivalents which comprise substituted amino acids having hydrophilic or hydrophobic indices that are within ± 2.5 , for example within ± 2.3 , such as within ± 2.1 , for example within ± 2.0 , such as within ± 1.8 , for example within ± 1.6 , such as within ± 1.5 , for example within ± 1.4 , such as within ± 1.3 for example within ± 1.2 , such as within ± 1.1 , for example within ± 1.0 , such as within ± 0.9 , for example within ± 0.8 , such as within ± 0.7 , for example within ± 0.6 , such as within ± 0.5 , for example within ± 0.4 , such as within ± 0.3 , for example within ± 0.25 , such as within ± 0.2 of the value of the amino acid it has substituted.

The importance of the hydrophilic and hydrophobic amino acid indices in conferring interactive biologic function on a protein is well understood in the art (Kyte & Doolittle, 1982 and Hopp, U.S. Pat. No. 4,554,101, each incorporated herein by reference).

The amino acid hydrophobic index values as used herein are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9);

alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5) (Kyte & Doolittle, 1982).

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The amino acid hydrophilicity values are: arginine (+3.0); lysine (+3.0); aspartate (+3.0.+-.1); glutamate (+3.0.+-.1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5.+-.1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3);

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phenylalanine (-2.5); tryptophan (-3.4) (U.S. 4,554,101).

Substitution of amino acids can therefore in one embodiment be made based upon their hydrophobicity and hydrophilicity values and the relative similarity of the amino acid side-chain substituents, including charge, size, and the like. Exemplary amino acid

15 substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

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In addition to the polypeptide compounds described herein, sterically similar compounds may be formulated to mimic the key portions of the peptide structure and that such compounds may also be used in the same manner as the peptides of the invention. This may be achieved by techniques of modelling and chemical designing known to those of skill in the art. For example, esterification and other alkylations may

20 be employed to modify the amino terminus of, e.g., a di-arginine peptide backbone, to mimic a tetra peptide structure. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

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Peptides with N-terminal alkylations and C-terminal esterifications are also encompassed within the present invention. Functional equivalents also comprise

30 glycosylated and covalent or aggregative conjugates, including dimers or unrelated chemical moieties. Such functional equivalents are prepared by linkage of functionalities to groups which are found in fragment including at any one or both of the N- and C-termini, by means known in the art.

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Functional equivalents may thus comprise fragments conjugated to aliphatic or acyl esters or amides of the carboxyl terminus, alkylamines or residues containing carboxyl side chains, e.g., conjugates to alkylamines at aspartic acid residues; O-acyl derivatives of hydroxyl group-containing residues and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, e.g. conjugates with Met-Leu-Phe. Derivatives of the acyl groups are selected from the group of alkyl-moieties (including C3 to C10 normal alkyl), thereby forming alkanoyl species, and carbocyclic or heterocyclic compounds, thereby forming aroyl species. The reactive groups preferably are difunctional compounds known per se for use in cross-linking proteins to insoluble matrices through reactive side groups.

Homologues of nucleic acid sequences within the scope of the present invention are nucleic acid sequences, which encodes an RNA and/or a protein with similar biological function, and which is either

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a) at least 50% identical, such as at least 60% identical, for example at least 70% identical, such as at least 75% identical, for example at least 80% identical, such as at least 85% identical, for example at least 90% identical, such as at least 95% identical

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b) or able to hybridise to the complementary strand of said nucleic acid sequence under stringent conditions.

Stringent conditions as used herein shall denote stringency as normally applied in connection with Southern blotting and hybridisation as described e.g. by Southern E. M., 1975, J. Mol. Biol. 98:503-517. For such purposes it is routine practise to include steps of prehybridization and hybridization. Such steps are normally performed using solutions containing 6x SSPE, 5% Denhardt's, 0.5% SDS, 50% formamide, 100 µg/ml denaturated salmon testis DNA (incubation for 18 hrs at 42°C), followed by washings with 2x SSC and 0.5% SDS (at room temperature and at 37°C), and a washing with 0.1x SSC and 0.5% SDS (incubation at 68°C for 30 min), as described by Sambrook et al., 1989, in "Molecular Cloning/A Laboratory Manual", Cold Spring Harbor), which is incorporated herein by reference.

Homologous of nucleic acid sequences also encompass nucleic acid sequences which comprise additions and/or deletions. Such additions and/or deletions may be internal or

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at the end. Additions and/or deletions may be of 1-5 nucleotides, such as 5 to 10 nucleotide, for example 10 to 50 nucleotides, such as 50 to 100 nucleotides, for example at least 100 nucleotides.

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Examples

Signal transducer and activator of transcription 3 (Stat3) is a well-described oncogene found constitutively activated in several cancer types (1). Folic acid is a component of the B vitamin complex that, when taken up by cells through the Reduced Folate Carrier (RFC) is essential for normal cell growth and replication (2). In addition to RFC, many cancer cells overexpress a glycoposphatidylinositol (GPI)-anchored Folate Receptor α (FR α) (3). The function of this receptor is still unknown and it has been suggested that transport of folate is not the primary function of this receptor in cancer cells (4). We show that folic acid activates Stat3 through FR α in a Janus Kinase (JAK)-dependent manner and hence propose a carcinogenic function of the FR α . These examples provide results that indicate that folic acid enhance the progression of already existing cancers through a signalling pathway mediated by the FR α .

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Cell Culture

HeLa (epithelial cervical carcinoma cells) (ATCC, CCL-2), HEK293 (embryonic kidney cells) (ATCC, CRL-1573), Ishikawa (endometrial adenocarcinoma cells) (Health Protection Agency, 99040201) and HT1080 (fibrosarcoma cells) (ATCC, CCL-121) cell lines were cultured in DMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. To reduce the level of active components, FBS was incubated with 2% active charcoal overnight (Sigma). Cells pre-treated for 4 hours with folate-free RPMI medium were treated for 25 minutes with IL-6 (Sigma) (1000 U/ml), folic acid (Pteroylglutamic acid) (Sigma) (250 μ g/ml) or folinic acid (N5-formyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid) (Sigma) (250 μ g/ml) in folate-free RPMI medium (Gibco). For dose-response analysis, cells were treated with 125 μ g/ml, 250 μ g/ml or 500 μ g/ml folic acid. For immunoblotting, cells were lysed in 62.5 mM Tris-HCl (pH 7.5), 2% SDS, 10% glycerol with Protease Inhibitor Cocktail P8340 (Sigma) (0,1%). For nuclear extractions, the NE-PER Nuclear and Cytoplasmic Extraction reagent (Pierce) was used and protein concentration was determined by Bio-Rad Protein Assay (Bio-

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Rad). For the AG490-experiments, cells were pre-treated for 30 minutes with 100 μ M JAK-inhibitor AG490 (α -Cyano-(3,4-dihydroxy)-N-benzylcinnamide) (Calbiochem) prior to treatment with IL-6, folic acid or folinic acid. For the receptor blocking experiments, cells were pre-treated for 30 minutes with 3 μ M AG1478 (Calbiochem) to inactivate the EGF receptor, or for 60 minutes with 8 μ g/ml anti-gp130 monoclonal antibody clone 28126 (R&D systems) in order to inactivate the IL-6/gp130 signalling pathway. Before application to SDSPAGE, protein extracts were briefly sonicated.

Immunoblotting

Immunoblotting was done in Tris-buffered saline (TBS) with 0.1 % Tween-20 and 5 % non-fat dry milk as blocking, and TBS with 0.1 % Tween-20 and 5 % BSA as antibody dilution buffer. Dilutions of 1:1000 of the primary antibodies were used: Stat3 (Santa Cruz Biotechnology), phosphoStat3 (Tyr705) (Cell Signalling Technology) and anti-FR α -Mov18/ZEL (Alexis Biochemicals). The incubation with primary antibodies was carried out overnight at 4°C. The secondary antibodies were HRP-conjugated goat-anti-rabbit (DakoCytomation) (1:5000) or goat-anti-mouse (Amersham Biosciences) (1:5000). Proteins were visualised by ECL Plus Western Blotting Detection System (GE Healthcare).

Stat3 Transcription Factor Assay

The Stat3 Transcription Factor Assay (Chemicon) was performed according to the manufacturers instructions. During the assay, the Capture Probe, a double stranded biotinylated oligonucleotide containing the STAT consensus sequences for STAT binding (5'-TTCCCGTAA-3' (hSIE67) and 5'-TTCCGGGAA-3' (ICAM-plRE)) was mixed with the nuclear extract. The mixture was transferred to a streptavidin-coated micro plate immobilizing the biotinylated Capture probe. Bound Stat3 transcription factor subunits were detected with specific Stat3 antibodies, and a HRP secondary antibody was used for detection in a spectrophotometric plate reader. Results from a biotinylated non-specific double stranded oligonucleotide used as a negative control was subtracted.

siRNA assay

HeLa cells were transiently transfected twice with 30 pmol GFP duplex oligo siRNA (Dharmacon) or 30 pmol FR α duplex oligo siRNA (a pool of three siRNAs: 5'-GAA GAA UGC CUG CUG UUC U-3'; 5'-GCA AUG GUG GGA AGA UUG U-3'; 5'-CCA CUG

UUC UGU GCA AUG A-3' from Santa Cruz Biotechnology) with 48 hours interval using Lipofectamine™ 2000 (Invitrogen). After additional 48 hours, the cells were treated for 25 minutes with IL-6 (Sigma) (1000 U/ml), folic acid (Sigma) (250 µg/ml) and folinic acid (Sigma) (250 µg/ml).

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Immunostaining

HeLa and HEK293 were grown on cover slips and fixed with 4 % paraformaldehyde (Bie and Berentsen) for 10 minutes at room temperature. The coverslips were blocked in 0.1 % Tween-20 in PBS with 3 % BSA and then incubated for one hour with anti-FRα-Mov18/ZEL (Alexis Biochemicals) (1:200). Cy3 conjugated anti-mouse IgG (Sigma) (1:250) was used as secondary antibody together with nuclear DAPI (Invitrogen) (1:1000) staining. Cells were visualized with a Zeiss DMR fluorescent microscope and photographed with a Leica DC200 CCD camera. The pictures were acquired and processed by Adobe Photoshop.

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Flow Cytometry

Cells were harvested by trypsinization and incubated for 30 minutes with anti-FRα-Mov18/ZEL (Alexis Biochemicals) (1:500). As a staining and washing buffer, PBS with 1% BSA was used. After incubation with secondary FITC-conjugated Antimouse IgG (Sigma) (1:200) for 20 minutes, the cells were fixed in 2 % PFA and Flow Cytometry was performed using a FASC Calibur Analyser (Becton Dickinson).

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RT-PCR

mRNA was purified from 1×10^4 cells using Dynabeads mRNA DIRECT Micro Kit (DynaL Biotech) and cDNA was prepared (MMLV reverse transcriptase, Epicentre). The PCR primers used were FRα (FOLR1) forward, 5'-ATG GCT CAG CGG ATG ACA AC-3', FRα (FOLR1) reverse, 5'-TCA GCT GAG CAG CCA CAG CA-3', GAPDH forward, 5'-GGT CGG AGT CAA CGG ATT T-3' and GAPDH reverse, 5'-CCA GCA TCG CCC CAC TTG G-3'. The PCRs were carried out in a Peltier Thermal Cycler from Merck Eurolab with 40 cycles of denaturation at 95°C for 10 minutes for the first cycle and then 30 seconds for the following cycles, annealing at 62°C for 30 seconds, and elongation at 72°C for 30 seconds.

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Example 1

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The Stat3 oncogene is constitutively active in several cancer types, for example ovarian, breast, prostate, lung, renal, colon, gastric and cervical cancers (1).

Stat3 contributes to tumorigenesis through stimulation of cell division, angiogenesis, and metastasis as well as inhibition of apoptosis (5). In addition, Stat3 has a direct inhibitory effect on the transcription of one of the most important defences against cancer, p53 (6). Since Stat3 is considered an important contributor to the oncogenic transformation in a number of cancer types, Stat3 is considered to be a putative molecular target in cancer therapy (1, 5-7).

Upon ligand binding to the specific cytokine receptors for interferon (IFN), epidermal growth factor (EGF), and interleukin 6 (IL-6), Stat3 associates with the receptors intracellularly and is phosphorylated on Tyr705 by one of the JAKs. Subsequently, Stat3 dimerizes, migrates to the nucleus and binds to SIE/GAS elements in the promoters of target genes thus activating transcription. The proteins encoded by these Stat3 activated genes lead to the carcinogenic effects observed in cancer cells (7).

Folate is a B vitamin that acts as single-carbon donor and is essential for nucleotide and methionine synthesis. The RFC, present on all cell surfaces, mediates up-take of folates from food, such as vegetables and liver (2). The FR α is highly expressed on the surface of a number of cancer cells including ovarian, endometrial, pancreas, breast and cervical cancers (3, 8). In general, more aggressive forms of cancer express higher amounts of FR α compared with their primary counterparts³. Except from a few fast growing cell types like placenta and colorectal cells⁸ most healthy tissues express non or negligible levels of FR α (3).

Although the precise pathway has not been delineated, FR α has been associated with internalising of folate *via* endocytosis (8, 9). Results, however, indicate that transport of folate is not the primary function of FR α in ovarian cancer cells, since folate-uptake in these cells primarily is mediated by RFC in spite of highly up-regulated FR α (4). The primary function of FR α is still considered unknown, but it has been proposed that FR α might affect cell proliferation *via* cell signalling pathways (8).

In contrast to RFC, FR α has a significantly higher affinity for the synthetic nonreduced

form of folate, folic acid, than for reduced folates (8). The presence of highly upregulated FR α on a number of cancer cell types combined with the high affinity for folic acid has made FR α a possible target for chemo- and immuno cancer therapy as well as for folate-targeted imaging (10).

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Since 1998 the US Food and Drug Administration has required that all flour and uncooked cereal-grain products are fortified with folic acid because of the overwhelming evidence of a protective effect of pre- and periconceptional folic acid supplementation against neural tube defects during pregnancy (11). However, an emerging body of evidence suggests that folic acid supplementation may enhance the development and progression of already existing, and undiagnosed premalignant and malignant lesions (11).

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To assess a potential carcinogenic effect of folic acid supplementation, the effect of folic acid (non-reduced form) and folinic acid (reduced form) on the activation of the Stat3 oncogene in HeLa cells was investigated (Fig. 1A). Interestingly, these results show that Stat3 is phosphorylated on Tyr705 and thus activated upon stimulation with folic acid and folinic acid. Dose-response analyses confirm that the amount of activated Stat3 increases with the concentrations of folic acid (Fig. 1B). In addition, it was also investigated whether Stat3, when activated by folic acid and folinic acid in HeLa cells, could bind to STAT consensus sequences. Figure 1C shows, that pStat3 binds to DNA promoter elements, thus acting as a transcription factor upon activation by both forms of folate.

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Stat3 is normally activated by receptor-mediated phosphorylation (7). It can therefore be speculated whether folic acid could bind to a receptor at the plasma membrane and initiate an intracellular signalling pathway leading to the activation of Stat3. Because FR α binds folic acid with a very high affinity and the function of FR α is largely unknown, it was investigated and confirmed that HeLa cells express FR α on the surface (Fig. 2A + 2C). The following cell lines; HEK293 (Fig. 2), Ishikawa and HT1080 (data not shown) were all tested and found FR α -negative.

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In order to investigate a potential role of FR α in folate-stimulated Stat3-activation, FR α -negative HEK293 cells were treated with folic acid and folinic acid (Fig. 2B). The results

show that Stat3-activity was not induced by folic acid and folinic acid in HEK293 cells, thus confirming that FR α is the mediator of the activation of Stat3 by folates.

To further demonstrate the involvement of FR α in the activation of Stat3 by folic acid, HeLa cells were treated with siRNA against FR α prior to induction with folic acid and folinic acid. Figure 3 shows, that the siRNA effectively blocks the FR α -expression (Fig. 3A + 3B), and inhibits the stimulation of Stat3 by folic acid and folinic acid (Fig. 3C). These results confirm that FR α through binding of folic acid and folinic acid initiates an intracellular signalling pathway leading to the activation of Stat3. A correlating occurrence of upregulated FR α and constitutively active Stat3 in many of the same cancers (1, 3, 8) further supports these results.

