



HU000028349T2

(19) **HU****MAGYARORSZÁG**
Szellemi Tulajdon Nemzeti Hivatala(11) Lajstromszám: **E 028 349**(13) **T2****EURÓPAI SZABADALOM**
SZÖVEGÉNEK FORDÍTÁSA(21) Magyar ügyszám: **E 12 179990**(51) Int. Cl.: **A61K 31/18** (2006.01)(22) A bejelentés napja: **2003. 11. 26.****A61K 3144/39** (2006.01)

(96) Az európai bejelentés bejelentési száma:

EP 20030179990

(97) Az európai bejelentés közzétételi adatai:

EP 2594265 A2 **2013. 05. 22.**

(97) Az európai szabadalom megadásának meghirdetési adatai:

EP 2594265 B1 **2015. 10. 28.**

(30) Elsőbbségi adatok:

429089 P **2002. 11. 26.** **US****489473 P** **2003. 07. 22.** **US****515104 P** **2003. 10. 28.** **US**

(73) Jogosult(ak):

INSTITUTE OF VIROLOGY, 842 46 Bratislava
(SK)**Supuran, Claudiu, 50122 Florence (IT)****Scozzafava, Andrea, 50134 Florence (IT)**

(72) Feltaláló(k):

Supuran, Claudiu, 50122 Florence (IT)**Scozzafava, Andrea, 50134 Florence (IT)****Pastorekova, Silvia, 84107 Bratislava (SK)****Pastorek, Jaromir, 84107 Bratislava (SK)**

(74) Képviselő:

Danubia Szabadalmi és Jogi Iroda Kft.,**Budapest**

(54)

CAIX-specifikus inhibitorok

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.



(11) **EP 2 594 265 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention
of the grant of the patent:
28.10.2015 Bulletin 2015/44

(51) Int Cl.:
A61K 31/18^(2006.01) A61K 31/4439^(2006.01)

(21) Application number: **12179990.2**

(22) Date of filing: **26.11.2003**

(54) **Ca ix-specific inhibitors**

CA-IX-spezifische Inhibitoren

Inhibiteurs spécifiques de CA IX

(84) Designated Contracting States:
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR
HU IE IT LI LU MC NL PT RO SE SI SK TR**

(30) Priority: **26.11.2002 US 429089 P
22.07.2003 US 489473 P
28.10.2003 US 515104 P**

(43) Date of publication of application:
22.05.2013 Bulletin 2013/21

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:
03787165.4 / 1 569 633

(73) Proprietors:
• **INSTITUTE OF VIROLOGY
842 46 Bratislava (SK)**
• **Supuran, Claudiu
50122 Florence (IT)**
• **Scozzafava, Andrea
50134 Florence (IT)**

(72) Inventors:
• **Supuran, Claudiu
50122 Florence (IT)**
• **Scozzafava, Andrea
50134 Florence (IT)**
• **Pastorekova, Silvia
84107 Bratislava (SK)**
• **Pastorek, Jaromir
84107 Bratislava (SK)**

(74) Representative: **Isarpatent
Patentanwälte Behnisch Barth Charles
Hassa Peckmann & Partner mbB
Friedrichstrasse 31
80801 München (DE)**

(56) References cited:
• **LIAO S-Y ET AL: "IDENTIFICATION OF THE
MN/CA9 PROTEIN AS A RELIABLE DIAGNOSTIC
BIOMARKER OF CLEAR CELL CARCINOMA OF
THE KIDNEY", CANCER RESEARCH, AMERICAN
ASSOCIATION FOR CANCER RESEARCH,
BALTIMORE, MD, US, vol. 57, 15 July 1997
(1997-07-15), pages 2827-2831, XP000941463,
ISSN: 0008-5472**
• **BARTOSOVA M ET AL: "Expression of carbonic
anhydrase IX in breast is associated with
malignant tissues and is related to
overexpression of c-erbB2", JOURNAL OF
PATHOLOGY, CHICHESTER, SUSSEX, GB, vol.
197, no. 3, 17 April 2002 (2002-04-17), pages
314-321, XP002971533, ISSN: 0022-3417**
• **WYKOFF C C ET AL: "Expression of the hypoxia-
inducible and tumor-associated carbonic
anhydrases in ductal carcinoma in situ of the
breast", AMERICAN JOURNAL OF PATHOLOGY,
PHILADELPHIA, PA, US, vol. 158, no. 3, March
2001 (2001-03), pages 1011-1019, XP002968992,
ISSN: 0002-9440**
• **SUPURAN ET AL: "Applications of carbonic
anhydrase inhibitors in therapy",
EXP.OPIN.THER.PATENTS, vol. 12, no. 2, 2002, -
2002, pages 217-242, XP002464857,**
• **SPUPURAN ET AL: "Carbonic anhydrase
inhibitors, Sulfonamides as antitumour agents",
BIOORBAN. AND MED, CHEM, vol. 9, 2001, - 2001,
pages 703-714, XP002464858,**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 2 594 265 B1

- VERMYLEN P ET AL: "Carbonic anhydrase IX antigen differentiates between preneoplastic malignant lesions in non-small cell lung carcinoma", EUROPEAN RESPIRATORY JOURNAL, MUNKSGAARD INTERNATIONAL PUBLISHERS, COPENHAGEN, DK, vol. 14, no. 4, October 1999 (1999-10), pages 806-811, XP002968996, ISSN: 0903-1936

DescriptionFIELD OF THE INVENTION

[0001] The present invention is in the general area of medical genetics and in the fields of chemistry, biochemical engineering, and oncology. More specifically, it relates to pharmaceutical compositions comprising organic and inorganic compounds, namely aromatic and heterocyclic sulfonamides, to treat preneoplastic and/or neoplastic diseases by specifically inhibiting the carbonic anhydrase activity of the oncoprotein now known alternatively as the MN protein, the MN/CA IX isoenzyme, the MN/G250 protein or simply MN/CA IX or CA IX or MN. The present invention therefore relates to pharmaceutical compositions for use in methods of treating preneoplastic and/or neoplastic diseases characterized by MN/CA IX overexpression by administering cell membrane-impermeant, inhibitors of MN/CA IX, preferably pyridinium derivatives of aromatic and heterocyclic sulfonamides.

BACKGROUND OF THE INVENTION

[0002] The instant inventors, Dr. Silvia Pastorekova and Dr. Jaromir Pastorek, with Dr. Jan Zavada ["Zavada et al."], discovered MN/CA IX, a cancer related cell surface protein originally named MN. [73, 123; Zavada et al., U.S. Patent No. 5,387,676 (Feb. 7, 1995).] Zavada et al., WO 93/18152 (published 16 September 1993) and Zavada et al., WO 95/34650 (published 21 December 1995) disclosed the discovery of the MN gene and protein and the strong association of MN gene expression and tumorigenicity led to the creation of methods that are both diagnostic/prognostic and therapeutic for cancer and precancerous conditions. Zavada et al. disclosed further aspects of the MN/CA IX protein and the MN/CA9 gene in Zavada et al., WO 00/24913 (published 4 May 2000).

[0003] Zavada et al. cloned and sequenced the MN cDNA and gene, and revealed that MN belongs to a carbonic anhydrase family of enzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and proton [66, 72]. MN protein (renamed to carbonic anhydrase IX, CA IX) is composed of an extracellular part containing a N-terminal proteoglycan-like region and a catalytically active carbonic anhydrase domain. It is anchored in the plasma membrane by a single transmembrane region and a short intracytoplasmic tail.

[0004] Expression of CA IX is restricted to only few normal tissues [74], but is tightly associated with tumors [123]. It is also regulated by cell density *in vitro* [52] and is strongly induced by tumor hypoxia both *in vitro* and *in vivo* [121]. Numerous clinical papers describe the value of CA IX as an indicator of poor prognosis. All CA IX-related studies performed so far support the assumption made in the original Zavada et al., U.S. Patent 5,387,676 that CA IX is useful as a diagnostic and/or prognostic tumor marker and as a therapeutic target.

[0005] MN/CA IX consists of an N-terminal proteoglycan-like domain that is unique among the CAs, a highly active CA catalytic domain, a single transmembrane region and a short intracytoplasmic tail [66, 72, 74, 116]. CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, ovarian, kidney, lung, esophagus, breast, colon, endometrial, bladder, colorectal, prostate, among many other human carcinomas, contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract [8, 11, 21, 35, 41, 48, 50, 51, 56, 66, 72, 74, 86, 110, 111, 113, 116, 121, 122].

[0006] Uemura et al. [112] reported in 1997 that the G250 antigen was identical to MN/CA IX, years after MN/CA IX had been discovered and sequenced by Zavada et al. [73, 123]; see also Pastorek et al. [72] and Opavsky et al. [66]. Uemura et al. [112] stated: "Sequence analysis and database searching revealed that G250 antigen is identical to MN a human tumor-associated antigen identified in cervical carcinoma (Pastorek et al., 1994)."

MN/CA 9 and MN/CA IX - Sequence Similarities

[0007] Figure 1 A-C shows the full-length MN/CA9 cDNA sequence of 1522 base pairs (bps) [SEQ ID NO: 1], and the full-length MN/CA IX amino acid (aa) sequence of 459 aa [SEQ ID NO: 2]. Figure 2A-F provides the 10,898 bp genomic sequence of MN/CA9 [SEQ ID NO: 3].

[0008] Computer analysis of the MN cDNA sequence was carried out using DNASIS and PROSIS (Pharmacia Software packages). GenBank, EMBL, Protein Identification Resource and SWISS-PROT databases were searched for all possible sequence similarities. In addition, a search for proteins sharing sequence similarities with MN was performed in the MIPS databank with the FastA program [75].

[0009] The proteoglycan-like domain [aa 53-111; SEQ ID NO: 4] which is between the signal peptide and the CA domain, shows significant homology (38% identity and 44% positivity) with a keratan sulphate attachment domain of a human large aggregating proteoglycan aggrecan [28].

[0010] The CA domain [aa 135-391; SEQ ID NO: 5] is spread over 265 aa and shows 38.9% amino acid identity with the human CA VI isoenzyme [5]. The homology between MN/CA IX and other isoenzymes is as follows: 35.2% with CA II in a 261 aa overlap [63], 31.8% with CA I in a 261 aa overlap [7], 31.6% with CA IV in a 266 aa overlap [65], and 30.5%

with CA III in a 259 aa overlap [55].

[0011] In addition to the CA domain, MN/CA IX has acquired both N-terminal and C-terminal extensions that are unrelated to the other CA isoenzymes. The amino acid sequence of the C-terminal part, consisting of the transmembrane anchor and the intracytoplasmic tail, shows no significant homology to any known protein sequence.

[0012] The MN gene (MN/CA9 or CA9) was clearly found to be a novel sequence derived from the human genome. The overall sequence homology between the cDNA MN/CA9 sequence and cDNA sequences encoding different CA isoenzymes is in a homology range of 48-50% which is considered by ones in the art to be low. Therefore, the MN/CA9 cDNA sequence is not closely related to any CA cDNA sequences.

[0013] Very few normal tissues have been found to express MN protein to any significant degree. Those MN-expressing normal tissues include the human gastric mucosa and gallbladder epithelium, and some other normal tissues of the alimentary tract. Paradoxically, MN gene expression has been found to be lost or reduced in carcinomas and other preneoplastic/neoplastic diseases in some tissues that normally express MN, e.g., gastric mucosa.

CA IX and Hypoxia

[0014] Strong association between CA IX expression and intratumoral hypoxia (either measured by microelectrodes, or detected by incorporation of a hypoxic marker pimonidazole, or by evaluation of extent of necrosis) has been demonstrated in the cervical, breast, head and neck, bladder and non-small cell lung carcinomas (NSCLC) [8, 11, 21, 35, 48, 56, 111, 122]. Moreover, in NSCLC and breast carcinomas, correlation between CA IX and a constellation of proteins involved in angiogenesis, apoptosis inhibition and cell-cell adhesion disruption has been observed, possibly contributing to strong relationship of this enzyme to a poor clinical outcome [8]. Hypoxia is linked with acidification of extracellular milieu that facilitates tumor invasion and CA IX is believed to play a role in this process via its catalytic activity [86]. Thus, inhibition of MN/CA IX by specific inhibitors is considered to constitute a novel approach to the treatment of cancers in which CA IX is expressed.

CAIs

[0015] Teicher et al. [106] reported that acetazolamide - the prototypical CA inhibitor (CAI) - functions as a modulator in anticancer therapies, in combination with different cytotoxic agents, such as alkylating agents; nucleoside analogs; platinum derivatives, among other such agents, to suppress tumor metastasis and to reduce the invasive capacity of several renal carcinoma cell lines (Caki-1, Caki-2, ACHN, and A-498). Such studies demonstrate that CAIs may be used in the management of tumors that overexpress one or more CA isozymes. It was hypothesized that the anticancer effects of acetazolamide (alone or in combination with such drugs) might be due to the acidification of the intratumoral environment ensuing after CA inhibition, although other mechanisms of action of this drug were not excluded [20]. Chegwidden et al. 2001 hypothesized that the *in vitro* inhibition of growth in cell cultures, of human lymphoma cells with two other potent, clinically used sulfonamide CAIs, methazolamide and ethoxzolamide, is probably due to a reduced provision of bicarbonate for nucleotide synthesis (HCO_3^- is the substrate of carbamoyl phosphate synthetase II) as a consequence of CA inhibition [20].

[0016] All the six classical CAIs (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, and dichlorophenamide) used in clinical medicine or as diagnostic tools, show some tumor growth inhibitory properties [18, 78, 101, 102].

[0017] The inventors, Dr. Claudia Supuran and Dr. Andrea Scozzafava, reported the design and *in vitro* antitumor activity of several classes of sulfonamide CAIs, shown to act as nanomolar inhibitors against the classical isozymes known to possess critical physiological roles, such as CA I, CA II and CA IV. Those compounds were also shown to exert potent inhibition of cell growth in several leukemia, non-small cell lung, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines, with GI_{50} values of 10 - 75 nM in some cases [77, 91, 92, 100].

[0018] Wingo et al. reported that three classic sulfonamide drugs (acetazolamide, ethoxzolamide and methoxzolamide) inhibited CA IX carbonic anhydrase activity with values of K_i in the nanomolar range [116]. However, until the present invention, no systematic structure-activity relationship study of sulfonamide inhibition of CA IX, alone or in comparison to other CA isozymes had been performed.