To further clarify the pathway for FR-activation of Stat3, the effect of the JAK-inhibitor, AG490, on the activation of Stat3 by folic acid and folinic acid was examined (Fig. 4). JAK is necessary for the activation of Stat3 by the IL-6 receptor (1). These results show that the activation of Stat3 by folic acid and folinic acid is depending on JAK, as Stat3-activation is inhibited upon co-stimulation with AG490, demonstrated in Fig. 4.

Cancer treatment strategies, using FR α as target, rely on the hypothesis that FR α contributes to cellular uptake of folic acid by endocytosis (8). However, the present results show that folic acid and folinic acid activate the Stat3 oncogene through FR α in a JAK-dependent manner, and hence suggest that cancer treatment strategies using FR α as target could participate in deactivation of Stat3 and in this way contribute to cell death.

Since FR α has the higher affinity for non-reduced forms of folate (8), the effect of folic acid compared with folinic acid could be expected to be more efficient. However, our results show that the effect of folic acid appeared only slightly more efficient than that of folinic acid, and both forms of folate seemed equally capable of activating Stat3.

It has serious implications that ingestion of folic acid as vitamin supplement or in fortified food might lead to or maintain Stat3 activation in FR α positive cancer cells. In fact this could explain the observation that folic acid supplementation may enhance the development and progression of already existing, and undiagnosed

pre-malignant and malignant lesions (8, 11, 12). However, the knowledge, provided in the present invention, of the molecular mechanisms involved in Stat3 oncogene activation by folic acid could lead to advantages in cancer treatment targeting Stat3. Ingestion of vitamin supplements containing folic acid may hamper on-going cancer treatments, but addition of FR α inhibitors simultaneously, might contribute to the treatment by preventing activation of Stat3.

Example 2

To assess a potential carcinogenic effect of folic acid supplementation, the effect of folic acid (non-reduced form) and folinic acid (reduced form) on the activation of the STAT3 oncogene in HeLa cells was investigated (Figure 5A). These results show that STAT3 is phosphorylated on Tyr705 and thus activated upon stimulation with folic acid and folinic acid. To reduce background caused by the occurrence of folates and other interfering components, cells were cultured in folate-depleted media supplemented with active charcoal pre-treated FBS. Cell extracts from HeLa, non-treated or treated with IL-6 were used as negative and positive controls respectively. Since type 1 IFN receptors can activate STAT3 as well as STAT1 (Tanabe et al., 2005 (17)), the effect of folic acid and folinic acid on the activation of STAT1 was also investigated (Figure 5B). The results show that treatment with folic acid and folinic acid does not stimulate STAT1 tyrosine phosphorylation (Y701) in HeLa cells. IFN α stimulated cells were used as a positive control for STAT1-activation. The lack of pSTAT1 expression in the non-treated control suggests that STAT1 is not constitutively active in HeLa cells, which confirms earlier studies (Krause et al., 2006 18()). Then it was investigated whether STAT3, when activated by folic acid and folinic acid in HeLa cells, could bind to STAT3 consensus sequences. Figure 5C shows that pSTAT3 indeed binds to DNA promoter elements, thus acting as a transcription factor upon activation by both forms of folate. The level of pSTAT3 binding in folic acid and folinic acid stimulated cells is lower than in the IL-6 treated cells. This correlates with the levels of pSTAT3 in the corresponding immunoblotting analyses. In addition, dose-response analyses of folic acid treated cells show that the amount of activated STAT3 increases with the concentrations of folic acid (Figure 5D).

To further clarify the pathway for the activation of STAT3, the effect of the JAK-inhibitor, AG490, on the activation of STAT3 by folic acid and folinic acid was examined (Figure 3). JAK2 is one of the mediators of STAT3 activation by IL-6 (Hodge et al., 2005 (1)), and it can be seen here that the activation of STAT3 by folic acid and folinic

acid is depending on JAK, as STAT3-activation is inhibited upon co-stimulation with AG490.

5 Since STAT3 is activated by receptor-mediated phosphorylation (Yu and Jove, 2004 (7)), it can be speculated whether folic acid could bind to one of its receptors at the plasma membrane and thus initiate an intracellular signalling pathway leading to the activation of STAT3. Because the glycosphosphatidylinositol (GPI)-anchored FR α binds folic acid with a very high affinity and the function of FR α is largely unknown, we investigated and confirmed that HeLa cells indeed express FR α on the surface (Figure 10 2A and 2B). In order to investigate whether FR α could be the mediator of folate-stimulated STAT3-activation, FR α -negative HEK293 cells were treated with folic acid and folinic acid (Figure 2C). The results show that STAT3-phosphorylation was not induced by folic acid and folinic acid in this FR α -negative cell line, which demonstrate that FR α was the mediator of STAT3 activation by folates. In addition, the observed IL-15 6 mediated STAT3-activation points to the fact that the lack of STAT3-activation by folic acid and folinic acid cannot be a result of dysfunction in the HEK293 STAT3 activation mechanism.

To further substantiate the involvement of FR α in the activation of STAT3 by folic acid and folinic acid, HeLa cells were treated with siRNA against FR α prior to induction with 20 folic acid and folinic acid. Figure 3 show that the siRNA effectively blocks the FR α -expression, and furthermore inhibits the stimulation of STAT3 by folic acid and folinic acid. These results are strong indications that FR α through binding of folic acid and folinic acid initiates an intracellular signalling pathway leading to the activation of 25 STAT3.

Since FR α is attached extracellularly to the cell by a GPI-anchor, the mechanism behind an FR α mediated STAT3-activation upon stimulation by folic acid and folinic acid was investigated. Based on knowledge of the traditional receptor-mediated STAT3 30 activation mechanism and the high affinity of folic acid and folinic acid for FR α , it seemed most likely that FR α - upon extracellular binding of folic acid and folinic acid - activated a transmembrane co-receptor on the cell surface that initiated an intracellular signalling pathway leading to the activation of STAT3. Therefore, we wanted to explore which co-receptor could be responsible for this signal transduction pathway. Known

STAT3 activating transmembrane receptors are the IL-6/gp130 receptor, the EGF receptor, and the type 1 IFN receptor. Since folic acid and folinic acid lacked the ability to activate STAT1 (Figure 5), the type 1 IFN receptor was excluded from further analysis. Therefore, we pre-treated HeLa cells with the EGF receptor inhibitor, AG1478, or with a monoclonal anti-gp130 antibody prior to addition of folic acid and folinic acid. The results seen in Figure 6 show that the EGF receptor is not involved, since addition of AG1478 did not influence the STAT3 activation induced by either folic acid or folinic acid (Figure 6A). In contrast, the IL-6 receptor gp130 seemed to be involved in the signal. Binding of anti-gp130 antibodies inhibited the STAT3 activation by folinic acid, whereas the activation by folic acid seemed to be increased by the same antibody (Figure 6B). A reduction in STAT3 activation was observed for IL-6 induced, anti-gp130 antibody treated HeLa cells compared with IL-6 induced cells not treated with anti-gp130 antibody. This complies with IL-6 mediating the activation of STAT3 through the gp130 receptor and validates the effectiveness of blocking this pathway with anti-gp130 antibody.

Discussion

Cancer treatment strategies, using FR α as target, rely on the hypothesis that FR α contributes to cellular uptake of folic acid by endocytosis (Kelemen, 2006 (8)). However, our results show that folic acid and folinic acid can activate the STAT3 oncogene through FR α in a JAK2-dependent manner, possibly using gp130 as a co-receptor.

The observation that STAT3, but not STAT1, is activated by folic acid and folinic acid not only correlates with the two STATs having opposing actions (Stephanou and Latchman, 2005 (19)), but also shows that STAT3 activation by folic acid and folinic acid is not mediated by the traditional STAT1-dependent JAK-STAT pathway.

Since FR α has a high affinity for non-reduced forms of folate (Kelemen, 2006 (8)), the effect of folic acid compared with folinic acid could be expected to be more efficient. However, our results show that the effect of folic acid appeared only marginally more efficient than that of folinic acid, since both forms of folate seemed equally capable of activating STAT3 in the used concentrations. In contrast, the blocking of the gp130 receptor by the monoclonal 28126 antibody showed that the STAT3 activation by folinic acid could be blocked, whereas the activation by folic acid was increased. The larger

reduced folate form folinic acid has a lower affinity for FR α compared with the smaller, non-reduced folate form folic acid. One explanation for the observed effect could be that the binding of the gp130 antibody to the receptor changed the conformation of the gp130 binding site for FR α and thus blocked the binding of the folinic acid:FR α complex, whereas the binding of the smaller folic acid:FR α complex was enhanced. These data suggest that the gp130 receptor could be involved in the signalling transduction pathway initiated by folate binding to the FR α receptor. Further analyses are required to determine how the interaction between FR α and gp130 is come about. This signal could be established with gp130 as a homodimer like in the case of IL-6 and IL-11 signals, or with gp130 as a heterodimer, as in the case of ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), leukemia inhibitory factor (LIF), neuropoietin (NPN), and oncostatin M (OSM) (Febbraio, 2007; Rose-John et al., 2006 (20,21)). Interestingly, it has been proposed that cardiotrophin-1 might act through a GPI-anchored receptor on the surface of neural cells (Pennica et al., 1996 (22)), although this was later questioned (Cheng et al., 1997 (23)).

A correlating occurrence of upregulated FR α and constitutively active STAT3 in several type of cancers (Hodge et al., 2005; Kelemen, 2006; Parker et al., 2005 (1,8,3)) could indicate that this signal transduction pathway might contribute to the development of cancer in vivo. Hence we suggest that cancer treatment strategies using FR α as target could participate in deactivation of STAT3 and in this way contribute to cell death. It has serious implications that ingestion of folic acid as vitamin supplement or in fortified food might lead to or maintain STAT3 activation in FR α positive cancer cells. In fact this could explain the observation that folic acid supplementation may enhance the development and progression of already existing, and undiagnosed premalignant and malignant lesions (Kelemen, 2006; Kim, 2004; Van Guelpen et al., 2006 (8,11,12)). However, knowledge of the molecular mechanisms involved in STAT3 oncogene activation by folic acid could lead to advantages in cancer treatment targeting STAT3. Ingestion of vitamin supplements containing folic acid may hamper on-going cancer treatments, but addition of FR α inhibitors simultaneously, might contribute to the treatment by preventing activation of STAT3.

Example 3

Identification of peptide inhibitors of folate receptor α by phage display

A soluble form of human Folate Receptor α (FR α) (aa#1-234) with a hexa-histidine-tag at the C-terminus (sFR-His) was cloned into pcDNA3.1 with the Directional Topo Expression kit. The residues #235-257 of the mature human FR α protein was deleted, so that an GPI anchor was not attached to the protein, and it was thus secreted from the cell into the growth medium.

Human embryo kidney (HEK) 293 cells were transfected with a pcDNA3.1-sFR-His construct using ExGene500. 48-72 hours after transfection, cell medium was harvested, and cell debris spun down. The medium containing the sFR-His protein was filtered and then applied to a HiTrap chelating HP 1 ml column using fast protein liquid chromatography (f.p.l.c.). sFR-His was eluted from the column with an increasing gradient concentration of imidazole. The sFR-His containing fractions were then diluted and applied to a cation column (Resource S) and the run-through was collected. This preparation containing sFR-His was used directly for screening a C7XC phage library.

The C7XC phage display screening was done according to G. Smith (Smith, G. P. and Petrenko, V. A. (1997) Phage display. Chem Rev. 97:391-410.) with some modifications. Briefly, each of the four bio-pannings were carried out in the following way. A maxisorp tube was coated with sFR-His, and then blocked with skimmed milk powder alternated with BSA. 1011 phages were added to the coated tube and they were allowed to bind to sFR-His. Then the non-bound phages were washed off with an increasing number of washing steps in each panning. The bound phages were eluted and propagated in the TG1 bacterial strain. After PEG precipitation, the enriched phages were ready for the next round of bio-panning. In the final panning, tetR bacterial colonies were selected and used for propagation of individual phage clones.

These individual phages were tested for their ability to bind immobilized sFR-His protein in an ELISA assay. 20 colonies were identified, which were able to bind sFR-His better than the blocking reagents, skimmed milk and/or BSA. These 20 phages were sequenced and 18 different peptide sequences binding to sFR-His identified.

In order to verify whether the individual peptides can indeed bind FR α , a titration of the binding of the individual phages may be performed on immobilized sFR-His protein in an ELISA assay.

Furthermore, peptides with the identified amino acid sequences can be synthesized in a Cys-XXXXXXX-Cys conformation (X being the identified amino acid sequences) and analyzed for their ability to bind sFR-His as well as to membrane bound FR α on the cell surface.

Also the ability of each of the peptides to inhibit folic acid and folinic acid induced STAT3 activation in FR expressing cell lines such as HeLa cells may be analyzed. And finally, the synthesized peptides can be tested for their ability to inhibit folic acid induced tumor growth in the human xenograft mouse model described below.

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*Example 4**A xenograft mouse model*

Nude NMRI mice (4-6 weeks of age) were injected s. c. with HeLa cells suspended in matrigel into each of the two flanks. Three to four days after injection the mice were divided into groups, and one group was continuing the normal diet (2 mg/kg folic acid), whereas the other group were put on a folate rich diet (8 mg/kg folic acid). After two weeks, a visible difference of tumor size between the two groups were observed, the group on high folate diet having larger tumors than the mice on normal diet. The number of tumors developed will be counted and the size of the tumors measured. The content of folic acid in the blood stream will be measured.

10

15

Sequences

Human FR α

Human Gene FOLR1 Description and Page Index:

5 **Description:** folate receptor 1 precursor

10 **RefSeq Summary:** The protein encoded by this gene is a member of the folate receptor (FOLR) family. Members of this gene family have a high affinity for folic acid and for several reduced folic acid derivatives, and mediate delivery of 5-methyltetrahydrofolate to the interior of cells. This gene is composed of 7 exons; exons 1 through 4 encode the 5' UTR and exons 4 through 7 encode the open reading frame. Due to the presence of 2 promoters, multiple transcription start sites, and alternative splicing of exons, several transcript variants are derived from this gene. These variants differ in the lengths of 5' and 3' UTR, but they encode an identical amino acid sequence.

Strand: + **Genomic Size:** 6765 **Exon Count:** 6 **Coding Exon Count:** 4

15 **Comments and Description Text from UniProt (Swiss-Prot/TrEMBL)**

ID: FOLR1_HUMAN

DESCRIPTION: Folate receptor alpha precursor (FR-alpha) (Folate receptor 1) (Folate receptor, adult) (Adult folate-binding protein) (FBP) (Ovarian tumor- associated antigen MOv18) (KB cells FBP).

20 **FUNCTION:** Binds to folate and reduced folic acid derivatives and mediates delivery of 5-methyltetrahydrofolate to the interior of cells.

SUBCELLULAR LOCATION: Cell membrane; lipid-anchor; GPI-anchor. Secreted protein (Probable).

25 **TISSUE SPECIFICITY:** Exclusively expressed in tissues of epithelial origin. Expression is increased in malignant tissues.

PTM: Eight disulfide bonds are present (Probable).

PTM: The secreted form is derived from the membrane-bound form either by cleavage of the GPI anchor, or/and by proteolysis catalyzed by a metalloprotease.

SIMILARITY: Belongs to the folate receptor family.

30

InterPro Domains: Graphical view of domain structure

IPR004269 - Folate receptor

Pfam Domains:

35 **PF03024 - Folate receptor family**

ModBase Predicted Comparative 3D Structure on P15328**Descriptions from all associated GenBank mRNAs**

5

J05013 - Human folate-binding protein (FBP) mRNA, complete cds.

BC002947 - Homo sapiens folate receptor 1 (adult), mRNA (cDNA clone MGC:10473 IMAGE:3956659), complete cds.

AK223527 - Homo sapiens mRNA for folate receptor 1 precursor variant, clone: FCC124E04.

10

M28099 - Human folate-binding protein (FBP) mRNA, complete cds.

X62753 - H.sapiens mRNA for adult folate binding protein.

U78793 - Human folate receptor alpha (hFR) mRNA, partial cds.

U78794 - Human folate receptor alpha (hFR) mRNA, alternatively spliced, partial cds.

S73490 - Homo sapiens folate receptor alpha isoform mRNA, partial cds.

15

CR542019 - Homo sapiens full open reading frame cDNA clone RZPDo834H0535D for gene FOLR1, folate receptor 1 (adult); complete cds, without stopcodon.

BT007158 - Homo sapiens folate receptor 1 (adult) mRNA, complete cds.

M25317 - Human folate binding protein (FBP) mRNA, 3' end.

M35069 - Human folate binding protein mRNA, partial cds.

20

AF000381 - Homo sapiens folate binding protein mRNA, partial cds.

S73474 - Homo sapiens folate receptor alpha isoform mRNA, partial cds.

DQ892744 - Synthetic construct clone IMAGE:100005374; FLH189297.01X;

RZPDo839D0874D folate receptor 1 (adult) (FOLR1) gene, encodes complete protein.

DQ895990 - Synthetic construct clone IMAGE:100010450; FLH189293.01L;

25

RZPDo839D0864D folate receptor 1 (adult) (FOLR1) gene, encodes complete protein.

Other Names for This Gene**Alternate Gene Symbols:** FOLR, FOLR1_HUMAN, NM_016724, NP_057936, P15328

30

UCSC ID: uc001orz.1**RefSeq Accession:** NM_016725**Protein:** P15328 (aka FOLR1_HUMAN)**CCDS:** CCDS8211.1

35

RefSeq Gene FOLR1:

RefSeq: NM_016725.1 Status: **Reviewed**

CCDS: CCDS8211.1

CDS: full length

OMIM: 136430

5 **Entrez Gene:** 2348

PubMed on Gene: FOLR1

PubMed on Product: folate receptor 1 precursor

GeneLynx FOLR1

GeneCards: FOLR1

10 **AceView:** FOLR1

Stanford SOURCE: NM_016725

Summary of FOLR1

15 The protein encoded by this gene is a member of the folate receptor (FOLR) family. Members of this gene family have a high affinity for folic acid and for several reduced folic acid derivatives, and mediate delivery of 5-methyltetrahydrofolate to the interior of cells. This gene is composed of 7 exons; exons 1 through 4 encode the 5' UTR and exons 4 through 7 encode the open reading frame. Due to the presence of 2 promoters, multiple transcription start sites, and
20 alternative splicing of exons, several transcript variants are derived from this gene. These variants differ in the lengths of 5' and 3' UTR, but they encode an identical amino acid sequence.

mRNA/Genomic Alignments

25

Position: chr11:71578250-71584989

Band: 11q13.4

Genomic Size: 6740

Strand: +

30 **Alternate Name:** FOLR1

CDS Start: complete

CDS End: complete

Data last updated: 2007-04-17

35

Description

The RefSeq Genes track shows known protein-coding genes taken from the NCBI mRNA reference sequences collection (RefSeq). On assemblies in which incremental GenBank
5 downloads are supported, the data underlying this track are updated nightly.

Methods

RefSeq mRNAs were aligned against the human genome using blat; those with an alignment of
10 less than 15% were discarded. When a single mRNA aligned in multiple places, the alignment having the highest base identity was identified. Only alignments having a base identity level within 0.1% of the best and at least 96% base identity with the genomic sequence were kept.

SEQ ID NO.: 1

15 FOLR1 (FR α) sequences:

Genomic DNA sequence from human genome browser.

1000 bp before predicted transcriptional start site.

Exons are designated by capitals letters.

```
>hg18_knownGene_uc001orz.1 range=chr11:71577250-71585014 5'pad=0  
20 3'pad=0 revComp=FALSE strand=+ repeatMasking=none  
ctgacataagaaaaaatattgtttccaaatatatagatctggtgatttta  
agttgacacttctcaggttggtcacaagattcaggtatggctcactggtgc  
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25 ggctccctgcctgcctcctccttggggtcctttcttgtggcattgcccag  
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30 tctgtggtaccaccatgggctcagagaataaaaaaatgaaggcttctgt  
cattgactggggtggagatggagggaagagttagcccagaatcacagggtg  
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CGCCCAATCCCAGGCTCCACTCCTGGGCTCCATTCCCACTCCCTGCCTGT
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TG TAGAGCAGAGCAGAAGCCTGAGCCAGACGGAGAGCCACCTCCTCTCCC
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ccgggcccagtggtctaaaggtcttccctcctctctacagGGTTTAAACAAG
TGCGCAGTGGGAGCTGCCTGCCAACCTTTCCATTTCTACTTCCCCACACC
CACTGTTCTGTGCAATGAAATCTGGACTCACTCCTACAAGGTCAGCAACT
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10 GGCAACCCCAATGAGGAGGTGGCGAGGTTCTATGCTGCAGCCATGAGTGG
GGCTGGGCCCTGGGCAGCCTGGCCTTTTCCTGCTTAGCCTGGCCCTAATGC
TGCTGTGGCTGCTCAGCTGACCTCCTTTTACCTTCTGATACCTGGAAATC
CCTGCCCTGTTTCAGCCCCACAGCTCCCAACTATTTGGTTCCTGCTCCATG
GTCGGGCCTCTGACAGCCACTTTGAATAAACCAGACACCGCACATGTGTC
15 TTGAGAATTATTTGG

SEQ ID NO.: 2

FOLR1 (FR α) mRNA sequence:

from human genome browser.

20 CDS is in capitals

>NM_016725.1

tggaggcctggctggtgctcacatacaataattaactgctgagtggcctt
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ctcctagggccactaaaccacagctgtcccctggaataaggcaagggggag
25 tgtagagcagagcagaagcctgagccagacggagagccacctcctctccc
agggacagac ATGGCTCAGCGGATGACAACACAGCTGCTGCTCCTTCTAG
TGTGGGTGGCTGTAGTAGGGGAGGCTCAGACAAGGATTGCATGGGCCAGG
ACTGAGCTTCTCAATGTCTGCATGAACGCCAAGCACCACAAGGAAAAGCC
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35 CACCTCCTACACCTGCAAGAGCAACTGGCACAAGGGCTGGAAGTGGACTT
CAGGGTTTAAACAAGTGCAGTGGGAGCTGCCTGCCAACCTTTCCATTTCT
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CAAGGTCAGCAACTACAGCCGAGGGAGTGGCCGCTGCATCCAGATGTGGT
TCGACCCAGCCCAGGGCAACCCCAATGAGGAGGTGGCGAGGTTCTATGCT
40 GCAGCCATGAGTGGGGCTGGGCCCTGGGCAGCCTGGCCTTTTCCTGCTTAG

CCTGGCCCTAATGCTGCTGTGGCTGCTCAGCTGA cctcctttttaccttct
 gatacctggaaatccctgcccctgttcagccccacagctcccaactatttg
 gttcctgctccatggtcgggcctctgacagccactttgaataaaccagac
 accg

5

SEQ ID NO.: 3

FOLR1 (FR α) amino acid sequence:

Predicted from human genome browser (correlated to swiss protein).

Protein variants exist and the alternative amino acid residues are indicated under the
 10 sequence (the variants are independent):

A putative signal peptide (1-24) is most likely cleaved off during translation. The
 cleavage site is indicated by an arrow.

The C-terminal tail constitute a propeptide, which is also cleaved off (position 236), also
 indicated by an arrow.

15 The amino acid 234 is attached to a GPI anchor (as indicated)

FR-alpha protein is a core protein of 29 kDa.

It has three putative glycosylation sites (marked Glc-Nac), and the protein is
 glycosylated so the apparent molecular weight is 60 kDa.

20 >NP_057937 length=257

NH3-MAQRM TTQLLLLLVWVAVVG EAQT RIAWARTE LLNVCMNAKHHKEKPGPE

↑

25 DKLHEQCRPWRKNACCSTNTS QEAHKDVSYLYRFNWNHCGEMAPACKRHF

GlcNac

30 IQDTCLYECSPNLGPW IQQVDQSWRKERV LNVP LCKEDCEQWWEDCRTSY

TCKSNWHKGWNWTSGFNKCAVGAACQPFHFYFPTPTVLCNEIWTHSYKVS

c |

S

GlnNac

35

NYSRGSGRCIQMWFDP AQGNPNEEVARFYAAAMSG AGPWAAWPFLLSLAL

|

| ↑

GlnNac

GPI anchor

MLLWLLS-COOH

5 SEQ ID NO.: 4

Bovine FR α amino acid sequence

The bovine form of FR α presumably contain a signal peptide, which is cleaved off, similar to the human form. Glycosylation most likely also occur as in the human variant of FR α as the same putative glycolysation sites are present in bovine FR α .

10

LOCUS P02702 241 aa linear

MAM 20-MAR-2007

DEFINITION Folate receptor alpha precursor (FR-alpha) (Folate receptor 1)

15

(Folate-binding protein 1) (Milk folate-binding protein) (FBP).

ACCESSION P02702

VERSION P02702 GI:110282963

DBSOURCE swissprot: locus FOLR1_BOVIN, accession P02702;

20

class: standard.

created: Jul 21, 1986.

sequence updated: Jul 11, 2006.

annotation updated: Mar 20, 2007.

xrefs: DN512948.1, BFBO

25

xrefs (non-sequence databases):

Ensembl:ENSBTAG00000027684,

KEGG:bta:516067, InterPro:IPR004269,

PANTHER:PTHR10517,

Pfam:PF03024

30

KEYWORDS Direct protein sequencing; Folate-binding; Glycoprotein; Milk

protein; Receptor; Signal.

SOURCE Bos taurus (cattle)

ORGANISM Bos taurus

35

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;

Mammalia; Eutheria; Laurasiatheria; Cetartiodactyla;

Ruminantia;

Pecora; Bovidae; Bovinae; Bos.

REFERENCE 1 (residues 1 to 241)
 AUTHORS Smith, T.P.L., Roberts, A.J., Echterkamp, S.E.,
 Chitko-McKown, C.G.,
 Wray, J.E. and Keele, J.W.

5 TITLE Direct Submission
 JOURNAL Submitted (??-MAR-2005)
 REMARK NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA].

REFERENCE 2 (residues 1 to 241)
 AUTHORS Svendsen, I., Hansen, S.I., Holm, J. and Lyngbye, J.

10 TITLE The complete amino acid sequence of the folate-
 binding protein from
 cow's milk
 JOURNAL Carlsberg Res. Commun. 49, 123-131 (1984)
 REMARK PROTEIN SEQUENCE OF 20-241.

15 TISSUE=Milk

REFERENCE 3 (residues 1 to 241)
 AUTHORS Svendsen, I., Martin, B., Pedersen, T.G., Hansen, S.I.,
 Holm, J. and
 Lyngbye, J.

20 TITLE Isolation and characterization of the folate-binding
 protein from
 cow's milk
 JOURNAL Carlsberg Res. Commun. 44, 89-99 (1979)
 REMARK PROTEIN SEQUENCE OF 20-81; 91-121 AND 211-241.

25 TISSUE=Milk

COMMENT On Jul 11, 2006 this sequence version replaced
 gi:120460.
 [FUNCTION] Binds to folate and reduced folic acid
 derivatives and
 30 mediates delivery of 5-methyltetrahydrofolate to the
 interior of
 cells.
 [PTM] Eight disulfide bonds are present.
 [SIMILARITY] Belongs to the folate receptor family.

35 FEATURES Location/Qualifiers
 source 1..241
 /organism="Bos taurus"
 /db_xref="taxon:9913"
 gene 1..241
 40 /gene="FOLR1"
 Protein 1..241

```

/ gene="FOLR1"
/ product="Folate receptor alpha precursor"
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/ gene="FOLR1"
5 / region_name="Signal"
/ experiment="experimental evidence, no
additional details
recorded"
      Region 20..241
10 / gene="FOLR1"
/ region_name="Mature chain"
/ experiment="experimental evidence, no
additional details
recorded"
15 / note="Folate receptor alpha.
/ FTId=PRO_0000147394."
      Region 27..240
/ gene="FOLR1"
/ region_name="Folate_rec"
20 / note="Folate receptor family. This family
includes the
folate receptor which binds to folate and
reduced folic
acid derivatives and mediates delivery of
25 5-methyltetrahydrofolate to the interior of
cells;
pfam03024"
/ db_xref="CDD:66681"
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/ site_type="glycosylation"
/ experiment="experimental evidence, no
additional details
recorded"
35 / note="N-linked (GlcNAc...)."
      Site 160
/ gene="FOLR1"
/ site_type="glycosylation"
/ experiment="experimental evidence, no
40 additional details
recorded"

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/note="N-linked (GlcNAc...)."

Region 174..175

/gene="FOLR1"

/region_name="Conflict"

5 /experiment="experimental evidence, no
 additional details
 recorded"

/note="HC -> CH (in Ref. 1)."

ORIGIN

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 121 qrwrkervlg vplckedcqs wwedcrtsyt cksnwhkgwn wtsgynqcpv kaahcrfdy
 181 fptpaalcne iwshsykvsn ysrgsgrciq mwfdpfqgnp neevaryae nptsgstpqq
 241 i

15

SEQ ID NO.: 5

The PCR primer FOLR1 forward

ATGGCTCAGCGGATGACAAC

20

SEQ ID NO.: 6

FOLR1 reverse

TCAGCTGAGCAGCCACAGCA

25

SEQ ID NO.: 7

GAPDH forward

GGTCGGAGTCAACGGATTT

30

SEQ ID NO.: 8

GAPDH reverse

CCAGCATCGCCCCACTTGG

siRNA sequences

Folate receptor α siRNA. The sequences for FR α siRNA (human) is a pool of three separate strands, SEQ ID NOs: 9-11.

35

SEQ ID NO.: 9

Sense (A): GAAGAAUGCCUGCUGUUCUtt

SEQ ID NO.: 10

Sense (B): GCAAUGGUGGGAAGAUUGUtt

SEQ ID NO.: 11

5 Sense (C): CCACUGUUCUGUGCAAUGAtt

SEQ ID NOs.: 12-14

Human FR beta

Human Gene FOLR2 Description and Page Index

10

Description: folate receptor 2 precursor

RefSeq Summary: The protein encoded by this gene is a member of the folate receptor (FOLR) family. Members of this gene family have a high affinity for folic acid and for several reduced folic acid derivatives, and mediate delivery of 5-methyltetrahydrofolate to the interior of cells.

15 This protein has a 68% and 79% sequence homology with the FOLR1 and FOLR3 proteins, respectively. The FOLR2 protein was originally thought to exist only in placenta, but is also detected in spleen, bone marrow, and thymus.

Strand: + **Genomic Size:** 5148 **Exon Count:** 5 **Coding Exon Count:** 4

20

InterPro Domains: Graphical view of domain structure

IPR004269 - Folate receptor

Pfam Domains:

25 **PF03024 - Folate receptor family**

ModBase Predicted Comparative 3D Structure on Q6GTE8

Descriptions from all associated GenBank mRNAs

30

AK222539 - Homo sapiens mRNA for folate receptor 2 precursor variant, clone: adSE01171.

AK222643 - Homo sapiens mRNA for folate receptor 2 precursor variant, clone: CBL05233.

AF000380 - Homo sapiens folate binding protein mRNA, complete cds.

BC058036 - Homo sapiens folate receptor 2 (fetal), mRNA (cDNA clone MGC:61912

35 IMAGE:6662871), complete cds.

BC027894 - Homo sapiens cDNA clone IMAGE:5225025, partial cds.

J02876 - Human placental folate binding protein mRNA, complete cds.

BC115366 - Homo sapiens folate receptor 2 (fetal), mRNA (cDNA clone IMAGE:40021750), partial cds.