[0019] Certain pyridinium derivatives of aromatic/heterocyclic sulfonamides have shown nanomolar affinities both for CA II, as well as CA IV, and more importantly, they were unable to cross the plasma membranes *in vivo* [17].

[0020] Sterling et al. [85] investigated the functional and physical relationship between the downregulated in adenoma bicarbonate transporter and CA II, by using membrane-impermeant sulfonamide inhibitors (in addition to the classical inhibitors such as acetazolamide), which could clearly discriminate between the contribution of the cytosolic and membrane-associated isozymes in these physiological processes.

CAS

[0021] Carbonic anhydrases (CAs) form a large family of genes encoding zinc metalloenzymes of great physiological importance. As catalysts of reversible hydration of carbon dioxide, these enzymes participate in a variety of biological processes, including respiration, calcification, acid-base balance, bone resorption, formation of aqueous humor, cerebrospinal fluid, saliva and gastric acid [reviewed in Dodgson et al. (27)]. CAs are widely distributed in different living organisms. In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) have been described, with very different subcellular localization and tissue distribution [40, 93, 95, 94, 102]. Basically, there are several cytosolic forms (CA I-III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), one mitochondrial form (CA V) as well as a secreted CA isozyme, CA VI [40, 93, 94, 95, 102].

[0022] It has been shown that some tumor cells predominantly express only some membrane-associated CA isozymes, such as CA IX and CA XII [2, 67, 68, 78, 87, 93, 95]. Occasionally, nuclear localization of some isoenzymes has been noted [64, 69, 70]. Not much is presently known about the cellular localization of the other isozymes.

[0023] CAs and CA-related proteins show extensive diversity in their tissue distribution, levels, and putative or established biological functions [105]. Some of the CAs are expressed in almost all tissues (CA II), while the expression of others appears to be more restricted (e.g., CA VI and CA VII in salivary glands [32, 69, 71]. The CAs and CA-related proteins also differ in kinetic properties and susceptibility to inhibitors [82].

[0024] Most of the clinically used sulfonamides mentioned above are systemically acting inhibitors showing several undesired side effects due to inhibition of many of the different CA isozymes present in the target tissue/organ (14 isoforms are presently known in humans) [93, 94, 95, 102]. Therefore, many attempts to design and synthesize new sulfonamides were recently reported, in order to avoid such side effects [13, 17, 42, 62, 80, 99, 100]. At least four CA isozymes (CA IV, CA IX, CA XII and CA XIV) are associated to cell membranes, with the enzyme active site generally oriented extracellularly [93, 94, 95, 102]. Some of these isozymes were shown to play pivotal physiological roles (such as for example CA IV and XII in the eye, lungs and kidneys, CA IX in the gastric mucosa and many tumor cells) [3, 18, 22, 29, 49, 67, 68, 83, 93, 94, 95, 102], whereas the function of other such isozymes (CA XIV) is for the moment less well understood [93, 95]. Due to the extracellular location of these isozymes, if membrane-impermeant CA inhibitors (CAIs) could be designed, only membrane-associated CAs would be affected.

[0025] The first approach towards introducing the membrane-impermeability to CAIs from the historical point of view was that of attaching aromatic/heterocyclic sulfonamides to polymers, such as polyethyleneglycol, aminoethyl-dextran, or dextran [39, 60, 107]. Such compounds, possessing molecular weights in the range of 3.5 - 99 kDa, prepared in that way, showed indeed membrane-impermeability due to their high molecular weights, and selectively inhibited *in vivo* only CA IV and not the cytosolic isozymes (primarily CA II), being used in several renal and pulmonary physiological studies [39, 60, 107]. Due to their macromolecular nature, such inhibitors could not be developed as drugs/diagnostic tools, since *in vivo* they induced potent allergic reactions [39, 60, 93, 95, 107]. A second approach for achieving membrane-impermeability is that of using highly polar, salt-like compounds. Only one such sulfonamide has until recently been used in physiological studies, QAS (quaternary ammonium sulphanilamide), which has been reported to inhibit only extracellular CAs in a variety of arthropods (such as the crab *Callinectes sapidus*) and fish [57]. The main drawback of QAS is its high toxicity in higher vertebrates [57].

[0026] Enzyme activity of carbonic anhydrases (including that of CA IX) can be efficiently blocked by sulfonamide inhibitors. That fact has been therapeutically exploited in diseases caused by excessive activities of certain CA isoforms (e.g. CA II in glaucoma). There is also an experimental evidence that sulfonamides may block tumor cell proliferation and invasion *in vitro* and tumor growth *in vivo*, but the targets of those sulfonamides have not been identified yet. However, the sulfonamides available so far indiscriminately inhibit various CA isoenzymes (14 are presently known in humans) that are localized in different subcellular compartments and play diverse biological roles. This lack of selectivity compromises the clinical utilization of these compounds (due to undesired side effects caused by concurrent inhibition of many CA isoforms) and represents a main drawback also for the sulfonamide application against CA IX in anticancer therapy.

[0027] Thus, there is a need in the art for membrane-impermeant, potent CA IX inhibitors, which would become doubly selective inhibitors for CA IX. The inventors have previously made and described some of the membrane-impermeant molecules described here; however, they were characterized only for their ability to inhibit CA I, CA II and CA IV. While others have studied effects of selective inhibition of extracellular CA by membrane impermeant agents in retinal pigmented epithelia or muscle [34, 120], these agents have not been characterized for their ability to inhibit CA IX. Since CA IX is one of the few extracellular carbonic anhydrases, a membrane-impermeant selective inhibitor of CA IX would be doubly selective for this enzyme and thereby avoid side effects associated with nonspecific CA inhibition.

SUMMARY OF THE INVENTION

[0028] The inventors approached the problem of lack of selectivity of CAIs by taking advantage of features that dis-

tinguish CA IX from the other CA isoforms. First of all, CA IX is an integral plasma membrane protein with an active site exposed on the extracellular side. In this respect, it is similar to some CAs (CA IV, CA XII and CA XIV) but differs from all other isoforms. Among these membrane-bound isoenzymes, CA IX shows some differences in the amino acid sequence of the catalytic domain that may influence the topology of the active site cavity and hence the interaction with sulfonamides. In addition, unlike the other CA isoforms, CA IX is expressed preferentially in hypoxic areas of tumors with poor prognosis.

[0029] The inventors evaluated inhibition profiles of CA IX with a series of aromatic and heterocyclic compounds and found that some of them inhibit CA IX more efficiently than the other widely distributed isoforms CA I, II and IV. Several nanomolar CA IX inhibitors have been detected both among the aromatic and the heterocyclic compounds. This finding is very promising for the design of CA IX-specific inhibitors by modification of their physico-chemical properties such as charge, size and bioreductivity to conform the characteristic properties of CA IX.

[0030] The inventors found that some of the more bulky compounds that strongly inhibited CA IX were very weak inhibitors of CA I, II and IV, possibly due to the fact that the CA IX active site cavity is larger than that of the other investigated isoenzymes. The compounds of such type, identified by screening as disclosed herein, based on the selective inhibition of tumor-associated isoform CA IX may be particularly preferred CA IX specific inhibitors, that could be used in new anticancer therapies and in the diagnostic/prognostic methods of this invention.

[0031] The inventors have shown that CA IX is capable of reducing E-cadherin-mediated cell-cell adhesion that may be important for increased invasion capacity of the cells [103]. CA IX was found by the inventors also to contribute to acidification of extracellular pH in hypoxia but not in normoxia (*unpublished data*). The latter result indicates that hypoxia up-regulates both expression level and enzyme activity of CA IX, that is, hypoxia activates the CA catalytic activity of CA IX. That is a very important finding because intratumoral hypoxia is a clinically relevant factor increasing aggressiveness of tumor cells and reducing success of therapy. Hypoxia is usually accompanied by acidification of extracellular microenvironment, which facilitates tumor invasion and metastasis. CA IX appears to participate in this phenomenon by catalyzing hydration of carbon dioxide to generate bicarbonate ions that are then transported into cell interior and protons that acidify extracellular pH. Therefore, inhibition of the CA IX catalytic activity resulting in reduced extracellular acidification may have direct anticancer effects or may modulate efficiency of those conventional chemotherapeutic drugs whose uptake is pH-dependent.

[0032] The instant invention is related to (1) the recognition that certain carbonic anhydrase inhibitors (CAIs), preferably sulfonamides, selectively target the cancer-related, hypoxia-induced MN/CA IX; (2) the use of such CAIs, preferably sulfonamides, as lead compounds for the design and synthesis of MN/CA IX-specific inhibitors (not claimed herein); (3) the employment of said MN/CA IX-specific inhibitors for anticancer therapy based upon the inhibition of MN/CA IX-mediated acidification of tumor microenvironments; and (4) the use of the specificity of potent MN/CA IX-specific inhibitors for diagnostic/prognostic methods including imaging methods, such as scintigraphy, and for gene therapy (not claimed herein). The CA IX-specific inhibitors described herein can be used for the development of drugs possessing anticancer properties and to modulate conventional chemotherapy for preneoplastic and neoplastic disease characterized by CA IX expression, particularly CA IX overexpression (not specifically claimed herein).

[0033] The invention concerns a pharmaceutical composition for use in a method of treating a preneoplastic and/or neoplastic disease associated with MN/CA IX overexpression in a patient, said method comprising:

- (a) determining if MN/CA IX is overexpressed in a sample taken from said patient, at a level above that for a control sample; and
- (b) if said determining step (a) indicates that MN/CA IX is overexpressed in said sample administering to said patient a composition comprising a specific inhibitor of activated MN/CA IX, wherein said inhibitor of activated MN/CA IX is an MN/CA IX-specific sulfonamide selected from aromatic sulfonamides and heterocyclic sulfonamides selected from the group consisting of compounds 1, 6, 11 - 13, 16 - 19, 22 - 26, 28, 29, 39, 40, 55, 58, 59, 62 and 70, and wherein said MN/CA IX-specific sulfonamide is determined to be a potent and specific inhibitor of MN/CA IX; wherein said MN/CA IX-specific sulfonamide is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay comprising determining the inhibition constant K_i of said sulfonamide, wherein if said inhibition constant K_i is determined to be less than about 50 nanomolar, said MN/CA IX-specific sulfonamide is determined to be a potent inhibitor of MN/CA IX enzymatic activity; and wherein said sulfonamide is determined to be a specific inhibitor of MN/CA IX if said sulfonamide is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of at least one of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV; and wherein said administration of said composition comprising a specific inhibitor of activated MN/CA IX treats the preneoplastic and/or neoplastic disease associated with MN/CA IX overexpression in a patient by inhibiting the activity of MN/CA IX.

[0034] Said compound is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate. Said mammal is preferably human, and said K_i is preferably less than about 35 nanomolar, more preferably less than about 25 nanomolar, and still more preferably less than about 10 nanomolar.

[0035] The composition is used for treating precancer and/or cancer, or inhibiting the growth of precancerous and/or cancerous cells in a mammalian subject, wherein said precancer and cancer are characterized by the overexpression of MN/CA IX. The composition is used for inhibiting the growth of such precancerous or cancerous mammalian cells overexpressing MN/CA IX comprising contacting said cells with a CA IX-specific inhibitor of this invention.

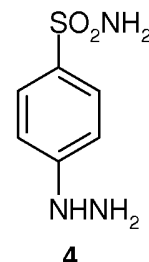
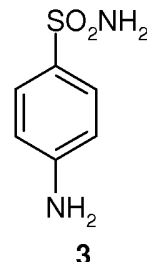
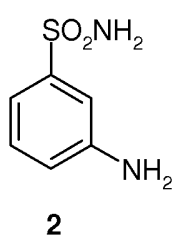
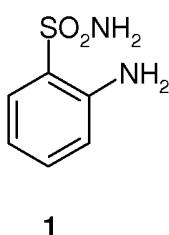
[0036] The CA IX-specific inhibitors of this invention can be administered in a therapeutically effective amount, preferably dispersed in a physiologically acceptable nontoxic liquid vehicle. Different routes of administration may be preferred depending on the site or type of preneoplastic/neoplastic disease, for example, solid or non-solid tumor or metastasis. In general, parenteral administration would be preferred to avoid undesired effects of systemic treatment, for example, those that could be occasioned by binding of the inhibitors to the gastrointestinal mucosa. Injection into or into the vicinity of the preneoplastic/neoplastic disease would be generally preferred. For example, such injections could be intravenous, intraperitoneal, rectal, subcutaneous, intramuscular, intraorbital, intracapsular, intraspinal, intrasternal, intramedullary, intralesional, intradermal, among other routes of injection. Also, other modes of administration, for example, by suppository or topically, can be used as would be appropriate to the target disease. The pharmaceutical formulation would be designed in accordance with known standards as suitable for the route of administration.