5

Other Names for This Gene

Alternate Gene Symbols: NM_000803, NP_000794, Q6GTE8, Q6GTE8_HUMAN

UCSC ID: uc001ose.1

10

RefSeq Accession: NM_000803

Protein: Q6GTE8 CCDS: CCDS8212.1

15

RefSeq Gene FOLR2

RefSeq: NM_000803.2 **Status:** Reviewed

CCDS: CCDS8212.1

CDS: full length

OMIM: 136425

20

Entrez Gene: 2350

PubMed on Gene: FOLR2

PubMed on Product: folate receptor 2 precursor

GeneLynx FOLR2

GeneCards: FOLR2

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AceView: FOLR2

Stanford SOURCE: NM_000803

Summary of FOLR2

30

The protein encoded by this gene is a member of the folate receptor (FOLR) family. Members of this gene family have a high affinity for folic acid and for several reduced folic acid derivatives, and mediate delivery of 5-methyltetrahydrofolate to the interior of cells. This protein has a 68% and 79% sequence homology with the FOLR1 and FOLR3 proteins, respectively. The FOLR2 protein was originally thought to exist only in placenta, but is also detected in spleen, bone marrow, and thymus. Publication Note: This RefSeq record includes a

35

subset of the publications that are available for this gene. Please see the Entrez Gene record to access additional publications.

mRNA/Genomic Alignments

5

Position: chr11:71605491-71610635

Band: 11q13.4

Genomic Size: 5145

Strand: +

10

Alternate Name: FOLR2

CDS Start: complete

CDS End: complete

Data last updated: 2007-04-17

15

Description

The RefSeq Genes track shows known protein-coding genes taken from the NCBI mRNA reference sequences collection (RefSeq). On assemblies in which incremental GenBank downloads are supported, the data underlying this track are updated nightly.

20

Methods

RefSeq mRNAs were aligned against the human genome using blat; those with an alignment of less than 15% were discarded. When a single mRNA aligned in multiple places, the alignment having the highest base identity was identified. Only alignments having a base identity level within 0.1% of the best and at least 96% base identity with the genomic sequence were kept.

25

FOLR2 (FR β) sequences:

30

SEQ ID NO.: 12

Genomic DNA sequence from human genome browser.

1000 bp before predicted transcriptional start site.

Exons in capitals.

>hg18_knownGene_uc001ose.1 range=chr11:71604491-71610638 5'pad=0
3'pad=0 revComp=FALSE strand=+ repeatMasking=none

35

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CCTCTGGAGTCACTCATAACAAGTCAGCAACTACAGCCGAGGGAGCGGCC
35 GCTGCATCCAGATGTGGTTTGATTTCAGCCCAGGGCAACCCCAACGAGGAA
GTGGCGAGGTTCTATGCTGCAGCCATGCATGTGAATGCTGGTGAGATGCT
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GGCTCCTTGGCTGAGTTCAGTCCTCCAGACTACCTGCCCTCAGCTTGGA
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40 TGCTTCTATTAGTCACCTAACCTCTGTACCCAGTCTGTTGCTGCTCCA
TGGTGGGGCCAAGAGTCACTTCTAATAAACAGACTGTTTTCTAATAAT

SEQ ID NO.: 13

FOLR2 (FR-beta) mRNA sequence:

5 from human genome browser.

CDS is in capitals

>NM_000803.2

```
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GCCAAGCACCACAAGACAAAGCCAGGTCTGAGGACAAGCTGCATGACCA
15 ATGCAGTCCCCTGGAAGAAGAATGCCTGCTGCACAGCCAGCACCAGCCAGG
AGCTGCACAAGGACACCTCCCGCCTGTACAACTTTAACTGGGACCACTGC
GGCAAGATGGAGCCCGCCTGCAAGCGCCACTTCATCCAGGACACCTGTCT
CTATGAGTGCTCACCCAACCTGGGGCCCTGGATCCAGCAGGTGAATCAGA
CGTGGCGAAAAGAACGCTTCCCTGGATGTGCCCTTATGCAAAGAGGACTGT
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GCACAGAGGATGGGACTGGACCTCAGGAGTTAACAAGTGCCCAGCTGGGG
CTCTCTGCCGCACCTTTGAGTCCTACTTCCCCACTCCAGCTGCCCTTTGT
GAAGGCCTCTGGAGTCACTCATAACAAGGTCAGCAACTACAGCCGAGGGAG
CGGCCGCTGCATCCAGATGTGGTTTGATTGAGCCAGGGCAACCCCAACG
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aaaaaaaaaaaaaaaaaaaa
```

SEQ ID NO.: 14

35 FOLR2 (FR-beta) amino acid sequence:

Predicted from human genome browser (correlated to swiss protein).

A putative signal peptide (1-17) is most likely cleaved off during translation. The cleavage site is indicated by an arrow.

The amino acid 230 is attached to a GPI anchor (as indicated)

No glycosylation reported.

>NP_000794 length=255

5 NH3-MVWKWMPLLLLLVCVA TMCSAQDRTDLLNVCMDAKHHKTKPGPEDKLHDQ
↑

CSPWKKNACCTASTSQELHKDTSRLYNFNWDHCGKMEPACKRHF IQDTCL
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10 HRGWDWTSGV NKPAGALC RTFESYFPTPAALCEGLWSHSYKVS NYSRGS
GRCIQMWFD SAQGNPNEEVARFYAAAMHV NAGEMLHGTGGLLLSLALMLQ
|
GPI-anchor
LWLLG-COOH
15

SEQ ID NOs.: 15-17

Human FR gamma

Human Gene FOLR3 Description and Page Index

20

Description: folate receptor 3 precursor

RefSeq Summary: This gene encodes a member of the folate receptor (FOLR) family, members of which have a high affinity for folic acid and for several reduced folic acid derivatives, and mediate delivery of 5-methyltetrahydrofolate to the interior of cells. This gene includes two polymorphic variants; the shorter one has two base deletion in the CDS, resulting in a truncated polypeptide, compared to the longer one. Both protein products are constitutively secreted in hematopoietic tissues and are potential serum marker for certain hematopoietic malignancies. The longer protein has a 71% and 79% sequence homology with the FOLR1 and FOLR2 proteins, respectively.

30 **Strand:** + **Genomic Size:** 4164 **Exon Count:** 6 **Coding Exon Count:** 5

Descriptions from all associated GenBank mRNAs

BC030285 - Homo sapiens cDNA clone IMAGE:5228679, partial cds.
35 U08470 - Human FR-gamma' mRNA, complete cds.
U08471 - Human folate receptor 3 mRNA, complete cds.
Z32564 - H.sapiens FRGAMMA mRNA (819bp) for folate receptor.

Z32633 - H.sapiens FRGAMMA' mRNA for folate receptor (817bp).

BC126398 - Homo sapiens cDNA clone IMAGE:8992114, containing frame-shift errors.

Other Names for This Gene

5

Alternate Gene Symbols: NM_000804, NP_000795

UCSC ID: uc001orx.1

RefSeq Accession: NM_000804

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RefSeq Gene FOLR3

RefSeq: NM_000804.2 **Status:** Reviewed

CDS: full length

15

OMIM: 602469

Entrez Gene: 2352

PubMed on Gene: FOLR3

PubMed on Product: folate receptor 3 precursor

GeneLynx FOLR3

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GeneCards: FOLR3

AceView: FOLR3

Stanford SOURCE: NM_000804

Summary of FOLR3

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This gene encodes a member of the folate receptor (FOLR) family, members of which have a high affinity for folic acid and for several reduced folic acid derivatives, and mediate delivery of 5-methyltetrahydrofolate to the interior of cells. This gene includes two polymorphic variants; the shorter one has two base deletion in the CDS, resulting in a truncated polypeptide, compared to the longer one. Both protein products are constitutively secreted in hematopoietic tissues and are potential serum marker for certain hematopoietic malignancies. The longer protein has a 71% and 79% sequence homology with the FOLR1 and FOLR2 proteins, respectively.

30

mRNA/Genomic Alignments

35

Position: chr11:71524419-71528582

Band: 11q13.4

Genomic Size: 4164

Strand: +

Alternate Name: FOLR3

5 **CDS Start:** complete

CDS End: complete

Data last updated: 2007-04-17

10 **Description**

The RefSeq Genes track shows known protein-coding genes taken from the NCBI mRNA reference sequences collection (RefSeq). On assemblies in which incremental GenBank downloads are supported, the data underlying this track are updated nightly.

15

Methods

20

RefSeq mRNAs were aligned against the human genome using blat; those with an alignment of less than 15% were discarded. When a single mRNA aligned in multiple places, the alignment having the highest base identity was identified. Only alignments having a base identity level within 0.1% of the best and at least 96% base identity with the genomic sequence were kept.

FOLR3 (FR β) sequences:

SEQ ID NO.: 15

25

Genomic DNA sequence from human genome browser.

1000 bp before predicted transcriptional start site.

Exons in capitals.

30

```
>hg18_refGene_NM_000804 range=chr11:71523419-71528582 5'pad=0
3'pad=0 revComp=FALSE strand=+ repeatMasking=none
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20 AGGAGTGCGCGGGCCAGGACGGACCTGCTCAATGTCTGCATGAACGCCAA
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TGTGCCCACCACTTCATTTGCTTTTCAGAACCTTCTAGAGCACATCCTCC
TTGCCAGGAAATGGTGTGGATGTAGACCTTTGAGAGAGACAGATGACTAT
35 CATTCTCAGGGCCATGAGCTATATGAGAGTGATGATATTTGTTGAGCCCT
TACTATAGCAAGGGAGTTCTTCTCATTGTACTCAGTAACTCTTTTGGAGG
CAACAACCCTTGACCCTGACAGGCAGGACCCATGTCTGCCAAACCCTAAG
ACCCATGATGTGCAAGGGGTCTTGCAGGAAGACCAAGAGTTGGAACATCC
AAGGAAAAGCAAGTGTGAAGTCGGGCTGGCAGGGAAGCATGTTCTGTGTC
40 AGCCGGCACTGGGCGTGGGCCAGGGTGTGGGAGGTGGGTAGGTCTGGCTC
CCCTCCCATGGATTTCCCTATTGTTTCTCCTGGGTGCTCAGGCCTGTCAC

GCCTCTGCCATCACTTGACCCTAGGTGCAAGGGTTCAGCCCAGAAATTTT
ATGCAATTGATTCATGATTTCTCAGGTTTTCTGAGTCCTGGCCTAGAGTG
ACTTCCCAAGAAAAACTCCACCATTTCTGCTTGTCTTACCTGCCTTGTA
TTTACCTTTCTAGGATTGCCTTTTCCACATTTAGTCAAGTCTAGGTTTCA
5 ACCCACGTGCAGGCTATAGCTCCTTCGTTCTCCACCACTCTCAGGATCTA
TCTAGAGTCTCCCCACCTGGACCTCCAGACCCTGGGAGAGCCAGACCAGC
CCCTTGACCTCCACCCTCCCCACAACCTGGGCCAGGTTCTCTCTCTCCC
TGTCTCAGTTATAATTTTTTTTTTTTTTAATTTGAGGCAGAGTTTCGCT
CTTGTGCCCAGGCTGGAATGCAATGGCATGATCTTGGCTCACTGCAACC
10 TCTGCCTCCTGGGTTCAAGTGATTCTCCTGCCTCTGCCTCCTGAGTAGCT
GGGATTACAGGCGCTTACCCTGTGCCTGGCTAATTTTTTGGTATTTTTTA
GTAGAGACAGGGTTTCTCTGTGTTGGTCAGGCTGGTCTCGAACTTCTAAC
CTCAGGTGATCCGCCCGCTCCTTAAATCTTAACCTCACTGTTTACCATG
GGTGTAGCTTACTTAACTCTGTAAAAATGGGGTAAGGATTCGTACTGG
15 GTTGTGAGAGGATAAAGCGCAAAAGCCTCAGGGACTTTGCACCTATGGT
TTTCTATGCCTAGAGTGTTCTTTGTCTCCCTTCTACACACAGCCCACCCA
CCCACTAAAACCATAACCCTCCCTGAGGGTAGAGGTGTTATTTGTTTTCTT
CACTGTGGTGTTCTTAGGACCTAGCACAGTGCCTGATGTATAATCAGCAC
TCAGTTAATATTGGCTGGATGCAAAATGAATAATATAAATAAGCTGAATA
20 ACATGAAATAGGCCGAACGCGGTGACTCACGCCTGTAATCCCAACACTTT
GGGAGGCCAAGGAGGGTGGATCACCTGAGGTCAGGAGTTCGAGACCAGCC
TGGCAAACATGGTGAACCCCGTCTCTACTAAAAATACAAAATTAGCTGG
GCATGGTGGCACGTGCCTGTAATCCCAGCTACTTGGGAGGCTGAGGCAGG
AGAATTGCTTGAACCCGGGAGGTGGAGGTTGCAGTGAGCCAAGATCACGC
25 CACTGCACTCCAGTCTGGGCAACAGGAGCGAACTCTGTCTCAAAAAAAA
GTTAGTTTAATAACATGAGATCACTTTTTTAAACCGTTAAGAGCTGTACTA
CTAATAATTACTCTCTCAGACAGTGGCTCTCCCTCATCTCCTATATCCCG
TGGATTACCCTCTGTTAAAAGCCAAAATTAAGCAGGCATGGTGGCTCACG
CCTCTAATCTTAGTATTTTGGGAAGCTGACATGAGCCAAGGAGTTTGAGA
30 CCAGCCTAGGCAACATAGTGAGACCCCATCTCTACAAAATACTTTATAT
TAGCCAGGCATGGTGGCATGTGCCTGAATTCCAGCTCCTCGGGAGGCTGA
GGTGGGAGGATTATTTGAGCCCAGGATGTTGAGGCTGCAGTGAGCTATGA
TCACACCACTGCGCTCCAGCCTGGTCAACAGAGCAAGACCCTGTCTCAAA
TAAATAAATGAATAAATAGGGCGGAAAGCACCAATATTGTAATTGCCTCC
35 GTCCCCAGGTGGGAGCTCCTCAAGGGCCCTCCCCAGGAAGTGTTCTCTG
GATGACCTACCTGGGGCAGAGGAGCCAGAATATGGAGGAGATGGCTGTGG
TGGGGAGAGACTTAGTCCTGTGTCTTCCCCACCCAGTGCAGTCCCTGGAA
GAAGAATGCCTGCTGCACGGCCAGCACCAGCCAGGAGCTGCACAAGGACA
CCTCCCGCCTGTACAACCTTTAACTGGGATCACTGTGGTAAGATGGAACCC
40 ACCTGCAAGCGCCACTTTATCCAGGACAGCTGTCTCTGAGTGCTCACCCA
ACCTGGGGCCCTGGATCCGGCAGGTATGAGTGCTGTTCCCAAAACATTA

ACCTCAGCAGAGGGCGGAGCCTGCCAGTTGCTGGCAGGGAGGGCTTGGTC
CAGGAATTCGGGTCTGAGGGTGGTGGACGCCCTGCCCCCTCCCACAGCTC
TGGTCCCCCTTCAAGGGTAAAGCTGCTGAGATACGTGGCTGACAGGAGTAT
TCTGTCTCCTCCCCACTCAGGTCAACCAGAGCTGGCGCAAAGAGCGCATT
5 CTGAACGTGCCCCCTGTGCAAAGAGGACTGTGAGCGCTGGTGGGAGGACTG
TCGCACCTCCTACACCTGCAAAAGCAACTGGCACAAAGGCTGGAATTGGA
CCTCAGGTGAGGACCTGAGGAGATAAGATGAGGAGTGGGAGTGGGGCTTT
GGGGTTGGGAGGGGTGCGGTCTGGCCCAGAAGCTAAGGGTCTTACGTTCT
CCTCCCTCAGGGATTAATGAGTGTCCGGCCGGGGCCCTCTGCAGCACCTT
10 TGAGTCCTACTTCCCCACTCCAGCCGCCCTTTGTGAAGGCCTCTGGAGCC
ACTCCTTCAAGGTCAGCAACTATAGTCGAGGGAGCGGCCGCTGCATCCAG
ATGTGGTTTGA CT CAGCCCAGGGCAACCCCAATGAGGAGGTGGCCAAGTT
CTATGCTGCGGCCATGAATGCTGGGGCCCCGTCTCGTGGGATTATTGATT
CCTGAtccaagaagggtcctctgggggttcttccaacaacctatttctaata
15 gacaaatccacatg

SEQ ID NO.: 16

FOLR3 (FR-gamma) mRNA sequence:

from human genome browser.

20 CDS is in capitals
>NM_000804.2
agagcctggacctacagecgtgttggtggaggtcctgcctccaggaatag
ATGGACATGGCCTGGCAGATGATGCAGCTGCTGCTTCTGGCTTTGGTGAC
TGCTGCGGGGAGTGCCCAGCCCAGGAGTGCGCGGGCCAGGACGGACCTGC
25 TCAATGTCTGCATGAACGCCAAGCACCAAGACACAGCCCAGCCCCGAG
GACGAGCTGTATGGCCAGTGCAGTCCCTGGAAGAAGAATGCCTGCTGCAC
GGCCAGCACCCAGCCAGGAGCTGCACAAGGACACCTCCCGCCTGTACAACT
TTAACTGGGATCACTGTGGTAAGATGGAACCCACCTGCAAGCGCCACTTT
ATCCAGGACAGCTGTCTCTATGAGTGCTCACCCAACCTGGGGCCCTGGAT
30 CCGGCAGGTCAACCAGAGCTGGCGCAAAGAGCGCATTCTGAACGTGCCCC
TGTGCAAAGAGGACTGTGAGCGCTGGTGGGAGGACTGTGCACCTCCTAC
ACCTGCAAAAGCAACTGGCACAAAGGCTGGAATTGGACCTCAGGGATTAA
TGAGTGTCCGGCCGGGGCCCTCTGCAGCACCTTTGAGTCCTACTTCCCCA
CTCCAGCCGCCCTTTGTGAAGGCCTCTGGAGCCACTCCTTCAAGGTCAGC
35 AACTATAGTCGAGGGAGCGGCCGCTGCATCCAGATGTGGTTTGACTCAGC
CCAGGGCAACCCCAATGAGGAGGTGGCCAAGTTCTATGCTGCGGCCATGA
ATGCTGGGGCCCCGTCTCGTGGGATTATTGATTCCCTGA tccaagaagggt
cctctgggggttcttccaacaacctatttctaatagacaaatccacatgaaa
aaaaaaa
40

SEQ ID NO.: 17

FOLR3 (FR-gamma) amino acid sequence:

Predicted from human genome browser (correlated to swiss protein).

A putative signal peptide (1-25) is most likely cleaved off during translation. The

5 cleavage site is indicated by an arrow.

It has three putative glycosylation sites (marked Glc-Nac).

No GPI anchor reported

>NP_000795 length=245

10 NH3-MDMAWQMMQLLLLALVTAAGSAQPR SARARTDLLNVCMNAKHHKTQPSPE

↑

DELYGQCSPWKKNACCTASTSQELHKDTSRLYNFNWDHCGKMEPTCKRHF

15

IQDSCLYECSPNLGPWIRQVNQSWRKERILNVPLCKEDCERWWEDCRTSY

Glc-Nac

TCKSNWHKGNWNTSGINECPAGALCSTFESYFPTPAALCEGLWSHSFKVS

20

Glc-Nac

NYSRGSGRCIQMWFDSAQGNPNEEVAKFYAAAMNAGAPSRGIIDS-COOH

Glc-Nac

25

SEQ ID NO.: 18

Peptide F10: LLLGDQL

SEQ ID NO.: 19

30 Peptide B12: KDLFGYQ

SEQ ID NO.: 20

Peptide A10: KVLDSAT

35 SEQ ID NO.: 21

Peptide C10: GKNPNHP

- SEQ ID NO.: 22
Peptide C5: GSGVPSL
- 5 SEQ ID NO.: 23
Peptide C9: ASNVQSH
- SEQ ID NO.: 24
Peptide D11: LGLPSGD
- 10 SEQ ID NO.: 25
Peptide B11: MDHSPER
- SEQ ID NO.: 26
Peptide D12: SGYTMSS
- 15 SEQ ID NO.: 27
Peptide H11: PYLNTVL
- SEQ ID NO.: 28
Peptide G10: AMGSMIW
- 20 SEQ ID NO.: 29
Peptide F1: SVISNMA
- 25 SEQ ID NO.: 30
Peptide G7: LLNLVNN
- SEQ ID NO.: 31
Peptide E8: LHSRGFA
- 30 SEQ ID NO.: 32
Peptide D7: WLLSAKT
- SEQ ID NO.: 33
Peptide A11: SGGLWPF
- 35 SEQ ID NO.: 34
Peptide E12: SLYSSKA

SEQ ID NO.: 35

Peptide B12: MLGAGWH

5

SEQ ID NO.: 36

A10: KVLDSAT

>gi|145701030|ref|NP_001077416.1| transmembrane
protease, serine 4 isoform 3 [Homo sapiens]
MLQDPDSQPLNSLDVKPLRKPRIPMETFRKVGIPIIIIALLSLASIIIVVLIK
VILDKYYFLCGQPLHF
IPRKQLCDGELDCPLGEDEEHCVKSFPEGPAVAVRLSKDRSTLQVLDSATGNWF
SACFDNFTEALAEATAC
RQMGYSRAVEIGPDQDL DVVEITENSQELMRNSSGPCLSGSLVSLHCLACGKS
LKTPRVVGEEASVDS
WPWQVSIQYDKQHVC GGSILDPHWVLTAAHCFRKHTDVFNWKVRAGSDKLGSFP
SLAVAKIIIIIEFNPMY
PKDNDIALMKLQFPLTFSGTVRPICLPFFDEELTPATPLWIIIGWGFTKQNGGKM
SDILLQASVQVIDSTR
CNADDAYQGEVTEKMM CAGIPEGGVDT CQGDSGGPLMYQSDQWHVVGIVSWGYG
CGGPSTPGVYTKVSAY
LNWIYNVWKAEL (115-119)
(Peptide sequence in Cys-rich domain, not in TM)

25

SEQ ID NO.: 37

C9: ASNVQSH

>gi|27262659|ref|NP_005202.2| colony stimulating
factor 1 receptor precursor [Homo sapiens]
MGPGVLLLLLVATAWHGQGIPVIEPSVPELVVKPGATVTLRCVGN GSVEWDGPP
SPHWTLYSDGSSSILS
TNNATFQNTGTYRCTEPGDPLGGSAAIHLYVKD PARPWNVLAQE VVVVFEDQDAL
LPCLLTDPVLEAGVSL
VRVRGRPLMRHTNYSFSPWHGFTIHRAKFIQSQDYQCSALMGGRKVM SISISIRLK
VQKVIPGPPALTLVPA
ELVRIRGEAAQIVCSASSVDVNF DVFLQHNNTKLAIPQQSDFHNNRYQKVLTLN
LDQVDFQHAGNYSCVA
SNVQGKHSTSMFFRVVESAYLNLSS EQNLIQEVTVGEGLNLKVMVEAYPGLQGF
NWTYLGPFSDHQPEPK
LANATTKD TYRHTFTLSLPRLKPSEAGRYSFLARNPGGWRALTFELTLRYPPEV
SVIWT FINGSGTLLCA