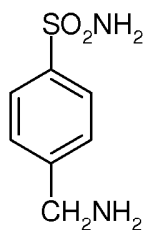
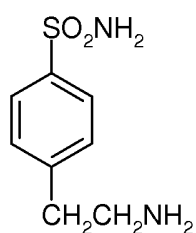
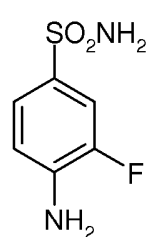
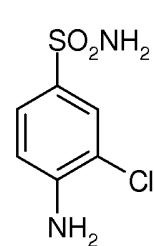
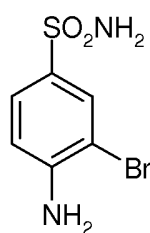
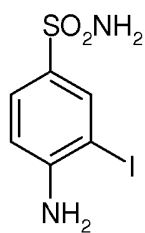
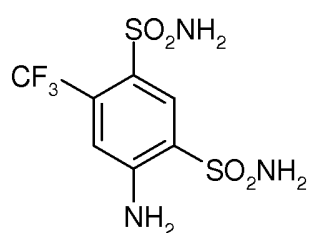
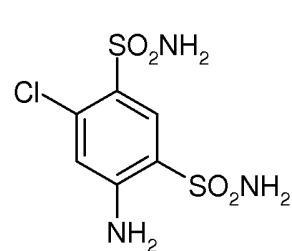
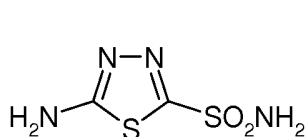
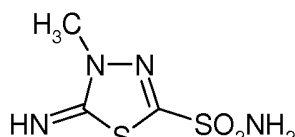
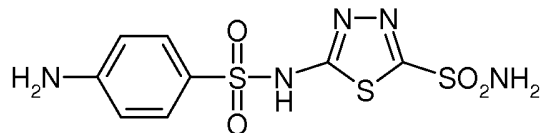
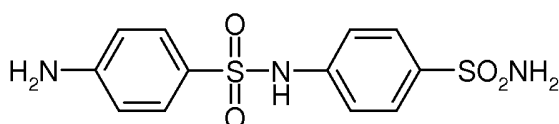
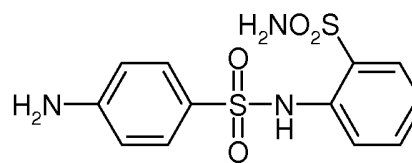
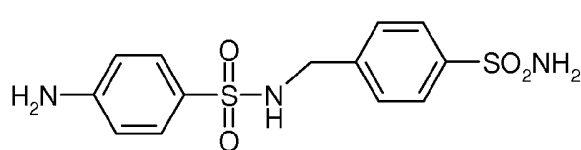
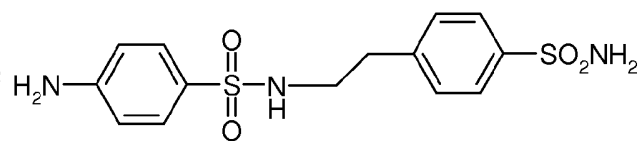
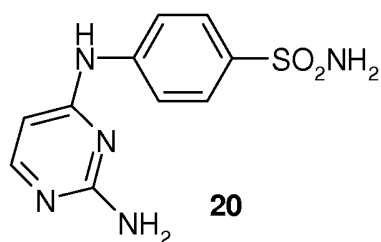
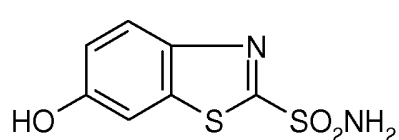
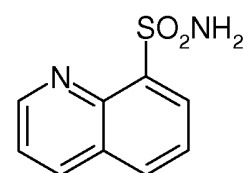
[0037] The CA IX-specific inhibitors of this invention are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of a carbonic anhydrase selected from the group consisting of CA I, CA II and CA IV. More preferably, the CA IX-specific inhibitors are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of at least two carbonic anhydrases selected from the group consisting of CA I, CA II and CA IV. Still more preferably, the CA IX-specific inhibitors are more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV.

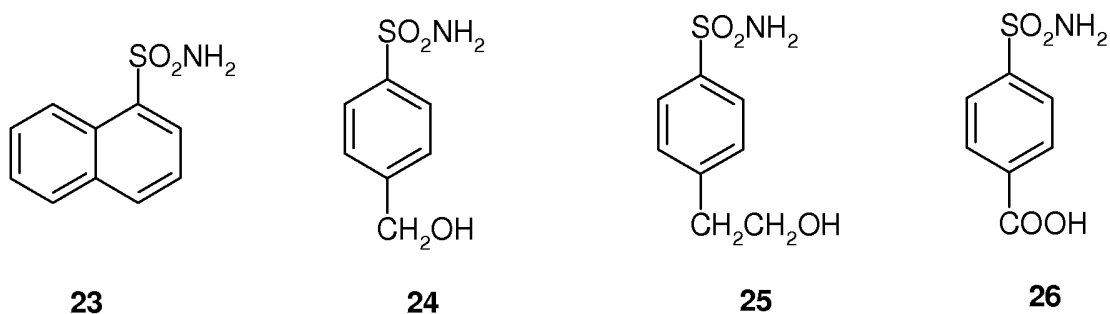
[0038] However, since CA II is a particularly abundant and significant CA, that is cytosolic, it is important when the CA IX-specific inhibitors of this invention are not membrane-impermeant, that they may be more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of CA II. A method comprising the following steps provides an exemplary screening assay that can be used to determine the K_i of a compound in inhibiting the enzymatic activity of CA II:

- a) preparing serial dilutions of said compound and serial dilutions of CA II;
- b) preincubating a dilution of said compound with a dilution of CA II for ten minutes at 20°C;
- c) combining said preincubated mixture of said compound and said CA II with a substrate solution, consisting essentially of 4-nitrophenylacetate in anhydrous acetonitrile, in a reaction vessel for a period of 1 to 3 minutes at 25°C;
- d) concurrently measuring the optical density, at the absorbance maximum of 400 nm, of the contents of said reaction vessel, using a spectrophotometer; and
- e) determining the inhibition constant K_i of said compound.

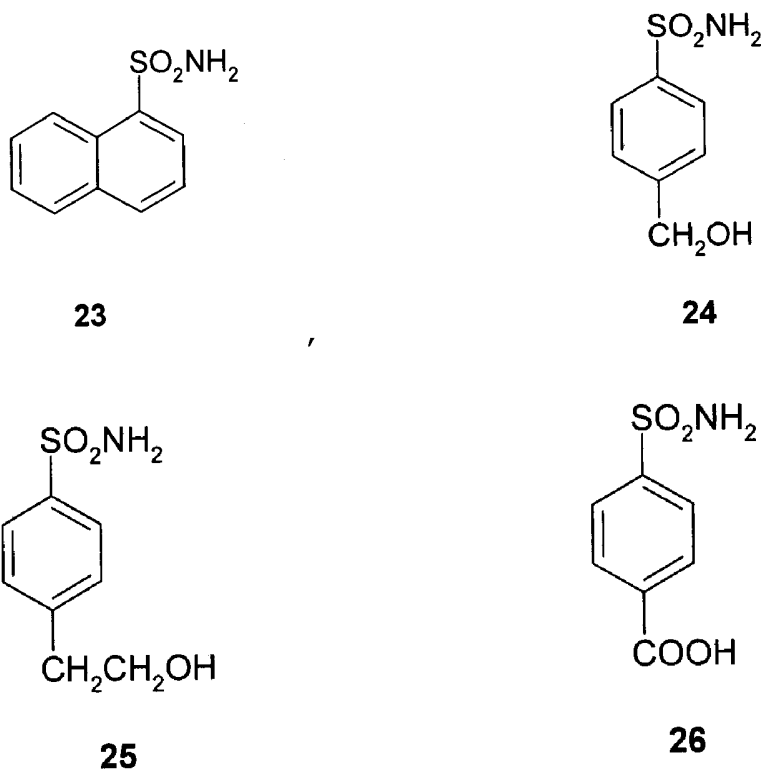
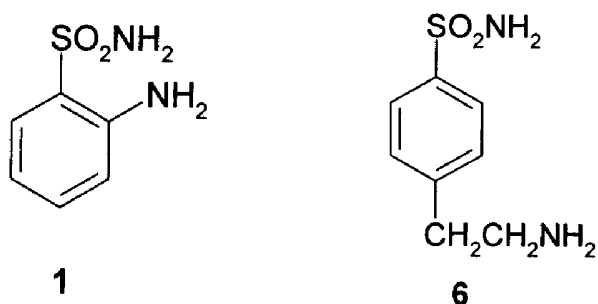
[0039] The aromatic sulfonamide or heterocyclic sulfonamide CA IX-specific inhibitors of this invention are selected from compounds 1, 6, 11 - 13, 16 - 19, 22 - 26, 28, 29, 39, 40, 55, 58, 59, 62 and 70 of the following group of compounds. All other compounds are mentioned for comparison only but are not claimed.



**5****6****7****8****9****10****11****12****13****14****15****16****17****18****19****20****21****22**



[0040] Exemplary preferred aromatic sulfonamide CA IX-specific inhibitors are selected from the group consisting of:

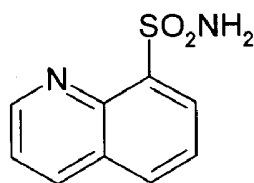


A preferred aromatic sulfonamide CA IX-specific inhibitor can be that wherein a halogen atom is bonded to at least one carbon atom in the aromatic ring of said aromatic sulfonamide.

[0041] Preferred heterocyclic sulfonamide CA IX-specific inhibitors can be substituted heterocyclic sulfonamides, wherein said substituted heterocyclic sulfonamide comprises a heterocyclic ring structure bearing a sulfonamide moiety bonded to said ring structure and optionally bearing one or more substituents independently selected from a group

consisting of halogeno, nitro, and an alkylamino group, wherein the alkyl radical of said alkylamino group comprises 1 to 4 carbon atoms. Preferred heterocyclic sulfonamide CA IX-specific inhibitors may be halogenated.

[0042] Further preferred heterocyclic sulfonamide CA IX-specific inhibitor is



22

[0043] Said membrane-impermeant compounds are determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay comprising:

- a) preparing serial dilutions of said membrane-impermeant compound and serial dilutions of MN/CA IX protein or a fragment of the MN/CA IX protein that comprises the carbonic anhydrase domain;
- b) preincubating a dilution of said membrane-impermeant compound with a dilution of said MN/CA IX protein or said MN/CA IX protein fragment for ten minutes at 20°C;
- c) combining said preincubated mixture of said diluted compound and said diluted MN/CA IX protein or protein fragment with a substrate, consisting essentially of a saturated CO₂ solution, phenol red to 0.2mM, Na₂SO₄ to 0.1 M, and Hepes buffer (pH 7.5) to 10mM, in a reaction vessel for a period of 10 to 100 seconds at 20°C;
- d) concurrently measuring the optical density, at the absorbance maximum of 557 nm, of the contents of said reaction vessel, using a stopped flow spectrophotometer; and
- e) determining the inhibition constant K_i of said membrane-impermeant compound,

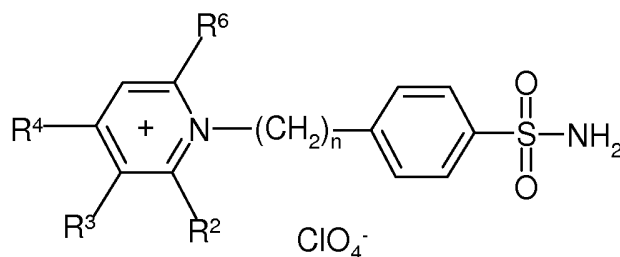
wherein if said inhibition constant K_i is determined to be less than about 50 nanomolar, said membrane-impermeant compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity. The mammal is preferably a human, and the K_i is preferably less than 35 nM, more preferably less than about 25 nM, and still more preferably less than about 10 nanomolar.

[0044] Such membrane-impermeant CA IX-specific inhibitor compounds are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of a carbonic anhydrase selected from the group consisting of CA I, CA II and CA IV, and still more preferably more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of at least two carbonic anhydrases selected from the group consisting of CA I, CA II and CA IV. Further more preferably, said membrane-impermeant CA IX-specific inhibitor compounds are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV. Since both CA IX and CA IV are membrane bound CAs, it is particularly important that the membrane-impermeant CA IX-specific inhibitor compounds are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of CA IV.

[0045] A method comprising the following steps provides an exemplary screening assay that can be used to determine the K_i of a compound inhibiting the enzymatic activity of CA IV:

- a) preparing serial dilutions of said membrane-impermeant compound and serial dilutions of CA IV;
- b) preincubating a dilution of said membrane-impermeant compound with a dilution of CA IV for ten minutes at 20°C;
- c) combining said preincubated mixture of said compound and said CA IV with a substrate solution, consisting essentially of 4-nitrophenylacetate in anhydrous acetonitrile, in a reaction vessel for a period of 1 to 3 minutes at 25°C;
- d) concurrently measuring the optical density, at the absorbance maximum of 400 nm, of the contents of said reaction vessel using a spectrophotometer; and
- e) determining the inhibition constant K_i of said membrane-impermeant compound.

[0046] The membrane-impermeant CA IX-specific inhibitor compounds described herein are pyridinium derivatives of aromatic sulfonamides selected from the group consisting of sulfanilamide, homosulfanilamide and 4-aminoethylbenzenesulfonamide. Some pyridinium derivatives of aromatic sulfonamides can have the general formula of:



wherein

n is 0, 1, or 2;

R₂, R₃, R₄ and R₆ are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties. Further preferred are such compounds wherein

R₂ is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *tert*-butyl and phenyl;

R₃ is selected from the group consisting of hydrogen and methyl;

R₄ is selected from the group consisting of hydrogen, methyl and phenyl; and

R₆ is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, and phenyl. Still further preferred are such compounds wherein

R₃ is hydrogen;

R₄ and R₆ are phenyl;

when n is 0, R₂ is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, and phenyl; and

when n is 1 or 2, R₂ is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *tert*-butyl, and phenyl. Other preferred such compounds include those wherein

R₃ is hydrogen;

R₄ is phenyl; and

when n is 0, R₂ and R₆ are the same and are selected from the group consisting of methyl, ethyl, *n*-propyl, and *iso*-propyl; and

when n is 1 or 2, R₂ and R₆ are the same and are selected from the group consisting of methyl, ethyl, *n*-propyl and *iso*-propyl. Other preferred compounds include those wherein R₂, R₃, R₄ and R₆ are methyl. Still further preferred are such CA IX-specific inhibitor compounds wherein

when n is 0, 1 or 2, R₂, R₄ and R₆ are methyl, and R₃ is hydrogen; or

when n is 1 or 2, R₂ is *iso*-propyl, R₃ is hydrogen, R₄ is methyl, and R₆ is methyl or *iso*-propyl; or

when n is 1 or 2, R₂ and R₆ are phenyl, and R₃ and R₄ are hydrogen. Still more preferred such compounds are those wherein

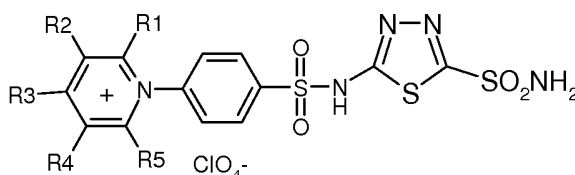
when n is 2, R₂ and R₆ are methyl, R₃ is hydrogen, and R₄ is phenyl; or

when n is 2, R₂ and R₆ are ethyl, R₃ is hydrogen, and R₄ is phenyl; or

when n is 2, R₂, R₃, R₄ and R₆ are methyl.

[0047] When said CA IX-specific inhibitors are membrane-impermeant pyridinium derivatives of a heterocyclic sulfonamides, a preferred compound is a pyridinium derivative of aminobenzolamide.

[0048] Other CA IX-specific inhibitor compounds disclosed herein that are pyridinium derivatives of heterocyclic sulfonamides may have the general formula of:



wherein R₁, R₂, R₃, R₄ and R₅ are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties. Further preferred are such compounds wherein

R₁ is selected from the group consisting of methyl, ethyl, *iso*-propyl, *n*-propyl, *n*-butyl, *tert*-butyl and phenyl;

R₂ is selected from the group consisting of hydrogen and methyl;

R₃ is selected from the group consisting of hydrogen, methyl, *n*-nonyl, and phenyl;

R₄ is selected from the group consisting of hydrogen and methyl; and

R5 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, *tert*-butyl, *n*-nonyl, and phenyl. Further preferred are such compounds wherein

R2 and R4 are hydrogen;

R3 is methyl; and

R1 and R5 are the same and selected from the group consisting of methyl, *iso*-propyl, and *tert*-butyl. Still further preferred are such compounds wherein R2 and R4 are hydrogen;

R3 is phenyl; and

R1 and R5 are the same and selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, and phenyl. Additionally are preferred such compounds wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, and n-butyl;

R2 and R4 are hydrogen; and

R3 and R5 are phenyl. Other preferred such compounds are those wherein

R2 and R4 are hydrogen, R3 is hydrogen or methyl, and R1 and R5 are phenyl; or

R1, R2, and R5 are methyl, R3 is phenyl, and R4 is hydrogen; or

R1 and R4 are methyl, R2 is hydrogen, and R3 and R5 are *n*-nonyl.

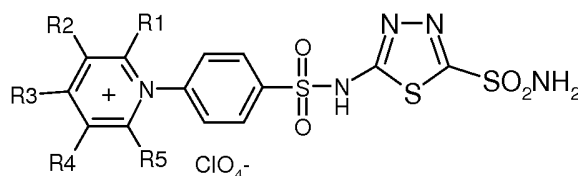
Also preferred such compounds are those wherein

R1 is methyl or iso-propyl, R3 and R5 are methyl, and R2 and R4 are hydrogen; or

R1 and R5 are the same and are methyl or ethyl, R2 and R4 are hydrogen, and R3 is phenyl; or

R1, R2, R3 and R5 are methyl, and R4 is hydrogen.

[0049] Further compounds described herein include pyridinium derivatives of heterocyclic sulfonamides with the general formula of:



wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, *n*-propyl, *n*-butyl, *tert*-butyl and phenyl;

R2 is selected from the group consisting of hydrogen and methyl;

R3 is selected from the group consisting of hydrogen, methyl, *n*-nonyl and phenyl;

R4 is selected from the group consisting of hydrogen and methyl; and

R5 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, *tert*-butyl, *n*-nonyl and phenyl, except that

R1 cannot be methyl when R2 and R4 are hydrogen and R3 and R5 are methyl; and

R1 cannot be methyl when R2 and R4 are hydrogen, R3 is phenyl and R5 is methyl; and

R1 cannot be phenyl when R2 and R4 are hydrogen and R3 and R5 are phenyl. Preferred such pyridinium derivatives of heterocyclic sulfonamides include those wherein

R2 and R4 are hydrogen;

R3 is methyl; and

R1 and R5 are the same and selected from the group consisting of *isopropyl* and *tert*-butyl, and those wherein R2 and R4 are hydrogen;

R3 is phenyl; and

R1 and R5 are the same and selected from the group consisting of ethyl, iso-propyl, n-propyl, and n-butyl, and further preferably those wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, and *tert*-butyl;

R2 and R4 are hydrogen; and

R3 and R5 are phenyl. Still further preferred are those pyridinium derivatives of heterocyclic sulfonamides, wherein

R1 is iso-propyl, R3 and R5 are methyl, and R2 and R4 are hydrogen; or

R2 and R4 are hydrogen, R3 is hydrogen or methyl, and R1 and R5 are phenyl; or

R1, R2, and R5 are methyl, R3 is phenyl, and R4 is hydrogen; or

R1, R2, R3 and R5 are methyl and R4 is hydrogen; or

R1 and R4 are methyl, R2 is hydrogen and R3 and R5 are *n*-nonyl.

[0050] In another therapeutic aspect of the invention, the CA IX-specific inhibitors can be conjugated to radioisotopes for administration. Also, the CA IX-specific inhibitors can be administered concurrently and/or sequentially with radiation and/or with a therapeutically effective amount in a physiologically acceptable formulation of one or more of the following compounds selected from the group consisting of: conventional anticancer drugs, chemotherapeutic agents, different inhibitors of cancer-related pathways, bioreductive drugs, CA IX-specific antibodies and CA IX-specific antibody fragments that are biologically active. Preferably said CA IX-specific antibodies and/or CA IX-specific antibody fragments are humanized or fully human, and may be attached to a cytotoxic entity.

[0051] Another therapeutic aspect described herein but not claimed in this invention concerns methods of treating a mammal for a precancerous or cancerous disease, wherein said disease is characterized by overexpression of MN/CA IX protein, comprising administering to said mammal a therapeutically effective amount in a physiologically acceptable formulation of a vector conjugated to a potent CA IX-specific inhibitor, wherein said vector expresses a wild-type gene that is absent from or mutated in a CA IX expressing cell, that is precancerous or cancerous, and wherein the wild type gene product has an anticancer effect in said cell; or wherein said vector comprises a gene that expresses a cytotoxic protein. An exemplary wild-type gene would be the von Hippel-Lindau gene known to be directly involved in the constitutive expression of CA IX in renal cell carcinoma.

[0052] Preferably said vector comprises a MN/CA IX promoter or a MN/CA IX promoter fragment, wherein said promoter or promoter fragment comprises one or more hypoxia response elements (HREs), and wherein said promoter or promoter fragment is operably linked to said wild-type gene or to said gene that expresses a cytotoxic protein. Preferably the CA IX-specific inhibitor conjugated to the vector has a K_i as determined above in the CO_2 saturation assay to be less than about 50 nM, more preferably less than about 35 nM, still more preferably less than about 25 nM and still further more preferably less than about 10 nM. Preferably, said potent MN/CA IX inhibitor is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate.

Still in another aspect described herein but not forming part of this invention concerns methods that are diagnostic or diagnostic and prognostic for precancer or cancer. For example, such methods may comprise contacting a mammalian sample with a CA IX-specific inhibitor conjugated to a label or a visualizing means, and detecting or detecting and quantifying binding of said CA IX-specific inhibitor to cells in said sample by detecting or detecting and quantifying said label or said visualizing means on cells in said sample, wherein said detection or said detection and quantitation at a level above that for a control sample is indicative of precancerous or cancerous cells that overexpress CA IX in said sample.

[0053] Such methods can be of particular diagnostic and prognostic importance by detecting or detecting and quantitating CA IX activated by hypoxic conditions. Hypoxia combined with CA IX overexpression indicates that the mammal from whom the sample was taken is considered to have a poorer prognosis, and decisions on treatment for said mammal are made in view of the presence of said hypoxic conditions. MN/CA IX as a hypoxia marker is useful in general in making therapeutic decisions. For example, a cancer patient whose tumor is known to express MN/CA IX at an abnormally high level would not be a candidate for certain kinds of chemotherapy and radiotherapy, but would be a candidate for hypoxia-selective chemotherapy.

[0054] Brown, J.M. [16] points out at page 157 that "solid tumours are considerably less well oxygenated than normal tissues. This leads to resistance to radiotherapy and anticancer chemotherapy, as well as predisposing to increased tumour metastases." Brown explains how tumor hypoxia can be exploited in cancer treatment. One strategy to exploit tumor hypoxia for cancer treatment proposed by Brown [16] is to use drugs that are toxic only under hypoxic conditions. Exemplary and preferred drugs that could be used under that strategy include tirapazamine and AQ4N, a di-N-oxide analogue of mitozantrone.

[0055] A second mode of exploiting hypoxia proposed by Brown [16] is by gene therapy strategies developed to take advantage of the selective induction of HIF-1. Brown notes that a tumor-specific delivery system can be developed wherein a promoter that is highly responsive to HIF-1 would drive the expression of a conditionally lethal gene under hypoxic but not normoxic conditions. The MN/CA IX promoter is just such a promoter highly responsive to hypoxia, as well as MN/CA IX promoter fragments comprising one or more HREs. "Expression of an enzyme not normally found in the human body could, under the control of a hypoxia-responsive promoter [the MN/Ca IX promoter], convert a nontoxic pro-drug into a toxic drug in the tumour." [Brown [16], page 160.] Exemplary is the use of the bacterial cytosine deaminase, which converts the nontoxic 5-fluorocytosine to the anticancer drug 5-fluorouracil (5FU) cited by Brown to Trinh et al. [109].

[0056] Ratcliffe et al., U.S. Patent Nos. 5,942,434 and 6,265,390 explain how anti-cancer drugs become activated under hypoxia [119], but that the use of a drug activation system, wherein the enzyme that activates the drug is significantly increased under hypoxia, results in much enhanced therapeutic effect.

[0057] This description further includes methods for imaging tumors and/or metastases that express CA IX in a patient comprising the administration of a CA IX-specific inhibitor linked to an imaging agent to said patient. A preferred imaging method would encompass scintigraphy. These methods are, however, not claimed as part of the invention.

[0058] The assays described herein and not forming part of the invention are both diagnostic and/or prognostic, i.e.,

diagnostic/prognostic. The term "diagnostic/ prognostic" is herein defined to encompass the following processes either individually or cumulatively depending upon the clinical context: determining the presence of disease, determining the nature of a disease, distinguishing one disease from another, forecasting as to the probable outcome of a disease state, determining the prospect as to recovery from a disease as indicated by the nature and symptoms of a case, monitoring the disease status of a patient, monitoring a patient for recurrence of disease, and/or determining the preferred therapeutic regimen for a patient. The diagnostic/prognostic methods of this invention are useful, for example, for screening populations for the presence of neoplastic or pre-neoplastic disease, determining the risk of developing neoplastic disease, diagnosing the presence of neoplastic and/or pre-neoplastic disease, monitoring the disease status of patients with neoplastic disease, and/or determining the prognosis for the course of neoplastic disease.

[0059] The present invention is useful for treating a wide variety of preneoplastic/neoplastic diseases including carcinomas, such as, mammary, colorectal, urinary tract, ovarian, uterine, cervical, endometrial, squamous cell and adenocarcinomas; head and neck cancers; mesodermal tumors, such as, neuroblastomas and retinoblastomas; sarcomas, such as osteosarcomas and Ewing's sarcoma; and melanomas. Of particular interest are gynecological cancers including ovarian, uterine, cervical, vaginal, vulval and endometrial cancers, particularly ovarian, uterine cervical and endometrial cancers. Also of particular interest are cancers of the breast, of gastrointestinal tract, of the stomach including esophagus, of the colon, of the kidney, of the prostate, of the liver, of the urinary tract including bladder, of the lung, and of the head and neck. Gynecologic cancers of particular interest are carcinomas of the uterine cervix, endometrium and ovaries; more particularly such gynecologic cancers include cervical squamous cell carcinomas, adenocarcinomas, adenocarcinomas as well as gynecologic precancerous conditions, such as metaplastic cervical tissues and condylomas.

[0060] The description discloses methods and compositions for evaluating the probability of the presence of malignant or pre-malignant cells, for example, in a group of cells freshly removed from a host which do, however, not form part of the presently claimed invention. Such an assay can be used to detect tumors, quantitate their growth, and help in the diagnosis and prognosis of disease. The assays can also be used to detect the presence of cancer metastasis, as well as confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy and/or radiation therapy. It can further be used to monitor cancer chemotherapy and tumor reappearance.

[0061] The presence of MN antigen can be detected and/or quantitated using a number of well-defined diagnostic assays. Those in the art can adapt any of the conventional immunoassay formats to detect and/or quantitate MN antigen as herein disclosed. The immunoassays of this invention can be embodied in test kits which comprise the potent CA IX-specific inhibitors of this invention, appropriately labeled and/or linked to a visualizing means, as known in the art. Such test kits can be in solid phase formats, but are not limited thereto, and can also be in liquid phase format, and can be based on immunohistochemical assays, ELISAs, particle assays, radiometric or fluorometric assays either unamplified or amplified, using, for example, avidin/biotin technology, among other assay formats.

[0062] CA IX-specific inhibitors of the invention are shown herein to treat transfected cells that constitutively express MN/CA IX compared to non-transfected cells with no MN/CA IX expression. The CA IX-specific inhibitors are shown to inhibit acidification of extracellular pH induced by MN/CA IX in cell cultures exposed to hypoxia.

[0063] Further, labeled exemplary CA IX-specific inhibitors, such as labeled sulfonamides, for example, conjugated to fluorescein isothiocyanate (FITC), are shown to bind to the surface of MN/CA IX transfected cells, and not to control cells, only in hypoxia but not in normoxia. Those experiments confirm that CA IX-specific inhibitors, such as the sulfonamide compounds described herein, can specifically target MN/CA IX under conditions characteristic of intratumoral microenvironments.

[0064] The CA IX-specific inhibitors described herein can be used diagnostically and prognostically for precancer and cancer, and to determine the status of a patient, and therapeutically, individually or in different combinations with conventional therapeutic regimens to treat precancers and/or cancer. The CA IX-specific inhibitors may also be used in cancer research. These embodiments do not form part of the present invention.

[0065] More particularly for treating precancer and/or cancer, the CA IX-specific inhibitors of this invention can be used to hinder cancer expansion and/or progression by blocking CA IX activity. The CA IX-specific inhibitors can be conjugated to radioisotopes for radiotherapy. The CA IX-specific inhibitors can be combined with CA IX-specific antibodies and a variety of conventional therapeutic drugs, different inhibitors of cancer-related pathways, bioreductive drugs, and/or radiotherapy, wherein different combinations of treatment regimens with the CA IX-specific inhibitors of this invention may increase overall treatment efficacy. Particularly, the CA IX-specific inhibitors of this invention may be combined with therapy using MN/CA IX-specific antibodies and/or CA IX-specific antibody fragments, preferably humanized CA IX-specific antibodies and/or biologically active fragments thereof, and more preferably fully human CA IX-specific antibodies and/or fully human CA IX-specific biologically active antibody fragments. Said CA IX-specific antibodies and biologically active CA IX-specific antibody fragments, preferably humanized and more preferably fully human, may be conjugated to a cytotoxic entity, for example, a cytotoxic protein, such as ricin A, among many other cytotoxic entities.