ASGYPQPNVTWLQCSGHTDRCDEAQVLQVWDDPYPEVLSQE PFHKVTVQSLLT V
ETLEHNQTYECRAHNS
VGSGSWAFIPISAGAH THPPDEF LFTPVVVACMSIMALLLLLLLLLLLLLYKYKQKP
KYQVRWKIIESYEGNS
YTFIDPTQLPYNEKWEFFRN NLQFGKTLGAGAFGKVVEATAFGLGKEDAVLKVA
VKMLKSTAHAD EKEAL
MSELKIMSHLGQHENIVNLLGACTHGGPVLVITEYCCYGDLLNFLRRKAEAMLG
PSLSPGQDPEGVDYK

45

NIHLEKKYVRRDSGFSSQGVDITYVEMRPVSTSSNDSFSEQDLDKEDGRPLELRD
LLHFSSQVAQGMAFLA
SKNCIHRDVAARNVLLTNGHVAKIGDFGLARDIMNDSNYIVKGNARLPVKWMAPE
ESIFDCVYTVQSDVWS
5 YGILLWEIFSLGLNPYPGILVNSKFYKLVKDGYPMAQPAFAPKNIYSIMQACWA
LEPTHRPTFQQICSL
QEQAQEDRRERDYNLPSSSRSGSGSGSSSELEEESSEHLTCCEQGDIAPLL
QPNNYQFC (280-284)
(Peptide in IGC2 domain, not in TM)

10

SEQ ID NO.: 38

D12: SGYTMSS

>gi|40254464|ref|NP_002294.2| leptin receptor isoform
1 [Homo sapiens]

15

MICQKFCVLLHWEFIYVITAFNLSYPITPWRFKLSCMPPNSTYDYFLLPAGLS
KNTSNSNGHYETAVEP
KFNSSGTHFSNLSKTTFHCCFRSEQDRNCSLCADNIEGKTFVSTVNSLVFQQID
ANWNIQCWLKGDCLKF
ICYVESLFFKNLFRNINYKVHLLYVLPEVLEDSPPLVPQKGSFQMVHCNCSVHECC
20 ECLVPVPTAKLNDTLL
MCLKITSGGVIFQSPLMSVQPINMVKPDPPLGLHMEITDDGNLKIWSPPPLVP
FPLQYQVKYSENSTTV
IREADKIVSATSLLVDSILPGSSYEYVQVRGKRLDGPGLIWSWSTPRVFTTQDVI
YFPPKILTSVGSNVSF

25

HCIYKKENKIVPSKEIVWWMNLAEKIPQSQYDVVSDHVSQVTFNLTNETKPRGK
FTYDAVYCCNEHECHH
RYAELYVIDVNINISCETDGYLTKMTCRWSTSTIQSLAESTLQRLYHRSSLYCS
DIPSIHPISEPKDCYL
QSDGFYECIFQPIFLLSGYTMWIRINHSLGSLDSPPTCVLPDSVVKPLPPSSVK
30 AEITINIGLLKISWEK
PVFPENNLQFQIRYGLSGKEVQWKMYEVYDAKSKSVSLPVPDLCAVYAVQVRCK
RLDGLGYWSNWSNPAY
TVVMDIKVPMRGPEFWRIINGDTMKKEKNVTLLWKPLMKNDLCSVQRYVINHH
TSCNGTWSEDEVGNHTK

35

FTFLWTEQAHTVTVLAINSIGASVANFNLTFSWPMSKVNIVQSL SAYPLNSSCV
IVSWILSPSDYKLMYF
IIEWKNLNEDEGEIKWLRISSSVKKYYIHDHFIPIEKYQFSLYPIFMEGVGKPKI
INSFTQDDIEKHQSDA
GLYVIVPVIISSSILLGTLTLLISHQRMKKLFWEDVPNPKNCSWAQGLNFQKPEP
40 FEHLFIKHTASVTCGP
LLEPETISEDISVDTSWKNKDEMMPPTTVVSLSTTDLEKGSVCISDQFNSVNF
SEAEGTEVTYEDESQR
QPFVKYATLISNSKPSETGEEQGLINSSVTKCFSSKNSPLKDSFSNSSWEIEAQ
AFFILSDQHPNII SPH

45

LTFSEGLDELLKLEGNFPEENNDKKSIIYLGVTSIKKRESGVLLTDKSRVSCPF
PAPCLFTDIRVLQDSC
SHFVENNINLGTSSKKTFFASYMPQFQTCSTQTHKIMENKMCDLTV (507-
511)

50

(Peptide sequence not in known domains, not in TM)

SEQ ID NO.: 39

F10: LLLGDQL

>gi|21314776|ref|NP_057029.8| solute carrier family
35, member C2 isoform a [Homo sapiens]
5 MGRWALDVAFLWKAVLTGLVLLYYCFSIGITFYNKWLTKSFHFPLEMTMLHLA
VIFLFSALSRALVQCS
SHRARVVLWADYLRRVAPTALATALDVGLSNWSFLYVTVSLYTMKSSAVLFI
LIFSLIFKLEELRAAL
VLVLLIAGGLFMFTYKSTQFNVEGFALVLGASFIGGIRWTLTQMLLQKAELGL
10 QNPIDTMFHLQPLMFL
GLFPLFAVFEGHLSTSEKIFRFQDTGLLLRVLGSLFLGGILAFGLGFSEFLLV
SRTSSLTSLIAGIFKE
VCTLLAAHLLGDQISLLNWLGFALCLSGISLHVALKALHSRGDGGPKALKGLG
SSPDLELLLRSSQREE GDNEEEYFVAQGGQ (290-294)
15 (Peptide sequence not in TMs)

SEQ ID NO.: 40

E8: LHSRGFA

>gi|21314776|ref|NP_057029.8| solute carrier family
35, member C2 isoform a [Homo sapiens]
20 MGRWALDVAFLWKAVLTGLVLLYYCFSIGITFYNKWLTKSFHFPLEMTMLHLA
VIFLFSALSRALVQCS
SHRARVVLWADYLRRVAPTALATALDVGLSNWSFLYVTVSLYTMKSSAVLFI
LIFSLIFKLEELRAAL
25 VLVLLIAGGLFMFTYKSTQFNVEGFALVLGASFIGGIRWTLTQMLLQKAELGL
QNPIDTMFHLQPLMFL
GLFPLFAVFEGHLSTSEKIFRFQDTGLLLRVLGSLFLGGILAFGLGFSEFLLV
SRTSSLTSLIAGIFKE
VCTLLAAHLLGDQISLLNWLGFALCLSGISLHVALKALHSRGDGGPKALKGLG
30 SSPDLELLLRSSQREE GDNEEEYFVAQGGQ (319-323)
(Same as for F10 peptide and also not in TM)

>gi|125661045|ref|NP_006855.2| leukocyte
immunoglobulin-like receptor, subfamily B, member 3
35 isoform 2 [Homo sapiens]
MTPALTALLCLGLSLGPRTRVQAGFPKPTLWAEPSVISWGSPVTIWCQGSQE
AQEYRLHKEGSPEPLD
RNNPLEPKNKARFSIPSMTEHHAGRYRCHYYSSAGWSEPSDPLEMVMTGAYSKP
TLSALPSPVVASGGNM
40 TLRCGSQKGYHHFVLMKEGEHQLPRTLDSQQ LHSRGFQALFPVGPVTPSHRWRF
TCYYYYTNTPWVWSHP
SDPLEILPSGVSRKPSLLTLQGPVLAPGQSLTLQCGSDVGYNRFVLYKEGERDF
LQRPGQQPQAGLSQAN
FTLGPVSPSNGGQYRCYGAHNLSSEWSAPSDPLNILMAGQIYDTVSLSAQPGPT
45 VASGENVTLLCQSWWQ
FDTFLLTKEGAHPPLRLRSMYGAHKYQAEFPMSPV TSAHAGTYRCYGSYSSNP
HLLSHPSEPLELVVSG
HSGGSSLPTGPSTPGLGRYLEVLIGVSVAFVLLLFLLLFLLLRRQRHSHKRT
SDQRKTD FQRPAGAAE

TEPKDRGLLRRSSPAADVQEEENLYAAVKDTQSEDRVELDSQSPHDEDPQAVTYA
PVKHSSPRREMASPPS
SLSGEFLDTKDRQVEEDRQMDTEAAASEASQDVTYAQLHSLTLRRKATEPPPSQ
EGEPPAEPSIYATLAI

5 H (172-177)
(Peptide in Ig domain)

SEQ ID NO.: 41

H11: PYLNTVL

10 >gi|13194199|ref|NP_075379.1| transmembrane 6
superfamily member 1 [Homo sapiens]
MSASAATGVFVLSLSAIPVTYVFNHLAAQHDSWTIVGVAALILFLVALLARVLV
KRKPPRDPLFYVYAVF
GFTSVVNLIIGLEQDGIIDGFMTHYLREGEPYLNTAYGHMICYWDGSAHYLMYL
15 VMVAIAWEETYRTIG
LYWVGSIIMSVVVFVPGNIVGKYGTRICPAFFLSIPYTCLPVWAGFRIYNQPSE
NYNYPISKVIEAQAKD
LLRRPFDLMLVCLLLATGFCLFRGLIALDCPSELCLRYTQFQEPYLLKDPAAYP
KIQMLAYMFYSVPYFV
20 TALYGLVVPGCWMPDITLIHAGGLAQAFSHIGASLHARTAYVYRVPEEAKIL
FLALNIVYGVLPQLLA YRCIYKPEFFIKTKAEKVE (101-105)
(Peptide sequence not in TMs)

25 SEQ ID NO.: 42

G10: AMGSMIW

>gi|47132557|ref|NP_997647.1| fibronectin 1 isoform 1
preproprotein [Homo sapiens]
MLRGPGPGLLLLAVQCLGTAVPSTGASKSKRQAQQMVQPQSPVAVSQSKPGCYD
30 NGKHYQINQQWERTYL
GNALVCTCYGGSRGFNCEKPEAEETCFDKYTGNTRYRGDTYERPKDSMIWDCT
CIGAGRGRISCTIANR
CHEGGQSYKIGDTWRRPHETGGYMLECVCLGNGKGEWTCKPIAEKCFDHAAGTS
YVVGETWEKPYQGWM
35 VDCTCLGEGSGRITCTSRNRCNDQDTRTSYRIGDTWSKKDNRGNLLQCICTGNG
RGEWK CERHTSVQTTS
SGSGPFTDVRAAVYQPQPHPPYGHCVTD SGVVYSVGMQWLKTQGNKQMLCT
CLGNGVSCQETAVTQT
YGGNSNGEPCVLPFTYNGRTFYSC TTEGRQDGHLCSTTSNYESQDQKYSFCTDH
40 TVLVQTRGGNSNGALC
HFPFLYNNHNYTDCTSEGRDNMKWCGTTQNYDADQKFGFCPMAAHEEICTTNE
GVMYRIGDQWDKQHDM
GHMMRCTCVGNRGEWTCIAYSQLRDQCI VDDITYNVNDTFHKRHEEGHMLNCT
CFGQGRGRWKCDPVDQ
45 CQDSETGTIFYQIGDSWEKYVHGVRYQCYCYGRGIGEWHCQPLQTYPSSSGPVEV
FITETPSQPN SHPIQW
NAPQPSHISKYILRWRPKNSVGRWKEATIPGHLNSYTIKGLKPGVVYEGQLISI
QQYGHQEVTRFDFTTT
STSTPVTSTNTVTGETTPFSPLVATSESVTEITASSFVVSWSASDTVSGFRVEY
50 ELSEEGDEPQYLDLPS

TATSVNIPDLLPGRKYIVNVYQISEDGEQSLILSTSQTAPDAPPDPTVDQVDD
TSIVVRWSRPQAPITG
YRIVYSPSVEGSSTELNLPETANSVTLSDLQPGVQYNITIYAVEENQESTPVVI
QQETTGTTPRSDTVPSP
5 RDLQFVEVTDVKVTIMWTPPESAVTGYRVDVIPVNLPGEHGQRLPISRNTFAEV
TGLSPGVTTYFVKVFAV
SHGRESKPLTAQQOTTKLDAPTNLQFVNETDSTVLVRWTPPRAQITGYRLTVGLT
RRGQPRQYNVGPSVSK
YPLRNLQPASEYTVSLVAIKGNQESPKATGVFTTLQPGSSIPPYNTEVTETTIV
10 ITWTPAPRIGFKLGVR
PSQGGEAPREVTSDSGSIVVSGLTPGVEYVYTIQVLRDGQERDAPIVNKVVTPL
SPPTNLHLEANPDTGV
LTVSWERSTTPDITGYRITTTPTNGQQGNSLEEVVHADQSSCTFDNLSPGLEYN
VSVYTVKDDKESVPIS
15 DTIIPEVPQLTDLSFVDITDSSIGLRWTPLNSSTIIGYRITVVAAGEGIPIFED
FVDSSVGYYTVTGLEP
GIDYDISVITLINGGESAPTTLTQQTAVPPPTDLRFTNIGPDTMRVTWAPPSI
DLTNFLVRYSPVKNEE
DVAELSI SPSDNAVVLTNLLPGTEYVVS SVSSVYEQHESTPLRGRQKTGLDSPTG
20 IDFSDITANSFTVHWI
APRATITGYRIRHHPEHFSGRPREDRVPHSRNSITLTNLTPGTEYVVSIVALNG
REESPLLIGQQSTVSD
VPRDLEVVAATPTSLLISWDAPAVTVRYRITYGETGGNSPVQEFTVPGSKSTA
TISGLKPGVDYTITVY
25 AVTGRGDSPASSKPISINYRTEIDKPSQM QVTDVQDNSISVKWLPSSSPVTGYR
VTTTPKNGPGPTKTKT
AGPDQTEMTIEGLQPTVEYVVS VYAQNPSGESQPLVQTAVTNIDRPKGLAFTDV
DVDSIKIAWESPQGQV
SRYRVTYSSPEDGIHELFPAPDGEEDTAELQGLRPGSEYTVSVVALHDDMESQP
30 LIGTQSTAIPAPTDLK
FTQVTPTSLSAQWTPPNVQLTGYRVRVTPKEKTGPMKEINLAPDSSSVVVSGLM
VATKYEVS VYALKDTL
TSRPAQGVVTTLENVSPRRARVTDATETTITISWRKTETITITGFQVDAVPANG
QTPIQRTIKPDVRSYT
35 ITGLQPGTDYKIYLYTLNDNARSSPVVIDASTAIDAPSNLRLATTPNSLLVSW
QPPRARITGYIIKYEK
PGSPPREVVRPRPRPGVTEATITGLEPGTEYTIYVIALKNNQKSEPLIGRKKTDE
LPQLVTLPHPNLHGPE
ILDVPSTVQKTPFVTHPGYDTGNGIQLPGTSGQQPSVGQQMIFEEHGFRRTPP
40 TTATPIRHRPRPYPPN
VGEEIQIGHIPREDVDYHLYPHGPGLNPNASTGQEALSQTTISWAPFQDTSEYI
ISCHPVGTDEEPLQFR
VPGTSTSATLTGLTRGATYNIIVEALKDQQRHKVREEVTVGNSVNEGLNQPTD
DSCFDPYTVSHYAVGD
45 EWERMSESGFKLLCQCLGFGSGHFRCDSSRWCHDNGVNYKIGEKWDRQGENGQM
MSCTCLGNGKGFEKCD
PHEATCYDDGKTYHVGEQWQKEYLGAICSCCTCFGGQRGWRCDNCRPPGGEPSPE
GTTGQSYNQYSQRYHQ RTNTNVNCPICFEMPLDVQADREDSRE (118-121)