[0066] Still further, a CA IX-specific inhibitor of this invention could be coupled to a vector for targeted delivery to CA IX-specific expressing cells for gene therapy (for example, with the wild-type von Hippel-Lindau gene), or for effecting

the expression of cytotoxic proteins, preferably wherein said vector comprises a MN/CA IX promoter or MN/CA IX promoter fragment comprising the MN/CA IX hypoxia response element (HRE) or a HRE of another gene, and more preferably wherein the CA IX promoter or CA IX promoter fragment comprises more than one HRE, wherein said HRE or HREs is or are either of MN/CA IX, and/or of other genes and/or of genetically engineered HRE consensus sequences in a preferred context.

[0067] Particularly, the CA IX-specific inhibitors described herein can be used diagnostically/prognostically to detect precancerous and/or cancerous cells by binding to CA IX, preferably to CA IX activated by hypoxic conditions, wherein said CA IX specific inhibitors are coupled to a label or to some visualizing means. Such detection, particularly of hypoxic conditions, and CA IX overexpression, can be helpful in determining effective treatment options, and in predicting treatment outcome and the prognosis of disease development. Further the CA IX-specific inhibitors when labeled or linked to an appropriate visualizing means can be used for imaging tumors and/or metastases that express CA IX. These embodiments do not form part of the present invention.

[0068] The CA IX-specific inhibitors described herein can also be used in basic and pre-clinical research. For example, the CA IX-specific inhibitors can be used to study the regulation of CA IX enzyme activity, to study the role of CA IX in tumor growth and metabolism, and to study the role of CA IX in response to treatment by drugs, radiation, inhibitors and other therapeutic regimens. These embodiments do not form part of the present invention.

[0069] Further methods are disclosed for the preparation of positively-charged, membrane-impermeant heterocyclic sulfonamide CA inhibitors with high affinity for the membrane-bound carbonic anhydrase CA IX. Particularly preferred CA IX-specific inhibitors are pyridinium derivatives of such aromatic and heterocyclic sulfonamides. The general structure of the preferred pyridinium derivatives of sulfonamides can be described as a pyridinium portion attached to the "tail" of an aromatic or heterocyclic sulphonamide portion of the compound. These embodiments do not form part of the present invention.

[0070] Further provided are screening assays for compounds that are useful for inhibiting the growth of a vertebrate, preferably mammalian, more preferably human, preneoplastic or neoplastic cell that abnormally expresses MN protein. Said screening assays comprise tests for the inhibition of the enzymatic activity of MN by said compounds. Additional assays provided herein test said compounds for their cell membrane impermeance. These embodiments do not form part of the present invention.

[0071] Aspects of the instant invention disclosed herein are described in more detail below.

References

[0072] The following references are cited throughout the application by numbers in italics keyed to the list below:

1. Abbate et al., J. Med. Chem., 45: 3583-3587 (2002).
2. Abbate et al., J. Enz. Inhib. Med. Chem., 18: 303-308 (2003a).
3. Abbate et al., Bioorg. Med. Chem. Lett., 13: In Press (2003b).
4. Abdine et al., J. Assoc. Off. Anal. Chem., 61: 695-701 (1978).
5. Aldred et al., Biochemistry, 30: 569-575 (1991).
6. Balaban et al., "Pyrylium Salts: Syntheses, Reactions and Physical Properties," In Advances in Heterocyclic Chemistry, Katritzky, A.R., Ed., Academic Press, New York, pp. 8-360 (1982).
7. Barlow et al., Nucl. Acids Res., 15: 2386 (1987).
8. Bartosova et al., J. Pathol., 197: 314-321 (2002).
9. Bayer, A., Ber. Dtsch. Chem. Ges., 43: 2337-2349 (1910).
10. Bayer and Piccard, Liebigs Ann. Chem., 384: 208-223 (1911).
11. Beasley et al., Cancer Res., 61: 5262-5267 (2001).
12. Behravan et al., Eur. J. Biochem, 190: 351-357 (1990).
13. Borrás et al., Bioorg. Med. Chem., 7: 2397-2406 (1999).
14. Briganti et al., Biochemistry, 36: 10384-10392 (1997).
15. Briganti et al., Inorg. Chim. Acta.: 275-276, 295-300 (1998).
16. Brown, J.M., "Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies," Molecular Medicine Today, 6: 157-162 (April 2000).
17. Casini et al., J. Med. Chem., 43: 4884-4892 (2000).
18. Casini et al., Curr. Cancer Drug Targets, 2: 55-75 (2002).
- 19.
- a) Casini et al., Bioorg. Med. Chem. Lett, 13: 841-845 (2003)
- b) Casini et al., Biorg. Med. Chem. Lett., 13: 2763-2769 (2003).

20. Chegwidden et al., "The Roles of carbonic anhydrase isozymes in cancer," *Gene Families: Studies of DNA, RNA, Enzymes and Proteins*, Xue et al., Eds., World Scientific, Singapore, pp. 157-169 (2001).
21. Chia et al., *J. Clin. Oncol.*, 19: 3660-3668 (2001).
22. Chirica et al., *Biochim. Biophys. Acta*, 1544: 55-63 (2001).
- 5 23. Clare and Supuran, *Eur. J. Med. Chem.*: 32: 311-319 (1997).
24. Clare and Supuran, *Eur. J. Med. Chem.*, 34: 463-474 (1999).
25. Cuthbert et al., *J. Physiol.*, 551 (Pt.1) 79-92, (2003).
26. Dinculescu and Balaban, *Rev. Roum. Chem.*, 25: 1505-1528 (1980).
27. Dodgson et al., *The Carbonic Anhydrases*, Plenum Press, New York-London, pp. 398 (1991).
- 10 28. Doege et al., *J. Biol. Chem.*, 266: 894-902 (1991).
29. Elleby et al., *Eur. J. Biochem.*, 268: 1613-1619 (2001).
30. Ferraroni et al., *Biochemistry*, 41: 6237-6244 (2002a).
31. Ferraroni et al., *Inorg. Chim. Acta*, 339: 135-144 (2002b).
32. Fleming et al., *J. Clin. Invest.*, 96: 2907-2913 (1995).
- 15 33. Franchi et al., *J. Enz. Inhib. Med. Chem.*, 18: 333-338 (2003).
34. Geers and Gros, *Physiol. Rev.* 80:681-715 (2000).
35. Giatromanolaki et al., *Cancer Res.*, 61: 7992-7998 (2001).
36. Goma, Z.S., *Biomed. Chromatogr.*, 7: 134-135 (1993).
37. Gruneberg et al., *Angew. Chem. Int. Ed.*, 40: 389-393 (2001).
- 20 38. Hakansson et al., *J. Mol. Biol.*, 227: 1192-1204 (1992).
39. Heming et al., *J. Appl. Physiol.*, 61: 1849-1856 (1986).
40. Hewett-Emmett, D., "Evolution and distribution of the carbonic anhydrase gene families," In *The Carbonic Anhydrases - New Horizons*, Chegwidden et al., Eds., Birkhauser Verlag: Basel, Switzerland, pp. 29-78 (2000).
41. Höckel and Vaupel, *J. Natl. Cancer Inst.*, 93: 266-276 (2001).
- 25 42. Ilies et al., *Bioorg. Med. Chem.*, 8: 2145-2155 (2000).
43. Ilies et al., *J. Med. Chem.*, 46: 2187-2196 (2003).
44. Khalifah, R.G., *J. Biol. Chem.*, 246: 2561-2573 (1971).
45. Khalifah et al., *Biochemistry*, 16: 2241-2247 (1977).
46. Kim et al., *J. Am. Chem. Soc.*, 122: 12125-12134 (2000).
- 30 47. Kim et al., *J. Am. Chem. Soc.*, 123: 9620-9627 (2001).
48. Koukourakis et al., *Clin. Cancer Res.*, 7: 3399-3403 (2001).
49. Krongkrai et al., *Int. J. Parasitol.*, 31: 661-668 (2001).
50. Liao et al., *Am. J. Pathol.*, 145: 598-609 (1994).
51. Liao et al., *Cancer Res.*, 57: 2827-2831 (1997).
- 35 52. Lieskovska et al., *Neoplasma*, 46: 17-24 (1999).
53. Lindskog and Coleman, *Proc. Natl. Acad. Sci. (USA)* 70: 2505-2508 (1964).
54. Lindskog et al., "Structure-function relations in human carbonic anhydrase II as studied by site-directed mutagenesis," in *Carbonic anhydrase - From biochemistry and genetics to physiology and clinical medicine*, Botre et al., Eds., VCH, Weinheim, pp. 1-13 (1991)].
- 40 55. Lloyd et al., *Genes. Dev.*, 1: 594-602 (1987).
56. Loncaster et al., *Cancer Res.*, 61: 6394-6399 (2001).
57. Maren, T.H., *Physiol. Rev.*, 47: 595-781 (1967).
58. Maren, T.H., "Benzolamide - a renal carbonic anhydrase inhibitor," In *Orphan Drugs*, Karch, T.E., Ed., M. Dekker, New York, pp. 89-115 (1982).
- 45 59. Maren et al., *Mol. Pharmacol.*, 44: 901-906 (1993).
60. Maren et al., *J. Pharmacol. Exp. Ther.*, 280: 98-104 (1997).
61. Mendelsohn and Lippman, "Growth Factors," pp. 114-133, IN: DeVita et al. (eds.), *Cancer: Principles and Practice of Oncology* (4th Ed.; Lippincott; Philadelphia, 1993).
62. Mincione et al., *Eur. J. Pharm. Sci.*, 9: 185-199 (1999).
- 50 63. Montgomery et al., *Nucl. Acids. Res.*, 15: 4687 (1987).
64. Mori et al., *Gastroenterol.*, 105: 820-826 (1993).
65. Okuyama et al., *PNAS (USA)* 89: 1315-1319 (1992).
66. Opavsky et al., *Genomics*, 33: 480-487 (1996).
67. Owa and Nagasu, *Exp. Opin. Ther. Patents*, 10: 1725-1740 (2000).
- 55 68. Owa et al., *J. Med. Chem.*, 42: 3789-3799 (1999).
69. Parkkila et al., *Gut*, 35: 646-650 (1994).
70. Parkkila et al., *Histochem. J.*, 27: 133-138 (1995).
71. Parkkila et al., *Hepatology*, 24: 104 (1996).

72. Pastorek et al., *Oncogene*, 9: 2788-2888 (1994).
73. Pastorekova et al., *Virology*, 187: 620-626 (1992).
74. Pastorekova et al., *Gastroenterology*, 112: 398-408 (1997).
75. Pearson and Lipman, *PNAS (USA)*, 85: 2444 (1988).
- 5 76. Pocker and Stone, *Biochemistry*, 6: 668-678 (1967).
77. Scozzafava and Supuran, *Bioorg. Med. Chem. Lett.*, 10: 117-1120 (2000).
78. Scozzafava et al., *Curr. Med. Chem.*, 10: 925-953 (2003).
79. Scozzafava et al., *J. Med. Chem.*, 42: 2641-2650 (1999).
80. Scozzafava et al., *J. Med. Chem.*, 42: 3690-3700 (1999).
- 10 81. Scozzafava et al., *J. Med. Chem.*, 43: 292-300 (2000).
82. Sly and Hu, *Annu. Rev. Biochem.*, 64: 375-401 (1995).
83. Smith and Ferry, *FEMS Microbiol. Rev.*, 24: 335-366 (2000).
84. Steiner et al., *Eur. J. Biochem.*, 59: 253-259 (1975).
85. Sterling et al., *Am. J. Physiol.-Cell Physiol.*, 283: C1522-C1529 (2002).
- 15 86. Stubbs et al., *Mol. Med. Toddy*, 6: 15-19 (2000).
87. Supuran, C.T., *Opin. Investig. Drugs*, 12: 283-287 (2003).
88. Supuran and Clare, *Eur J. Med. Chem.*, 30: 687-696 (1995).
89. Supuran and Clare, *Eur J. Med. Chem.*, 33: 489-500 (1998).
90. Supuran and Clare, *Eur. J. Med. Chem.*, 34: 41-50 (1999).
- 20 91. Supuran and Scozzafava, *Enzyme Inhib.*, 15: 597-610 (2000a).
92. Supuran and Scozzafava, *Eur. J. Med. Chem.*, 35: 867-874 (2000b).
93. Supuran and Scozzafava, *Exp. Opin. Ther. Patens*, 10: 575-600 (2000c).
94. Supuran and Scozzafava, *Curr. Med. Chem.- Imm., Endoc. Metab. Agents*, 1: 61-97 (2001).
95. Supuran and Scozzafava, *Exp. Opin. Ther. Patents*, 12: 217-242 (2002).
- 25 96. Supuran et al., *Eur. J. Med. Chem.*, 33: 577-594 (1998a).
97. Supuran et al., *Eur. J. Med. Chem.*, 33: 739-752 (1998b).
98. Supuran et al., *J. Enz. Inhib.*, 15: 381-401 (2000a).
99. Supuran et al., *J. Med. Chem.*, 35: 309-321 (2000b).
100. Supuran et al., *Bioorg. Med. Chem.*, 9: 703-714 (2001 a).
- 30 101. Supuran et al., *Curr. Med. Chem.- Imm., Endoc. Metab. Agents*, 1: 61-97 (2001 b)
102. Supuran et al., *Med. Res. Rev.*, 23: 146-189 (2003).
103. Svastova et al., *Experimental Cell Research*, 290: 332-345, (2003).
104. Symington, *J. Biol. Chem.*, 267: 25744 (1992).
105. Tashian, R. E., *Adv. in Genetics*, 30: 321-356 (1992)..
- 35 106. Teicher et al., *Anticancer Research*, 13: 1549-1556 (1993).
107. Tinker et al., *J. Pharmacol. Exp. Ther.*, 218: 600-607 (1981).
108. Toma and Balaban, *Tetrahedron, Suppl.* 7: 27-34 (1966).
109. Trinh et al., *Cancer Res.*, 55: 4808-4812 (1995).
110. Turner, et al., *Hum. Pathol.*, 28: 740-744 (1997).
- 40 111. Turner et al., *Br. J. Cancer*, 86: 1276-1282 (2002).
112. Uemura et al. [*J. Urology*, 1571 (4 Supp.): 377 (Abstract 1475) (April 16, 1997)]
113. Vermynen et al., *Eur. Respir. J.*, 14: 806-811 (1999);
114. Vullo et al., *Bioorg. Med. Chem. Lett.*, 13: 1005-1009 (2003a).
115. Vullo et al. *J. Enz. Inhib. Med. Chem.*, 18: 403-406 (2003b).
- 45 116. Wingo et al., *Biochem. Biophys. Res. Comm.*, 288: 666-669 (2001).
117. Winum et al., *J. Med. Chem.*, 46: 2197-2204 (2003).
118. Wistrand and Lindqvist, "Design of carbonic anhydrase inhibitors and the relationship between the pharmacodynamics and pharmacokinetics of acetazolamide," In *Carbonic Anhydrase - From Biochemistry and Genetics to Physiology and Clinical Medicine*, Botrè et al., Eds., VCH, Weinheim, pp. 352-378 (1991).
- 50 119. Workman and Stafford, *Cancer and Metastasis Reviews*, 12: 73-82 (1993)
120. Wu et al., *J. Membr. Biol.* 162: 31-38 (1998).
121. Wykoff et al., *Cancer Research*, 60: 7075-7083 (2000).
122. Wykoff et al., *Am. J. Pathol.*, 158: 1011-1019 (2001).
123. Zavada et al., *Int. J. Cancer*, 54: 268-274 (1993).

Abbreviations

[0073] The following abbreviations are used herein:

	aa	- amino acid
	AAZ	- acetazolamide
	ATCC	- American Type Culture Collection
	bp	- base pairs
5	BRL	- Bethesda Research Laboratories
	BRZ	- brinzolamide
	BSA	- bovine serum albumin
	CA	- carbonic anhydrase
	CAI	- carbonic anhydrase inhibitor
10	CAM	- cell adhesion molecule
	CARP	- carbonic anhydrase related protein
	Ci	- curie
	cm	- centimeter
	CNS	- central nervous system
15	cpm	- counts per minute
	C-terminus	- carboxyl-terminus
	°C	- degrees centigrade
	DCP	- dichlorophenamide
	DEAE	- diethylaminoethyl
20	DMEM	- Dulbecco modified Eagle medium
	ds	- double-stranded
	DZA	- dorzolamide
	EDTA	- ethylenediaminetetraacetate
	EZA	- ethoxzolamide
25	F	- fibroblasts
	FCS	- fetal calf serum
	FITC	- fluorescein isothiocyanate
	H	- HeLa cells
	IC	- intracellular
30	kb	- kilobase
	kbp	- kilobase pairs
	kd or kDa	- kilodaltons
	K _i	- inhibition constant
	KS	- keratan sulphate
35	LTR	- long terminal repeat
	M	- molar
	mA	- milliamperes
	MAb	- monoclonal antibody
	ME	- mercaptoethanol
40	MEM	- minimal essential medium
	min.	- minute(s)
	mg	- milligram
	ml	- milliliter
	mM	- millimolar
45	MMC	- mitomycin C
	mmol	- millimole
	MZA	- methazolamide
	N	- normal concentration
	NEG	- negative
50	ng	- nanogram
	nm	- nanometer
	nM	- nanomolar
	nt	- nucleotide
	N-terminus	- amino-terminus
55	ODN	- oligodeoxynucleotide
	ORF	- open reading frame
	PA	- Protein A
	PBS	- phosphate buffered saline

PCR	- polymerase chain reaction
PG	- proteoglycan
pI	- isoelectric point
PMA	- phorbol 12-myristate 13-acetate
5 POS	- positive
Py	- pyrimidine
QAS	- quaternary ammonium sulfonamide
QSAR	- quantitative structure-activity relationship(s)
RACE	- rapid amplification of cDNA ends
10 RCC	- renal cell carcinoma
RIA	- radioimmunoassay
RIP	- radioimmunoprecipitation
RIPA	- radioimmunoprecipitation assay
RNP	- RNase protection assay
15 RT-PCT	- reverse transcription polymerase chain reaction
SAC	- <i>Staphylococcus aureus</i> cells
SAR	- structure-activity relationship
sc	- subcutaneous
SDS	- sodium dodecyl sulfate
20 SDS-PAGE	- sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SINE	- short interspersed repeated sequence
SP	- signal peptide
SP-RIA	- solid-phase radioimmunoassay
TBE	- Tris-borate/EDTA electrophoresis buffer
25 TC	- tissue culture
TCA	- trichloroacetic acid
TC media	- tissue culture media
TC	- tissue culture
tk	- thymidine kinase
30 TM	- transmembrane
Tris	- tris (hydroxymethyl) aminomethane
μCi	- microcurie
μg	- microgram
μl	- microliter
35 μM	- micromolar

Cell Lines

[0074]

40	BL21 (DE3) --	<i>Escherichia coli</i> strain described by Lindskog's group (for CA I, II expression)[Lindskog et al., "Structure-function relations in human carbonic anhydrase II as studied by site-directed mutagenesis," in Carbonic anhydrase - From biochemistry and genetics to physiology and clinical medicine, Botre et al., Eds., VCH, Weinheim, pp. 1-13 (1991)]
45	BL21-GOLD --	<i>Escherichia coli</i> strain (from Stratagene) used for CA IX (DE3) expression)

Nucleotide and Amino Acid Sequence Symbols

[0075] The following symbols are used to represent nucleotides herein:

50		Base Symbol	Meaning
		A	adenine
		C	cytosine
55		G	guanine
		T	thymine
		U	uracil

(continued)

	Base Symbol	Meaning
	I	inosine
5	M	A or C
	R	A or G
	W	A or T/U
	S	C or G
10	Y	C or T/U
	K	G or T/U
	V	A or C or G
	H	A or C or T/U
	D	A or G or T/U
15	B	C or G or T/U
	N/X	A or C or G or T/U

[0076] There are twenty main amino acids, each of which is specified by a different arrangement of three adjacent nucleotides (triplet code or codon), and which are linked together in a specific order to form a characteristic protein. A three-letter or one-letter convention is used herein to identify said amino acids, as, for example, in Figure 1 as follows:

	Amino acid name	3 Ltr. Abbrev.	1 Ltr. Abbrev.
	Alanine	Ala	A
25	Arginine	Arg	R
	Asparagine	Asn	N
	Aspartic Acid	Asp	D
	Cysteine	Cys	C
	Glutamic Acid	Glu	E
30	Glutamine	Gln	Q
	Glycine	Gly	G
	Histidine	His	H
	Isoleucine	Ile	I
35	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
	Phenylalanine	Phe	F
	Proline	Pro	P
40	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
	Tyrosine	Tyr	Y
45	Valine	Val	V
	Unknown or other		X

BRIEF DESCRIPTION OF THE FIGURES

[0077]

Figure 1 A-C provides the nucleotide sequence for MN/CA IX full-length cDNA [SEQ ID NO: 1]. Figure 1 A-C also sets forth the predicted amino acid sequence [SEQ ID NO: 2] encoded by the cDNA.

Figure 2A-F provides a 10,898 bp complete genomic sequence of MN/CA9 [SEQ ID NO: 3]. The base count is as follows: 2654 A; 2739 C; 2645 G; and 2859 T. The 11 exons are in general shown in capital letters, but exon 1 is considered to begin at position 3507 as determined by RNase protection assay.

Figure 3 provides an exon-intron map of the human MN/CA9 gene. The positions and sizes of the exons (numbered,

cross-hatched boxes), Alu repeat elements (open boxes) and an LTR-related sequence (first unnumbered stippled box) are adjusted to the indicated scale. The exons corresponding to individual MN/CA IX protein domains are enclosed in dashed frames designated PG (proteoglycan-like domain), CA (carbonic anhydrase domain), TM (transmembrane anchor) and IC (intracytoplasmic tail). Below the map, the alignment of amino acid sequences illustrates the extent of homology between the MN/CA IX protein PG region (aa 53-111) [SEQ ID NO: 4] and the human aggrecan (aa 781-839) [SEQ ID NO: 5].

Figure 4 A-B shows the chemical structures of the 26 different sulfonamide compounds tested in Example 1.

Figure 5 shows the scheme for the general synthesis of compounds **71 - 91** of Example 3 (**Scheme 1**).

Figure 6 shows the scheme for the reaction between a pyrylium salt and an amine (**Scheme 2**), as described in Example 3.

DETAILED DESCRIPTION

[0078] The use of oncoproteins as targets for developing new cancer therapeutics is considered conventional by those of skill in the art. [See, e.g., Mendelsohn and Lippman [61]. However, the application of such approaches to MN is new. In comparison to other tumor-related molecules (e.g. growth factors and their receptors), MN has the unique property of being differentially expressed in preneoplastic/neoplastic and normal tissues, which are separated by an anatomic barrier.

[0079] The pyridinium derivatives of sulfonamides used in the present invention can be formed, for example, by creating bonds between pyrylium salts and aromatic or heterocyclic sulfonamide reagents, as described below. The aromatic or heterocyclic sulfonamide portion of a pyridinium salt of a sulfonamide compound can be called the "head," and the pyridinium portion can be called the "tail."

[0080] It can be appreciated by those of skill in the art that various other types of linkages can couple the pyridinium portion with the sulfonamide portion. It can further be appreciated that alternate methods, in addition to those disclosed herein, can be used to make the pyridinium derivatives of the present invention.

[0081] As used herein, "cancerous" and "neoplastic" have equivalent meanings, and "precancerous" and "preneoplastic" have equivalent meanings.

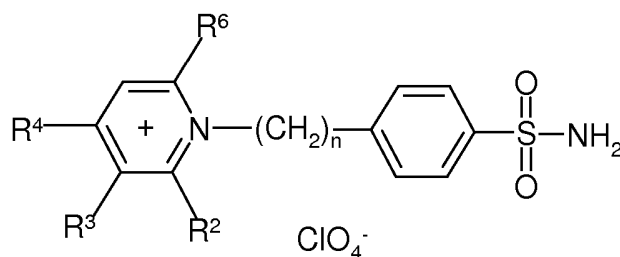
[0082] As used herein, the term "aromatic" when applied to sulphonamide structures means "comprising an aromatic ring, without an additional heterocyclic ring." The term "heterocyclic" when applied to sulphonamide structures means "comprising a heterocyclic ring, with or without an additional aromatic ring."

[0083] As used herein, the term "alkyl", alone or in combination, refers to a straight-chain or branched-chain alkyl radical containing from 1 to 12, preferably from 1 to 6 and more preferably from 1 to 4, carbon atoms. Examples of such radicals include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, iso-amyl, hexyl, decyl and the like.

[0084] The term "aryl", alone or in combination, means a phenyl or naphthyl radical which optionally carries one or more substituents selected from alkyl, alkoxy, halogen, hydroxy, amino, nitro, cyano, haloalkyl, carboxy, alkoxycarbonyl, cycloalkyl, heterocycloalkyl, amido, mono and dialkyl substituted amino, mono and dialkyl substituted amido and the like, such as phenyl, p-tolyl, 4-methoxyphenyl, 4-(tert-butoxy)phenyl, 3-methyl-4-methoxyphenyl, 4-fluorophenyl, 4-chlorophenyl, 3-nitrophenyl, 3-aminophenyl, 3-acetamidophenyl, 4-acetamidophenyl, 2-methyl-3-acetamidophenyl, 2-methyl-3-aminophenyl, 3-methyl-4-aminophenyl, 2-amino-3-methylphenyl, 2,4-dimethyl-3-aminophenyl, 4-hydroxyphenyl, 3-methyl-4-hydroxyphenyl, 1-naphthyl, 2-naphthyl, 3-amino-1-naphthyl, 2-methyl-3-amino-1-naphthyl, 6-amino-2-naphthyl, 4,6-dimethoxy-2-naphthyl and the like.

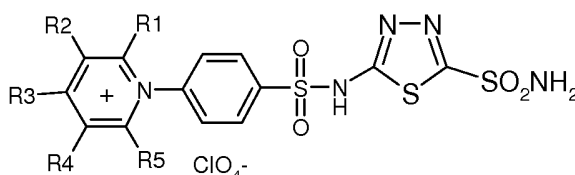
[0085] Sulfonamides described herein are aromatic and heterocyclic sulfonamides. The structures of representative sulfonamides of this group, designated **1-26**, are shown in Figure 4. Compounds mentioned herein but not covered by the claims are excluded from the scope of protection.

[0086] Sulfonamides described herein are pyridinium derivatives of aromatic sulfonamides and have the general formula (**A**) below,

**A**

wherein n is 0, 1, or 2; and R2, R3, R4 and R6 are each independently selected from the group consisting of hydrogen, alkyls and aryls. The structures of representative sulfonamides of this group, designated **27** through **70**, are shown as derivatives of the general structure (**A**), in Table 2.

[0087] Alternatively, further sulfonamides of the present invention are pyridinium derivatives of heterocyclic sulfonamides and have the general formula (**B**) below, wherein said pyridinium derivative of a heterocyclic sulfonamide has the general formula of:

**B**

wherein R1, R2, R3, R4 and R5 are each independently selected from the group consisting of hydrogen, alkyls and aryls. The structures of representative sulfonamides of this group, designated **71** through **91**, are shown as derivatives of the general structure (**B**), in Table 3.

[0088] Representative sulfonamide derivatives of the group of compounds represented by the general formulas (**A**) and (**B**) have CA IX inhibitory activity, and are potentially useful therapeutically as anticancer agents in treating MN-associated tumors.

[0089] Further, biologic activity of the identified sulfonamides will be tested in vitro by inhibition of the carbonic anhydrase enzymatic activity of the MN protein, by effects on cell morphology and growth characteristics of MN-related tumor cells (HeLa) and of control cells [104]. In vivo screening will be carried out in nude mice that have been injected with HeLa cells.

Representative Sulfonamide Inhibitors of CA IX

[0090] The sulfonamides investigated in Example 1 for the inhibition of the tumor-associated isozyme CA IX, of types **1-26** are shown in Figure 4A-B. Compounds **1-6**, **11-12**, **20** and **26** are commercially available, whereas **7-10** [43], **13-19** [24, 90, 97] and **21-25** [79] were prepared as reported earlier. The six clinically used compounds were also assayed. For Example 2 compounds (pyridinium derivatives of aromatic sulfonamides), reaction of sulfanilamide, homosulfanilamide or 4-(2-aminoethyl)-benzenesulfonamide with 2,6-di-, 2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts afforded the pyridinium salts **27-70** investigated here, by the general Bayer - Piccard synthesis [9, 10, 97].

[0091] As described in Example 3, a series of positively-charged sulfonamides, designated here as compounds **71-91**, were obtained by reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) with tri-/tetra-substituted pyrylium salts possessing alkyl-, aryl- or combinations of alkyl and aryl groups at the pyridinium ring (described below). Three of these compounds (**71**, **75**, and **87**) have been described elsewhere [25, 85]; all other compounds of this series are new.