(Peptide sequence in fibronectin domain, not in TM)
50

SEQ ID NO.: 43

F1: SVISNMA

>gi|50233846|ref|NP_001001961.1| olfactory receptor,
family 13, subfamily C, member 3 [Homo sapiens]

5 MIVQLICTVCFLAVNTFHVRSSEDFLKADDMGEINQTLVSEFLLLGLSGYPKIE
IVYFALILVMYLVILI
GNGVLIIASIFDSHFHTPMYFFLGNLSFLDICYTSSSV PSTLVSLISKRNISF
SGCAVQMFFGFAMGST
ECLLLGMMAFDRYVAICNPLRYPILSKVAYVLMASVSWLSGGINS AVQTLLAM
10 RLPFCGNNIINHFACE
ILAVLKLACADISLNIITMVISNMAFLVLPLMVIFFSYMFILY TILQMNSATGR
RKAFTCSAHLTVVII
FYGTIFFMYAKPKSQDLIGEEKLQALDKLISLFYGVVTPMLNPILYSLRNKDVK
AAVKYLLNKKPIH (230-235)
15 (Peptide sequence not in TMs)

SEQ ID NO.: 44

D7: WLLSAKT

>gi|32698841|ref|NP_872308.1| transmembrane protease,
serine 11B [Homo sapiens]

20 MYRHGISSQRSWPLWTTIFIFLGVAAILGVTIGLLVHFLAVEKTYYYQGDFHIS
GVTYNDNCENAASQAS
TNLSKDIETKMLNAFQNSSIYKEYVKSEVIKLLPNANGSNVQLQLKFKFPPAEG
VSMRTKIKAKLHQMLK
25 NNMASWNAVPA SIKLMEISKAASEMLTNNCCGRQVANSIITGNKIVNGKSSLEG
AWPWQASMQWKGRHYC
GASLISSRWLLSAAHCF AKKNNSKDWTVNF GVVVNKP YMTRKVQNIIFHENYSS
PGLHDDIALVQLAEEV
SFTEYIRKICLPEAKMKLSENDNVVVTGWGTYMNGSFPVILQE AFLKIIDNKI
30 CNASYAYS GFVTD SML
CAGFMSGEADACQND SGGPLAYPDSRNIWHLVGIVSWGDGCGKKNKPGVYTRVT
SYRNWITSKTGL (219-223)
(Peptide sequence in trypsin domain, not in TM)

35 SEQ ID NO.: 45

E12: SLYSSKA

>gi|126116583|ref|NP_001005410.2| Fc fragment of IgG,
low affinity IIc, receptor for isoform 2 [Homo
sapiens]

40 MGILSFLPVLATESDWADCKSPQPWGHMLLWTAVLFLAPVAGTPAAPPKAVLKL
EPQWINVLQEDSVTLT
CRGTHSPESDSIPWFHNGNLIPHTHTQPSYRFKANNND SGEYTCQTGQTSLSDPV
HLTVLSEWLVLQTPHL
EFQEGETIVLRCHSWKDKPLVKVTFFQNGKSKKFSRSDPNFSIPQANHSHSGDY
45 HCTGNIGYTLYSSKPV
TITVQAPSSSPMGIIIVAVVTGI AVAAI VAAVVALIYCRKKRISANSTD PVKAAQ
FEMLSCTHLDVK (204-208)
(Peptide sequence in Ig domain, not in TM)

SEQ ID NO.: 46

B12: MLGAGWH

>gi|16933559|ref|NP_060145.2| protocadherin LKC

5 precursor [Homo sapiens]

MAQLWLSCFLLPALVVSVAANVAPKFLANMTSVILPEDLPVGAQAFWLVAEDQD
NDPLTYGMSGPNAYFFAVTPKTGEVKLASALDYETLYTFKVTISVSDPYIQVQREMLVIVEDRNDNAPVF
QNTAFST SINETLPVG10 SVVFSVLAVDKDMGSAGMVVYSIEKVIPSTGDSEHLFRILANGSIVLNGSLSYN
NKSAFYQLELKACDLGGMYHNTFTIQCSLPVFLSISVVDQPDLPQFVREFYASVAEDAAGTSVLTVE
AVDGDKGINDPVIYSI15 SYSTRPGWFDIGADGVIRVNGSLDREQLLEADEEVQLQVTATETHLNIYGQEAK
VSIWVTVRVMDVNDHKPEFYNCSLPACTFTPEEAQVNFTGYVDEHASPRIPIDDLTMVVYDPDKGSNGTF
LLSLGGPDAAEAFSVSPERAAGSASVQVLVRVSALVDYERQTAMAVQVVATDSVSQNF SVAMVTIHLRDIN
DHRPTFPQSLYVLTVP20 EHSATGSVVTDSIHATDPDTGAWGQITYSLLPGNGADLFQVDPVSGTVTVRNGE
LLDRESQAVYYLT LQATDGGNLSSSTTLQIHLLDINDNAPVVSGSYNIFVQEEEGNVSVTIQAHDNDEPG
TNSRLLFNLLPGPYS25 HNFSLDPDTGLLRNLGPLDREAIDPALEGRIVLTVLVSDCGEPVLGTKVNVTTIT
VEDINDNLPIFNQSSYNFTVKEEDPGVLVG VVKAWDADQTEANNRISFSLSGSGANYFMIRGLVLGAGWA
EGYLRLPPDVSLDYETQPVFNLTVSAENPDPQGGETIVDVCVNVKDVNDNPPTLDVASLRGIRVAENGSO
HGQVAVVVASDVDTSA30 QLEIQLVNILCTKAGVDVGS LCGWF SVAANGSVYINQSKAIDYEACDLVTLVV
RACDLATDPGFQAYSNNGSLITIEDVNDNAPYFLPENKTFVIIPELVLPNREVASVRARDDDSGNNGVI
LFSILRVDFISKDGAT35 IPFQGVFSIFTSSEADV FAGSIQPVTS LDSTLQGT YQVTVQARDRPSLGPFLEA
TTTLNLFTVDQSYRSRLQFSTPKKEEVGANRQAINAALTQATR TTVYIVDIQDIDSAARARPHSYLDAYFV
FPNGSALTLD ELSVMIRNDQDSL TQLLQLGLVVLGSQESQESDLSKQLISVIIGLGVALLLVLVIMTMAF
VCVRKSYNRKLQAMKA40 AKEARKTAAGVMPSAPAIPGTNMYNTERANPMLNLPNKDLGLEYLSPSNDLDSV
SVNSLDDNSVDVDKNSQEIKEHRPPHTPPEPDPEPLSVVLLGRQAGASGQLEGPSYTNAGLDTTDL
(749-753)

45 Peptide sequence in Cadherin domain, not in TM)

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Stat3 inactivation by inhibition of the Folate receptor pathway**Claims**

- 5 1. A method for treating, ameliorating, or preventing a disorder comprising administration of a therapeutically effective amount of at least one folate receptor α inhibitor to an animal including a human being in need thereof.
- 10 2. A method of inducing apoptosis and/or cell cycle arrest in a cell, comprising contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.
- 15 3. A method of rendering a cell sensitive to an inducer of apoptosis, comprising contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.
- 20 4. The method according to any of the preceding claims, wherein said disorder is associated with 50-100 % increase in Stat3 activity.
- 25 5. The method according to any of the preceding claims, wherein said disorder is associated with a 50-100 % increase in phosphorylated Stat3.
6. The method according to any of the preceding claims comprising inhibiting Stat3 activity in a cell by contacting the cell with a therapeutically effective amount of folate receptor α inhibitor.
- 30 7. The method according to any of the preceding claims, further comprising contacting the cell with an inducer of apoptosis.
8. The method according claim 7, wherein said inducer of apoptosis is a chemotherapeutic agent.
9. The method according claim 7, wherein said inducer of apoptosis is radiation.
- 35 10. The method according any of the preceding claims, wherein said disorder is a hyperproliferative disease.

11. The method according claim 10, wherein said hyperproliferative disease is cancer.
- 5 12. The method according claim 11, wherein said cancer is selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, lung cancer, renal cancer, colon cancer, gastric cancer, and cervical cancer.
- 10 13. The method according claim 11, wherein said cancer is selected from the group consisting of breast cancer, ovarian cancer, lung cancer, and cervical cancer.
14. The method according claim 11, wherein said cancer is breast cancer.
15. The method according claim 11, wherein said cancer is prostate cancer.
- 15 16. The method according claim 10, wherein said hyperproliferative disease is psoriasis.
- 20 17. The method according to any of the preceding claims, wherein said folate receptor α inhibitor is selected from the group consisting of antibodies, polypeptides, peptides, peptide fragments, peptide aptamers, nucleic acid aptamers, small molecules, foline analogues, natural single domain antibodies, affibodies, affibody-antibody chimeras, and non-immunoglobulin folate receptor α inhibitors.
- 25 18. The method according to any of the preceding claims, wherein said folate receptor α inhibitor is a peptide.
- 30 19. The method according to claim 18, wherein said peptide is selected from the group consisting of SEQ ID NO: 18 to 35.
20. The method according to claim 19, wherein said peptide is SEQ ID NO: 18 or SEQ ID NO: 19.

21. The method according to claim 18, wherein said peptide is selected from the group consisting of SEQ ID NO: 36 to 46.
22. The method according to claim 18, wherein said peptide comprises a
5 consecutive amino acid sequence selected from a peptide selected from the group consisting of SEQ ID NO: 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, and 46.
23. The method according to claim 18, wherein said peptide is folate receptor α or a
10 fragment thereof.
24. The method according to claim 17, wherein said folate receptor α inhibitor is an antibody.
25. The method according to claim 24, wherein said antibody is developed from
15 rabbit by immunization of said rabbit with folate receptor α or fragments thereof.
26. The method according to any of claims 1 to 16, wherein said at least one folate receptor α inhibitor is siRNA.
20
27. The method according to claim 26, wherein said siRNA is selected from the group consisting of SEQ ID NOs.: 9, 10 and 11.