Heterocyclic Sulfonamide Inhibitors of CA IX: Synthesis of Pyridinium Derivatives of Aminobenzolamide

[0092] Chemistry: Reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) [97] with 2,6-di-, 2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts afforded the pyridinium salts **71-91** investigated

here, by the general synthesis of such derivatives with nucleophiles (Scheme 1 as shown in Figure 5) [6, 26, 108].

[0093] Preparation of compounds: A large number of positively-charged sulfonamides, prepared by reaction of amino-sulfonamides with pyrylium salts [23, 88, 89] were recently reported by this group, and generally tested as inhibitors of the "classical" isozymes CA I, II and IV [81, 96, 97, 98]. Based on QSAR studies on several series of CA inhibitors, including some positively-charged derivatives [23, 88, 89], it emerged that the enhancement of CA inhibitory activity is correlated with increased positive charges on the heterocyclic/aromatic ring incorporated in such molecules, as well as with "long" inhibitor molecules *per se* (i.e., molecules extending on the direction passing through the Zn(II) ion of the enzyme, the sulfonamide nitrogen atom and the long axis of the inhibitor) [23, 88, 89]. It appeared thus of interest to try to explore this result, designing positively-charged, long sulfonamide CAIs. Thus, we thought of attaching substituted-pyridinium moieties to an already potent and long-molecule CAI suitable for reaction with pyrylium salts, i.e., aminobenzolamide [97]. Indeed, this compound acts as a very potent CAI against isozymes I, II and IV (with inhibition constants in the low nanomolar range - see later in the text). The substitution pattern of the pyridinium ring was previously shown [81, 96, 97, 98] to be critical for the biological activity of this type of sulfonamide CAIs. Thus, a large series of 2,4,6-trialkylpyridinium-; 2,6-dialkyl-4-phenylpyridinium-; 2-alkyl-4,6-diphenylpyridinium-; 2,4,6-triphenylpyridinium-, together with various 2,6-disubstituted-pyridinium and 2,3,5,6-tetrasubstituted-pyridinium aminobenzolamide derivatives have been prepared by the reaction described in Scheme 1 (Shown in Figure 5).

[0094] Although apparently simple, the reaction between a pyrylium salt and an amine, leading to pyridinium salts, is in reality a complicated process (Scheme 2, shown in Figure 6), as established by detailed spectroscopic and kinetic data from Balaban's and Katritzky's groups [6, 26, 108]. Thus, the nucleophilic attack of a primary amine RNH_2 on pyrylium cations generally occurs in the α position, with the formation of intermediates of type **IV** (depicted in Figure 6), which by deprotonation in the presence of bases lead to the 2-amino-tetrahydropyran derivatives **V**. In many cases the deprotonation reaction is promoted by the amine itself, when this is basic enough (this being the reason why in many cases one works at molar ratios pyrylium : amine of 1:2 when pyridinium salts are prepared by this method), or by external catalysts added to the reaction mixture, such as triethylamine [6, 26, 108]. The derivatives **V** are generally unstable, being tautomers with the ketodieneamines **VI** which are the key intermediates for the conversion of pyryliums into pyridiniums [6, 26, 108]. In acidic media, in the rate-determining step of the whole process, ketodieneamines **VI** may be converted to the corresponding pyridinium salts **VII**, although other products, such as vinylogous amides with diverse structures have also been isolated in such reactions [6, 26, 108]. A supplementary complication appears when the moiety substituting the 2- and/or 6-position(s) of the pyrylium ring is methyl, cases in which a concurrent cyclisation with formation of the anilines **VIII** in addition to the pyridinium salts **VII**, may take place too [6, 26, 108]. These concurrent reactions mentioned above are generally important when the amine to be converted into the pyridinium salt possesses weak nucleophilicity or basicity. This happens to be the case of aminobenzolamide. In fact, reaction of aminobenzolamide with several pyrylium salts, performed in a variety of conditions (different solvents, such as low molecular weight alcohols (MeOH, EtOH, *i*-PrOH); DMF; methylene chloride; acetonitrile; diverse molar ratios of the reagents; temperatures from 25 to 150 °C; reaction times between 15 min and 48 hours, etc) led only to the isolation of the unreacted raw materials. The only conditions which led to the formation of the pyridinium salts **III** (depicted in Figure 5) were the following: anhydrous methanol in the presence of acetic anhydride as solvent and triethylamine as catalysts for the deprotonation of the intermediates **IV**. Acetic anhydride had the role of reacting with the water formed in the condensation reaction. This water may in fact act as a competitive nucleophile with aminobenzolamide when reacting with the pyrylium cation, and as a consequence the yields in pyridinium salts would dramatically be decreased. After the rapid formation of the ketodieneamine, catalyzed by triethylamine (and in the presence of the acetic anhydride as water scavenging agent), the cyclisation to the pyridinium ring (the rate-determining step) has been achieved by refluxation in the presence of acetic acid (2-5 hours). Still the yields were not always good, especially for the 2-methyl-containing derivatives.

Preparation of MN Proteins and/or Polypeptides

[0095] The terms "MN/CA IX" and "MN/CA9" are herein considered to be synonyms for MN. Also, the G250 antigen is considered to refer to MN protein/polypeptide [112].

[0096] Zavada et al., WO 93/18152 and/or WO 95/34650 disclose the MN cDNA sequence shown herein in Figure 1A-1C [SEQ ID NO: 1], the MN amino acid sequence [SEQ ID NO: 2] also shown in Figure 1A-1C, and the MN genomic sequence [SEQ ID NO: 3] shown herein in Figure 2A-2F. The MN gene is organized into 11 exons and 10 introns.

[0097] The first thirty seven amino acids of the MN protein shown in Figure 1A-1C is the putative MN signal peptide [SEQ ID NO: 6]. The MN protein has an extracellular domain [amino acids (aa) 38-414 of Figure 1A-1C [SEQ ID NO: 7], a transmembrane domain [aa 415-434; SEQ ID NO: 8] and an intracellular domain [aa 435-459; SEQ ID NO: 9]. The extracellular domain contains the proteoglycan-like domain [aa 53-111; SEQ ID NO: 4] and the carbonic anhydrase (CA) domain [aa 135-391; SEQ ID NO: 5].

[0098] The phrase "MN proteins and/or polypeptides" (MN proteins/polypeptides) is herein defined to mean proteins and/or polypeptides encoded by an MN gene or fragments thereof. An exemplary and preferred MN protein according

to this invention has the deduced amino acid sequence shown in Figure 1. Preferred MN proteins/polypeptides are those proteins and/or polypeptides that have substantial homology with the MN protein shown in Figure 1. For example, such substantially homologous MN proteins/polypeptides are those that are reactive with the MN-specific antibodies, preferably the Mab M75 or its equivalent. The VU-M75 hybridoma that secretes the M75 Mab was deposited at the ATCC under

HB 11128 on September 17, 1992.

[0099] A "polypeptide" or "peptide" is a chain of amino acids covalently bound by peptide linkages and is herein considered to be composed of 50 or less amino acids. A "protein" is herein defined to be a polypeptide composed of more than 50 amino acids. The term polypeptide encompasses the terms peptide and oligopeptide.

[0100] It can be appreciated that a protein or polypeptide produced by a neoplastic cell *in vivo* could be altered in sequence from that produced by a tumor cell in cell culture or by a transformed cell. Thus, MN proteins and/or polypeptides which have varying amino acid sequences including without limitation, amino acid substitutions, extensions, deletions, truncations and combinations thereof, fall within the scope of this invention. It can also be appreciated that a protein extant within body fluids is subject to degradative processes, such as, proteolytic processes; thus, MN proteins that are significantly truncated and MN polypeptides may be found in body fluids, such as, sera. The phrase "MN antigen" is used herein to encompass MN proteins and/or polypeptides.

[0101] It will further be appreciated that the amino acid sequence of MN proteins and polypeptides can be modified by genetic techniques. One or more amino acids can be deleted or substituted. Such amino acid changes may not cause any measurable change in the biological activity of the protein or polypeptide and result in proteins or polypeptides which are within the scope of this invention, as well as, MN muteins.

[0102] The MN proteins and polypeptides of this invention can be prepared in a variety of ways according to this invention, for example, recombinantly, synthetically or otherwise biologically, that is, by cleaving longer proteins and polypeptides enzymatically and/or chemically. A preferred method to prepare MN proteins is by a recombinant means. Particularly preferred methods of recombinantly producing MN proteins are described below. A representative method to prepare the MN proteins shown in Figure 1 or fragments thereof would be to insert the full-length or an appropriate fragment of MN cDNA into an appropriate expression vector as exemplified in the Materials and Methods section.

MN Gene

[0103] Figure 1 A-C provides the nucleotide sequence for a full-length MN cDNA clone [SEQ ID NO: 1] isolated as described in Zavada et al., WO 95/34650. Figure 2A-F provides a complete MN genomic sequence [SEQ ID NO: 3].

[0104] The ORF of the MN cDNA shown in Figure 1 has the coding capacity for a 459 amino acid protein with a calculated molecular weight of 49.7 kd. The overall amino acid composition of the MN/CA IX protein is rather acidic, and predicted to have a pI of 4.3. Analysis of native MN/CA IX protein from CGL3 cells by two-dimensional electrophoresis followed by immunoblotting has shown that in agreement with computer prediction, the MN/CA IX is an acidic protein existing in several isoelectric forms with pIs ranging from 4.7 to 6.3.

[0105] The CA domain is essential for induction of anchorage independence, whereas the TM anchor and IC tail are dispensable for that biological effect. The MN protein is also capable of causing plasma membrane ruffling in the transfected cells and appears to participate in their attachment to the solid support. The data evince the involvement of MN in the regulation of cell proliferation, adhesion and intercellular communication.

Enzymatic Screening Assays

[0106] Assays are provided herein for the screening of compounds for inhibition of the enzymatic activity of the MN protein. Such assays comprise the incubation of said compound with said MN protein and with a substrate selected from the group consisting of saturated CO₂ and 4-nitrophenylacetate, preferably saturated CO₂, and determination of the inhibition constant K_i of said compound, wherein said enzymatic activity of the MN protein is measured by the pH change of an indicator by stopped flow spectrophotometer.

[0107] Screening of representative heterocyclic and aromatic sulfonamides for inhibition of MN protein: From Example 1, it was found that the inhibition profile of isozyme CA IX is very different from that of the classical isozymes CA I and II (cytosolic) as well as CA IV (membrane-bound). The following particular features may be noted: (i) all the 32 sulfonamides investigated in Example 1 act as CA IX inhibitors, with inhibition constants in the range of 14-285 nM (the corresponding affinities for the other three isozymes vary in a much wider range, as seen from data of Table 1). Based on these data, it can be noted that CA IX is a sulfonamide avid CA, similarly to CA II, the isozyme considered up to now to be responsible for the majority of pharmacological effect of sulfonamides [22, 29, 83,93, 94, 95, 102]. Still, many other differences are observed between CA IX and other isozymes for which inhibitors were developed for clinical use; (ii) for CA I, II and IV, generally, aromatic sulfonamides behave as weaker inhibitors as compared to heterocyclic derivatives (compare 1-6, or DCP), as aromatic compounds, with 15, 21, AAZ, MZA, EZA, DZA or BRZ among others (as heterocyclic sulfonamides). In the case of CA IX, such a fine distinction is rather difficult to be made, since both aromatic (such as

1, 6, 11, 12, 17, 18, 22-26 derivatives, as well as heterocyclic compounds (such as **14, 15, 21**, and the clinically used sulfonamides - except dichlorophenamide) possess rather similar inhibition constants, in the range of 14-50 nM; (iii) orthanilamide derivatives (such as **1, 17** and **22**) behave as very potent CA IX inhibitors (K_i -s in the range of 20-33 nM), although they are weak or medium-weak inhibitors of CA I, II and IV; (iv) 1,3-benzene-disulfonamide derivatives (such as **11, 12** and **DCP**) are again strong CA IX inhibitors, with K_i -s in the range of 24-50 nM, although their CA II, I and IV inhibition profile is not particularly strong; (v) metanilamide **2**, sulfanilamide **3**, and 4-hydrazino-benzenesulfonamide **4** show CA IX inhibition data quite similar with those against CA II, whereas homosulfanilamide **5** and 4-aminoethylbenzenesulfonamide **6** act as better CA IX inhibitors as compared to CA II inhibition; (vi) the halogenosulfanilamides **7-10** are much weaker inhibitors of CA IX than of CA II, a finding difficult to interpret at this moment; (vii) the strongest CA II inhibitor among the investigated compounds, 4-aminobenzolamide **15** (K_i of 2 nM) is not the strongest CA IX inhibitor (K_i of 38 nM). Instead, the best CA IX inhibitor detected so far is the ethoxzolamide phenol **21** (K_i of 14 nM). It is interesting to note that **21** and **EZA** have the same affinity for CA II, whereas their affinity for CA IX is rather different, with the phenol more active than the ethoxy-derivative; (viii) among the clinically used compounds, the best inhibitor is acetazolamide, followed by methazolamide, ethoxzolamide and brinzolamide. The most ineffective (but appreciably inhibiting the isozyme IX) are dichlorophenamide and dorzolamide; (ix) sulfonamides **20** and **22-26** behave as very good CA IX inhibitors, with K_i -s in the range of 16-32 nM, being slightly more effective than the clinically used CAIs mentioned above, and among the best CA IX inhibitors detected so far. It is thus envisageable that such compounds may be used as lead molecules for obtaining more potent and eventually specific CA IX inhibitors, with applications as antitumor agents.