28. The method according to claim 27, wherein said siRNA is selected from the
25 group consisting of SEQ ID NOs.: 9 and 10.
29. The method according to claim 27, wherein said siRNA is SEQ ID NO.: 11.
30. A method for identifying a compound suitable for folate receptor α inhibition,
30 said method comprising the steps of bringing said compound in contact with a cell comprising the folate receptor α , and detecting the level of phosphorylated Stat3 in the presence and/or absence of said compound, wherein a decrease of phosphorylated Stat3 in the presence of said compound compared with the level of phosphorylated Stat3 in the absence of said compound is indicative of
35 an inhibitory effect of said compound on the folate receptor α .

31. The method according to claim 30, wherein said at least one folate receptor α inhibitor is as defined in any of claims 17 to 29.

- 5 32. A method for identifying a peptide suitable for folate receptor α inhibition, said method comprising the steps of
- a. immobilizing folate receptor α or a fragment thereof on a solid support,
 - b. expressing a peptide library in phages, which display said peptide on its surface,
 - 10 c. bringing said phages in contact with said immobilized folate receptor α or fragment thereof.
 - d. removing unbound phage,
 - e. eluting phages bound to said immobilized folate receptor α or fragment thereof, and
 - 15 f. analysing DNA comprised in said bound phages to obtain sequence of peptide suitable for folate receptor α inhibition.

33. A folate receptor α inhibitor for use as a medicament.

- 20 34. The folate receptor α inhibitor according to claim 33, wherein said folate receptor α is as defined in any of claims 18 to 29, or is identified by a method according to any of claims 30 to 32.

- 25 35. A folate receptor α inhibitor for treatment of a disorder as defined in any of claims 10 to 16.

- 30 36. The folate receptor α inhibitor according to claim 35, wherein said folate receptor α inhibitor is as defined in any of claims 17 to 29, or is identified by a method according to any of claims 30 to 32.

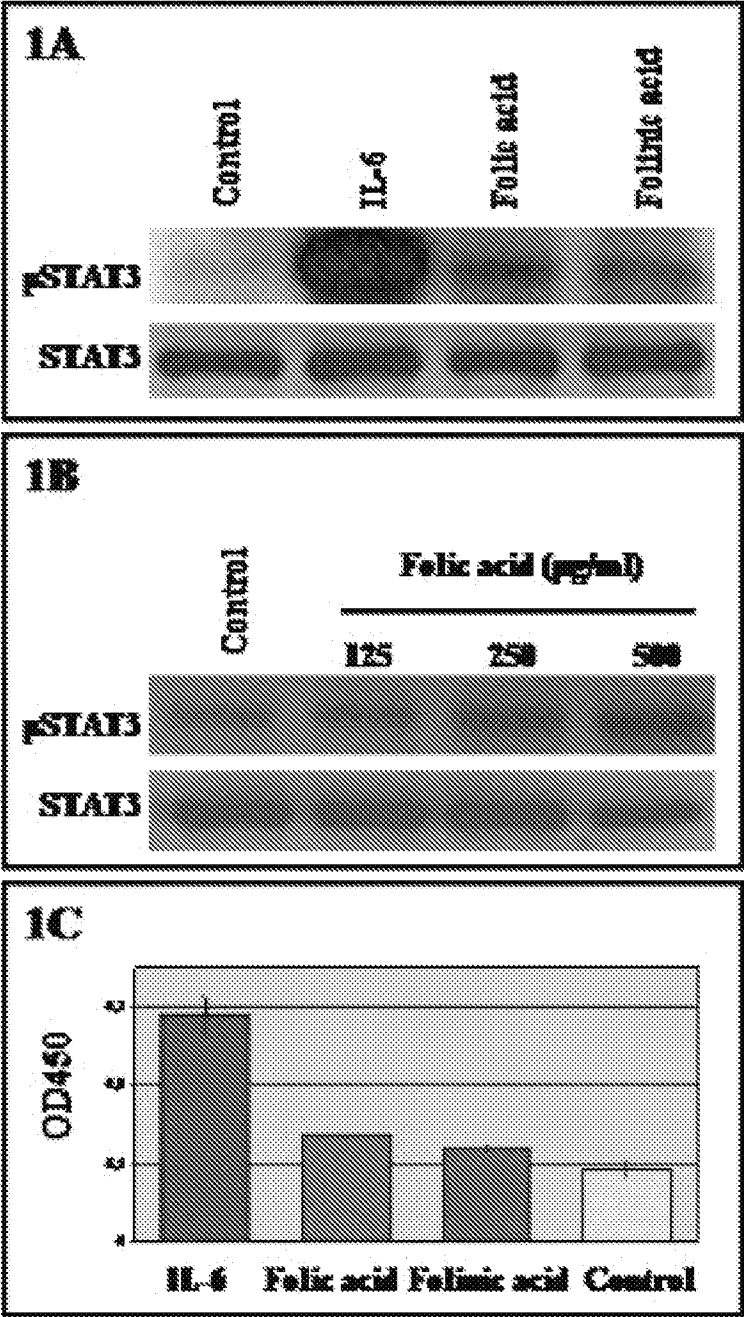
37. Use of a folate receptor α inhibitor for the manufacture of a medicament.

- 35 38. The use according to claim 37, for the preparation of a medicament for the treatment of a disease, as defined in any of claims 10 to 16.

39. The use according to any of claims 37 to 38, wherein said folate receptor α is as defined in any of claims 17 to 29, or is identified by a method according to any of claims 30 to 32.
- 5 40. A nucleotide sequence encoding at least one peptide as defined in claims 18 to 25.
41. A nucleotide sequence encoding at least one siRNA as defined in claims 26 to 29.
- 10 42. A recombinant vector comprising at least one nucleotide sequence as defined in claims 40 or 41.
43. A cell comprising a nucleotide sequence as defined in claims 40 or 41
15 integrated in said cells genome and/or carrying a recombinant vector according to claim 42 within said cell.
44. A pharmaceutical composition comprising a pharmaceutically effective amount of folate receptor α inhibitor and a pharmaceutically acceptable carrier.
- 20 45. The pharmaceutical composition according to claim 44, wherein said folate receptor α inhibitor is capable of binding to a polypeptide comprising at least one region of selected from SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3 or part thereof, thereby inhibiting binding of folate receptor α agonist.
- 25 46. The pharmaceutical composition according to claim 45, wherein said agonist is selected from the group consisting of foline and derivatives thereof.
47. The pharmaceutical composition according to any of claims 44 to 46 for
30 treatment of a disorder as defined in any of claims 10 to 16.
48. The pharmaceutical composition according to any of claims 44 to 47, wherein said folate receptor α is as defined in any of claims 17 to 29, or is identified by a method according to any of claims 30 to 32.
- 35

49. A kit comprising a pharmaceutically effective amount of folate receptor α inhibitor and instructions for administering said compound to an animal including a human being in need thereof.
- 5 50. The kit according to claim 49, wherein said folate receptor α inhibitor is as defined in any of claims 17 to 29, or is identified by a method according to any of claims 30 to 32.
- 10 51. The kit according to any of claims 49 or 50, further comprising an inducer of apoptosis.
52. The kit according to claim 51, wherein said inducer of apoptosis is a chemotherapeutic agent.
- 15 53. The kit according to any of claims 49 to 52, wherein said instructions are for administering said folate receptor α inhibitor to an animal having a disorder as defined in any of claims 10 to 16.

Figure 1



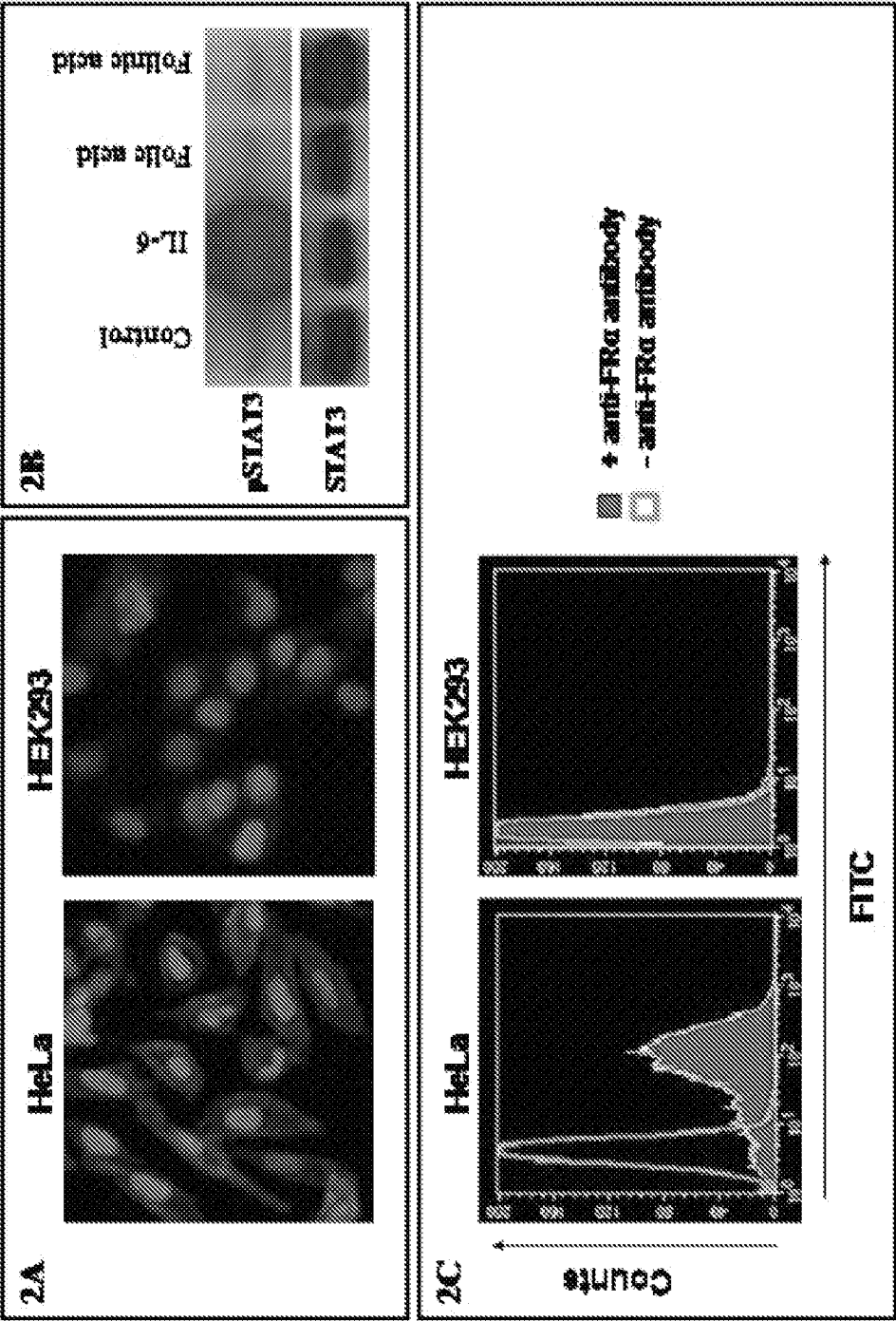


Figure 2

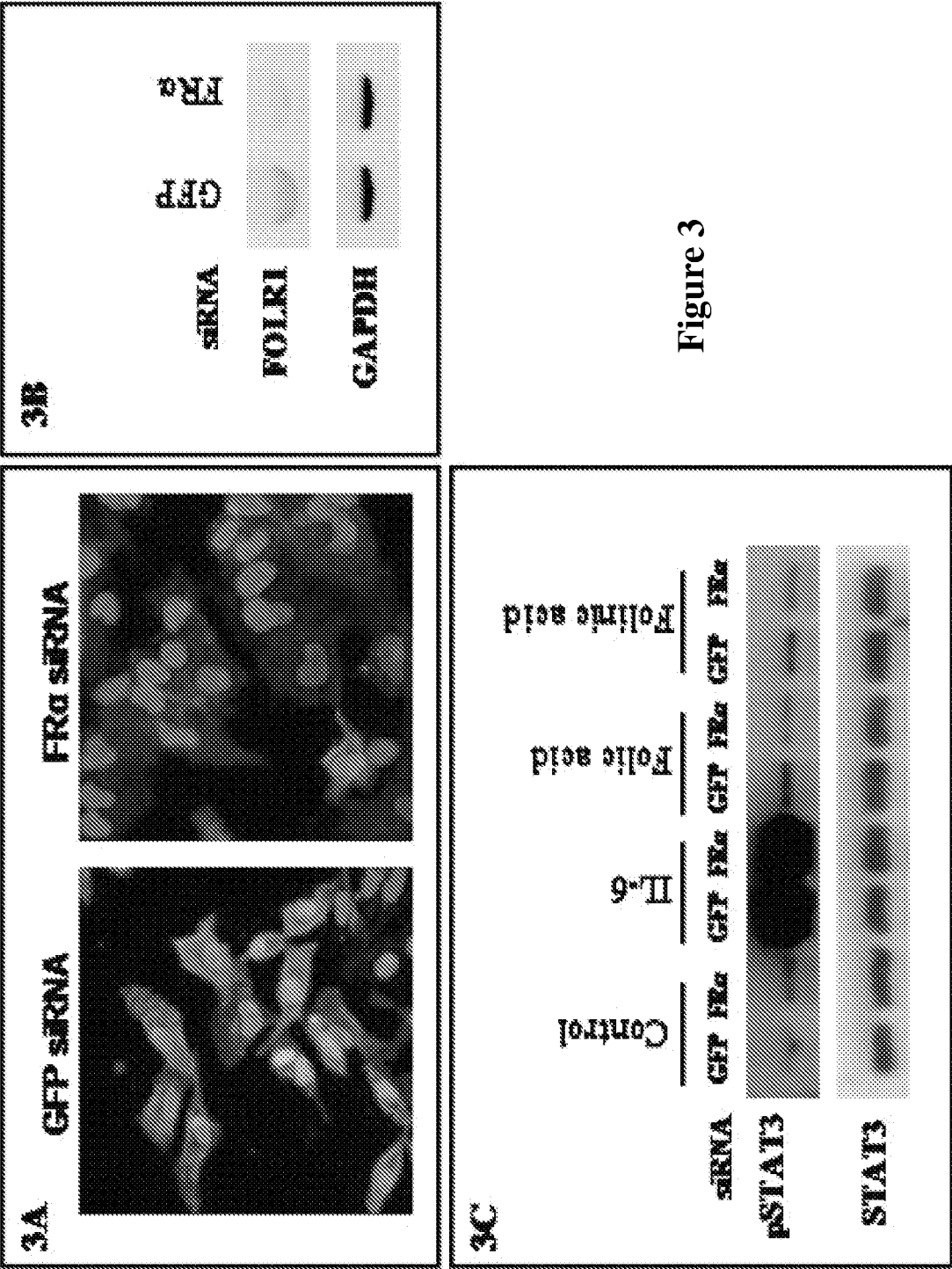


Figure 3

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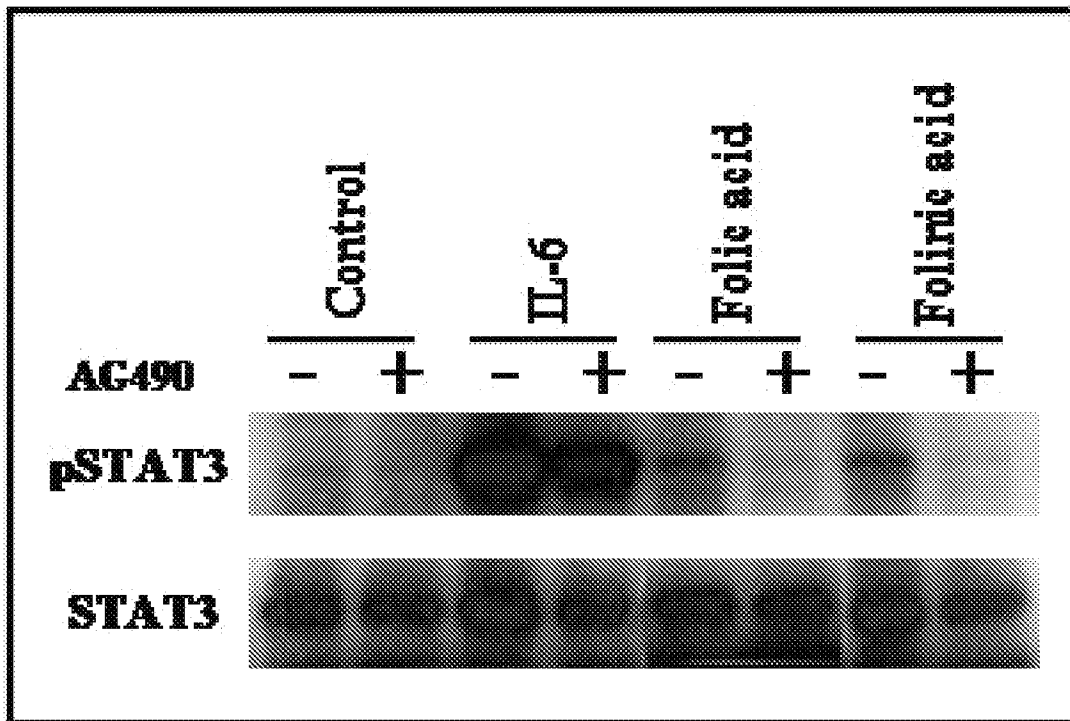
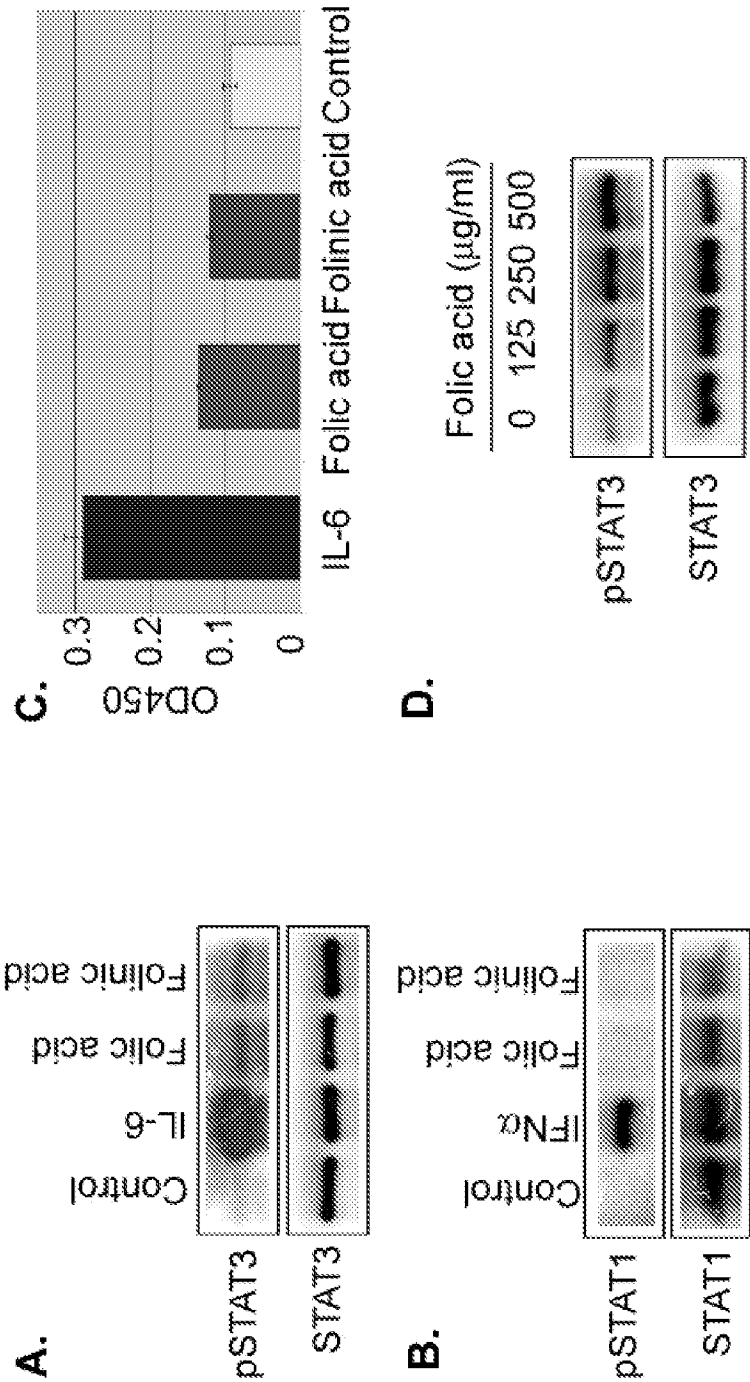


Figure 4

Figure 5



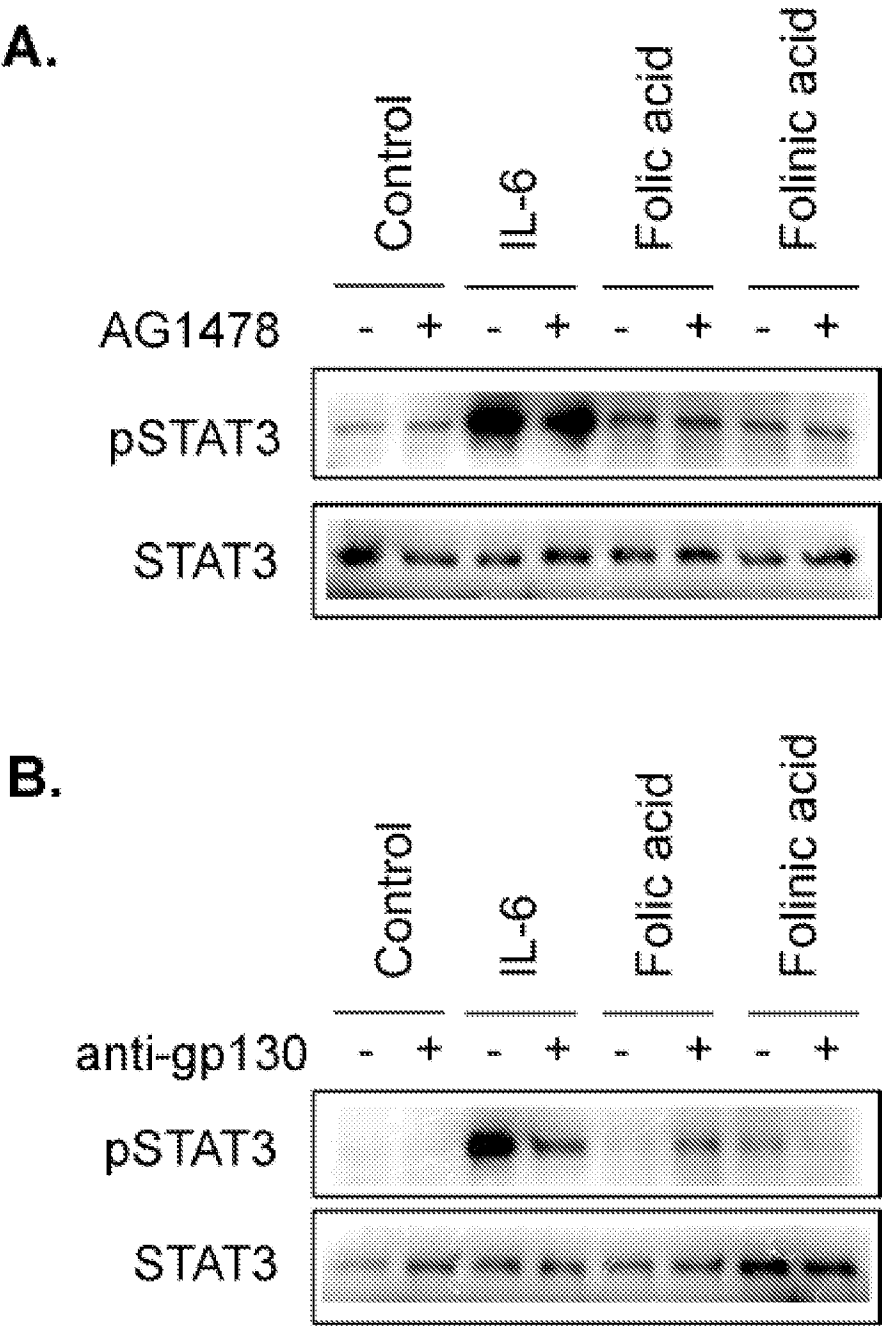


Figure 6

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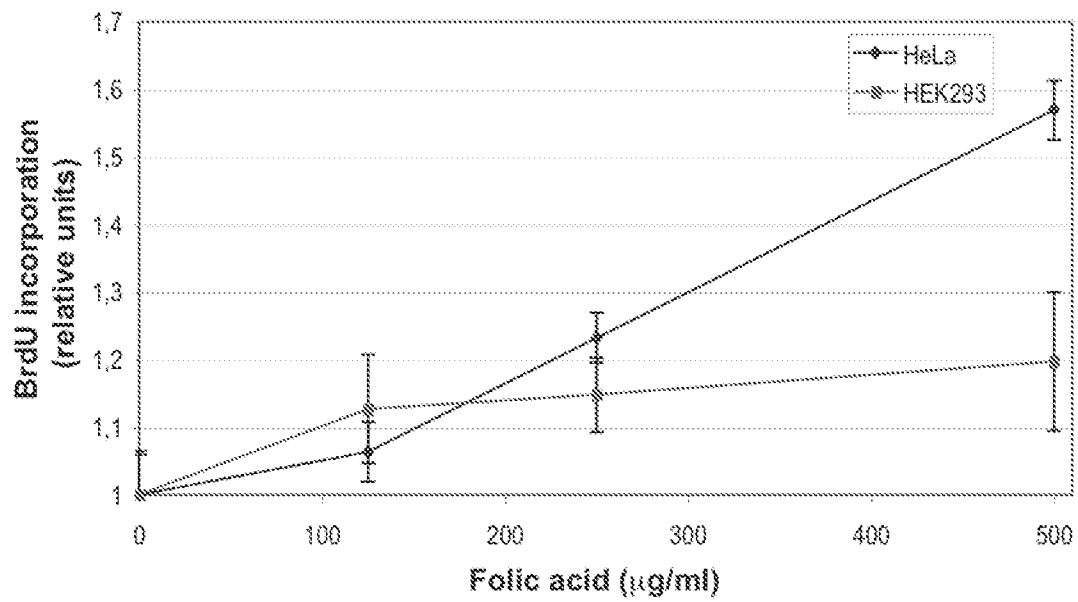
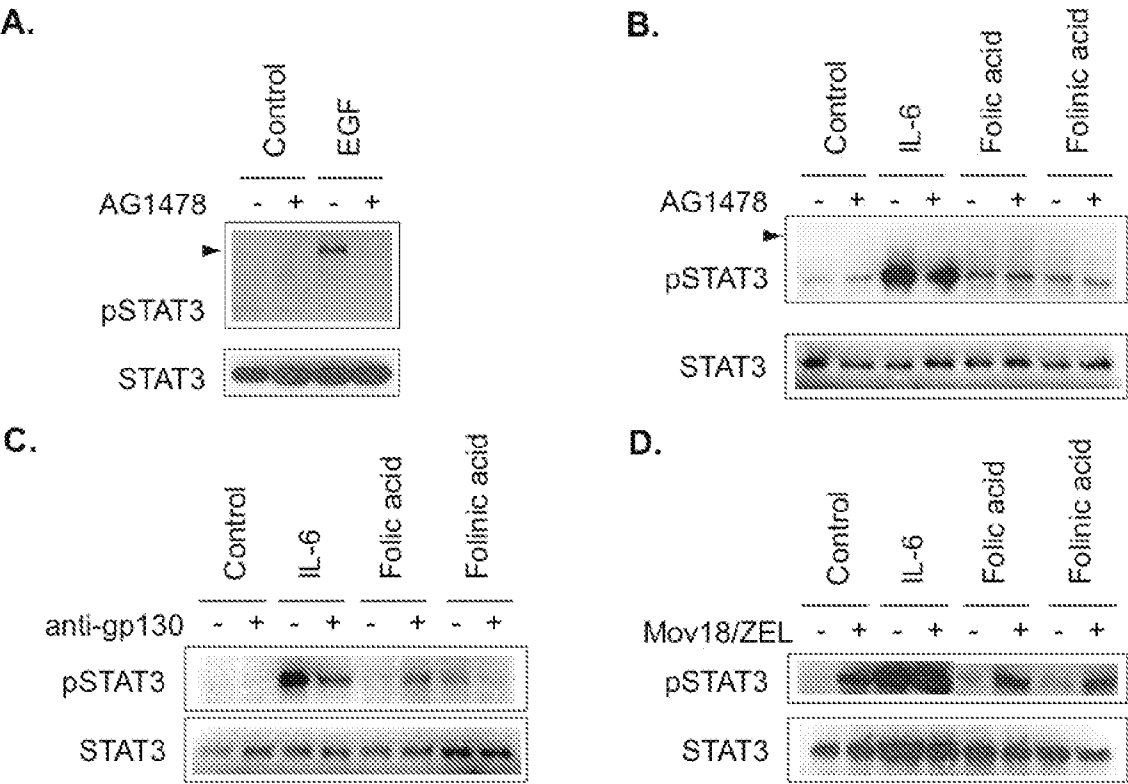
Figure 7

Figure 8



INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2008/050123

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/08 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBASE [Online] ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL; 9 March 2007 (2007-03-09), EBEL W ET AL: "Preclinical evaluation of MORAb-003, a humanized monoclonal antibody antagonizing folate receptor-alpha" XP002496400 Database accession no. EMB-2007129542 abstract & CANCER IMMUNITY 20070309 CH, vol. 7, 9 March 2007 (2007-03-09), ISSN: 1424-9634 -/--	1-17, 23-29, 32-53

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/DK2008/050123

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1 July 2003 (2003-07-01), THETI DAVINDER S ET AL: "Selective delivery of CB300638, a cyclopenta(g)quinazoline-based thymidylate synthase inhibitor into human tumor cell lines overexpressing the alpha-isoform of the folate receptor." XP002496401 Database accession no. PREV200300346207 abstract & CANCER RESEARCH, vol. 63, no. 13, 1 July 2003 (2003-07-01), pages 3612-3618, ISSN: 0008-5472	1-17, 23-29, 32-53
X	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; December 2005 (2005-12), GIBBS DAVID D ET AL: "BGC 945, a novel tumor-selective thymidylate synthase inhibitor targeted to alpha-folate receptor-overexpressing tumors" XP002496402 Database accession no. PREV200600149443 abstract & CANCER RESEARCH, vol. 65, no. 24, December 2005 (2005-12), pages 11721-11728, ISSN: 0008-5472	1-17, 23-29, 32-53
X	US 2005/009851 A1 (BAVETSIAS VASILIOS [GB] ET AL) 13 January 2005 (2005-01-13) paragraph 40, 1; claims 1-9	1-17, 23-29, 32-53
X	US 2003/050267 A1 (JHAVERI MONA SAVITRI [US] ET AL) 13 March 2003 (2003-03-13) abstract claims 1-30	1-17, 23-29, 32-53

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/DK2008/050123

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2005009851 A1	13-01-2005	NONE	
US 2003050267 A1	13-03-2003	US 2006178331 A1	10-08-2006