[0108] Screening of representative pyridinium derivatives of aromatic sulfonamides for inhibition of MN protein: From Example 2, wherein membrane-impermeant pyridinium derivatives of sulfonamides were tested for their ability to inhibit the enzymatic activity of CA IX, the following conclusions were drawn from data of Table 2: (i) for a given substitution pattern of the pyridinium ring, the 4-aminoethyl-benzenesulfonamide derivatives **55-70** were more active than the corresponding homosulfanilamide derivatives **39-54**, which in turn were more active than the corresponding sulfanilamides **27-38**. This behavior has also been observed for the other three investigated isozymes [96]; (ii) some of the derivatives possessing bulky substituents at the pyridinium ring (mainly phenyls, *tert*-butyls; *n*-butyl, *n*-propyl or *iso*-propyl), such as **34-37, 51** and **67**, were very ineffective CA IX inhibitors, showing inhibition constants > 500 nM; (iii) another group of compounds, including **27, 30-33, 44**, and **60** showed a moderate inhibitory power towards the tumor-associated isozyme IX, showing K_i values in the range of 160 - 450 nM. Most of these compounds are sulfanilamide derivatives (except **44** and **60**), and the substitution pattern at the pyridinium ring includes (with one exception, **27**) at least one phenyl group in 4, or two phenyls in the 2 and 4 positions. It should be noted that the corresponding homosulfanilamides and 4-aminoethylbenzene-sulfonamides incorporating the same substitution pattern as the compounds mentioned above (sulfanilamides), lead to much better CA IX inhibitors (see later in the text); (iv) a third group of derivatives, including **38, 45-50, 52, 53, 61, 63-66, 68** and **69**, showed good CA IX inhibitory properties, with K_i values in the range of 64 - 135 nM. As mentioned above, except for the tetramethyl-pyridinium-substituted derivative **38**, most of these compounds incorporate 4-phenyl-pyridinium or 2,4-diphenylpyridinium moieties, whereas the group in position 6 is generally quite variable (alkyls or phenyl are tolerated). The most interesting observation regarding this subtype of CA IX inhibitors is constituted by the fact that the 2,4,6-triphenyl-pyridinium- and 2,6-diphenyl-pyridinium derivatives of homosulfanilamide and 4-aminoethylbenzenesulfonamide (**52-53** and **68-69**) efficiently inhibit isozyme IX, although they act as very weak inhibitors for isozymes I, II and IV (Table 2). As it will be discussed shortly, this may be due to the fact that the hCA IX active site is larger than that of the other investigated isozymes, notably CA II, I and IV; (v) a last group of derivatives (**28-29; 39-43; 54; 55-59; 62** and **70**) showed very good CA IX inhibitory properties, these compounds possessing K_i values in the range of 6 - 54 nM, similarly to the clinically used inhibitors acetazolamide, methazolamide, dichlorophenamide and indisulam, for which the inhibition data are provided for comparison. It should be noted that three derivatives **58, 59** and **70** showed inhibition constants < 10 nM, these being the most potent CA IX inhibitors ever reported up to now. Correlated with their membrane-impermeability [96, 85], it may be assumed that *in vivo* such compounds may lead for the first time to a selective CA IX inhibition. Thus, the best substitution pattern at the pyridinium ring includes either only compact alkyls (**39-41, 54, 55** and **70**), or 2,6-dialkyl-4-phenyl-pyridinium moieties (all compounds mentioned above except **62**, which incorporates a 2-methyl-4,6-diphenylpyridinium ring); (vi) the number of the substituents at the pyridinium ring seems to be less important for the activity of this series of CAIs, since both di-, tri- or tetrasubstituted derivatives showed good inhibitory potency. The nature of these groups on the other hand - as discussed in detail above - is the most important parameter influencing CA inhibitory properties (together with the linker between the benzenesulfonamide moiety and the substituted pyridinium ring); (vii) the isozyme most similar to hCA IX regarding the affinity for these inhibitors was hCA II (which has 33 % homology with hCA IX) [Pastorek et al. (1994), *supra*] whereas the affinities of isozymes I and IV were rather different.

[0109] Screening of representative pyridinium derivatives of heterocyclic sulfonamides for inhibition of MN protein, and comparison with inhibition of other CA isozymes: Isozyme I. As seen from data of Table 3, all derivatives **71-91** reported here act as very efficient CAIs against this isozyme which is generally the most "resistant" to inhibitors of this type [30, 31, 100, 102]. Indeed, aminobenzolamide is already a highly potent CA I inhibitor (K_i of 6 nM), whereas inhibitors

71-91 show inhibition constants in the range of 3-12 nM, in contrast to the clinically used sulfonamide CAls which are much less effective inhibitors, with K_i values in the range of 30 - 1200 nM (Table 3). Thus, derivatives possessing several bulky groups (*i*-Pr; *t*-Bu; *n*-Pr; *n*-Bu; Ph, etc) substituting the pyridinium moiety, such as **73, 74, 77, 78, 82, 84, 85** showed a decreased inhibitory activity as compared to aminobenzolamide, with K_i values in the range of 7-12 nM (aminobenzolamide has a K_i of 6 nM against hCA I). The rest of the compounds were more efficient as compared to aminobenzolamide in inhibiting this isozyme, with K_i values in the range of 3-5 nM. Best CA I inhibitors were **75**, and **89-91** (K_i of 3 nM), all of which containing either only alkyl moieties or 4-Ph and other alkyl moieties substituting the pyridinium ring. These are probably the best CA I inhibitors ever reported up to now, since the clinically used CAls show much higher inhibition constants against isozyme I (Table 3).

[0110] *Isozyme II.* Aminobenzolamide is already a very potent CA II inhibitor, with an inhibition constant around 2 nM. Several of the new inhibitors, such as **74, 77, 78, 82-88** act as weaker CA II inhibitors as compared to aminobenzolamide, with K_i values in the range of 3.13-5.96 nM (but all these compounds act as potent inhibitors, being much more effective than the clinically used CAls acetazolamide, methazolamide, dichlorophenamide or indisulam - see Table 3). Again the substitution pattern at the pyridinium ring is the main discriminator of activity for these compounds: all the less active derivatives mentioned above incorporate at least two bulky/long aliphatic groups, mainly in positions 2- and 6- of the pyridinium ring (*n*-Pr; *t*-Bu; *n*-Bu; and Ph). The best CA II inhibitors among derivatives **71-91** were those incorporating more compact 2,6-substituents at the pyridinium ring (such as Me, Et) together with a 4-Me or 4-Phe moiety, or those incorporating only aliphatic such groups, such as **71-73, 75, 76, 79-81, 89-91**, which showed K_i values in the range of 0.20-1.61 nM (thus, for the best inhibitors a factor of 10 increase in inhibitory power as compared to aminobenzolamide). It should be mentioned that *iso*-propyl-substituted compounds (**73, 79**) are active as CA II inhibitors, although their activity against CA I was not so good.

[0111] *Isozyme IV.* Most sulfonamides show inhibitory activity against CA IV intermediate between those towards CA I (less susceptible) and CA II (very high affinity for sulfonamides). This is also the trend observed with the sulfonamides investigated here, derivatives of aminobenzolamide. Thus, the parent sulfonamide (shown in Figure 5) is a potent CA IV inhibitor, with a K_i value around 5 nM. The new derivatives of general formula (B) incorporating bulky pyridinium-ring substituents (such as **74, 77, 78, 82, 84-88, 90**) were less effective than aminobenzolamide, showing K_i values in the range of 5.2-10.3 nM, whereas the compounds showing the other substitution pattern mentioned above were better CA IV inhibitors, showing K_i values in the range of 2.0-4.7 nM.

[0112] *Isozyme IX.* Aminobenzolamide is less inhibitory against this isozyme (K_i of 38 nM) as compared to other isozymes discussed above. This behavior is difficult to explain at this point, since no X-ray crystal structure of this isozyme has been reported. A very encouraging result obtained with the new derivatives of general formula (B) reported here, was the observation that several of them show very high affinity for CA IX, with K_i values in the range of 3-9 nM (derivatives **71, 72, 75, 76**, and **89**). It may be seen that all of them incorporate aliphatic moieties (Me, Et and *i*-Pr) in positions 2- and 6- of the pyridinium ring, and either 4-Me or 4-Ph moieties. Only one compound is tetrasubstituted (**89**), again possessing only methyl moieties. The best CA IX inhibitor (and the best ever reported up to now) was **71**, which is almost 13 times more effective than benzolamide in inhibiting this isozyme. Another group of new derivatives, such as **73, 74, 77, 79, 80, 81, 83, 86-88, 90, 91**, showed effective CA IX inhibition, with K_i values in the range of 12-35 nM, being thus more effective than aminobenzolamide. They incorporate slightly bulkier groups as compared to the previously discussed ones. Again the less effective inhibitors (K_i values in the range of 40-43 nM) were those incorporating several bulky pyridinium substituents, such as **78, 84, 85** which contained either two *n*-Bu or one Ph and *n*-Bu/*t*-Bu in positions 2- and 6- of the pyridinium ring. Thus, SAR is now rather clear for this type of CAls: best CA IX inhibitors should contain either only small, compact aliphatic moieties substituting the pyridinium ring, or they tolerate a 4-Ph moiety, but the 2,6-substituents should again be small, compact aliphatic moieties. In this particular case, 2,4,6-trisubstituted-pyridinium derivatives were more effective CA IX inhibitors as compared to the tetrasubstituted derivatives.

[0113] Membrane impermeability of Heterocyclic Sulfonamide Inhibitors of CA IX. As seen from data of Table 4 of Example 3, incubation of human red cells (which contain high concentrations of isozymes I and II, i.e., 150 μ M hCA I and 20 μ M hCA II, but not the membrane-bound CA IV or CA IX) [118] with millimolar concentrations of different sulfonamide inhibitors, such as acetazolamide, or methazolamide, led to saturation of the two isozymes present in erythrocytes with inhibitor, already after short periods of incubation (30 min), whereas for benzolamide or aminobenzolamide, a similar effect is achieved after somehow longer periods (60 min) (Table 4). This is obviously due to the high diffusibility through membranes of the first three inhibitors, whereas benzolamide/aminobenzolamide with a pK_a of 3.2 for the second sulfonamido group [58] being present mainly as an (di)anion at the pH at which the experiment has been done (7.4), is already less diffusible and penetrates membranes in a longer time. Different cationic sulfonamides synthesized by us here, such as **71, 76, 89, 91**, in the same conditions, were detected only in very small amounts within the blood red cells, proving that they were unable to penetrate through the membranes, obviously due to their cationic nature. Even after incubation times as long as one hour (and longer, data not shown), only traces of such cationic sulfonamides were present inside the blood red cells, as proved by the three assay methods used for their identification in the cell lysate, which were in good agreement with each other (Table 4). This demonstrates that the proposed approach

for achieving membrane impermeability works well for the designed positively-charged sulfonamide CAIs of the general formula (B) (shown above), since the very small amount of sulfonamide detected may be due to contamination of the lysates with very small amount of membranes.

Design of Membrane-Impermeant Sulfonamide Inhibitors of CA IX

[0114] No X-ray crystal structure of isozyme IX is available up to now, in strong contrast with hCA II, for which many X-ray crystal structures are available (alone or in complexes with inhibitors and activators) [1, 2, 14, 15, 19a, 19b, 37, 38]. Examining the active site residues of these two isozymes and the architecture of hCA II, may help explain the above inhibition data and their relevance for CA IX specific inhibitors.

[0115] First of all, the zinc ligands and the proton shuttle residue of these two isozymes are identical [33, 43, 72, 100, 101, 102, 114, 115, 117]. An important difference is constituted by the amino acid in position 131, which is Phe for hCA II and Val for hCA IX. Phe 131 is known to be very important for the binding of sulfonamide inhibitors to hCA II [2, 46, 47]: in many cases this bulky side chain limits the space available for the inhibitor aromatic moieties, or it may participate in stacking interactions with groups present in it (for recent examples see refs. [2, 46, 47]. Thus, the presence of a less bulky such residue in hCA IX (i.e., a valine) which is also unavailable for participation to stacking interactions has as a consequence the fact that the hCA IX active site is larger than that of hCA II. A second potentially important residue is 132, which is Gly in hCA II and Asp in hCA IX. This residue is situated on the rim of the hydrophilic half of the entrance to the active site of hCA II (and presumably also of hCA IX) and it is critical for the interaction with inhibitors possessing elongated molecules, as recently shown by us [19b]. Strong hydrogen bonds involving the CONH moiety of Gly 132 were shown to stabilize the complex of this isozyme with a *p*-aminoethylbenzenesulfonamide derived inhibitor [19b]. In the case of hCA IX, the presence of aspartic acid in this position at the entrance of the active site may signify that: (i) stronger interactions with polar moieties of the inhibitor bound within the active site should be possible, since the COOH moiety possesses more donor atoms; (ii) this residue may have flexible conformations, fine-tuning in this way the interaction with inhibitors. Thus, the stronger hCA IX inhibition with some of these inhibitors (as compared to their affinity for isozyme II), such as for example 46-50, 52, 53, 55, 58, 62 and 68-70, might be explained just by the different interactions with the two active site residues mentioned above.

Therapeutic Use of MN-Specific Inhibitors

[0116] The MN-specific inhibitors of this invention, organic and/or inorganic, preferably organic, and as outlined above, may be used therapeutically in the treatment of neoplastic and/or pre-neoplastic disease, either alone or in combination with other chemotherapeutic drugs.

[0117] The MN-specific inhibitors can be administered in a therapeutically effective amount, preferably dispersed in a physiologically acceptable, non-toxic liquid vehicle.

Materials and Methods

[0118] General. Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400-4000 cm⁻¹ Perkin-Elmer 16PC FTIR spectrometer; ¹H-NMR spectra: Varian 300CXP apparatus (chemical shifts are expressed as δ values relative to Me₄Si as standard); Elemental analysis: Carlo Erba Instrument CHNS Elemental Analyzer, Model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Pyrylium salts were prepared by literature procedures, generally by olefin (or their precursors) bisacylation, as described in the literature [6, 26, 108], whereas aminobenzolamide as described earlier [97]. Other sulfonamides used as standards were commercially available.

General procedure for the preparation of compounds 71-91 (Pyridinium derivatives of aminobenzolamide) (not falling under the invention)

[0119] An amount of 2.9 mM of aminobenzolamide [97] and 2.9 mM of pyrylium salt II (depicted in Figure 5) were suspended in 5 mL of anhydrous methanol and poured into a stirred mixture of 14.5 mM of triethylamine and 5.8 mM of acetic anhydride. After five minutes of stirring, another 10 mL of methanol were added to the reaction mixture, which was heated to reflux for 15 min. Then 14.5 mM of acetic acid was added and heating was continued for 2-5 hours. The role of the acetic anhydride is to react with the water formed during the condensation reaction between the pyrylium salt and the aromatic amine, in order to shift the equilibrium towards the formation of the pyridinium salts of the general formula (B) (shown above). In the case of aminobenzolamide, this procedure is the only one which gave acceptable yields in pyridinium salts, probably due to the deactivating effect of the sulfamoylaminothiadiazole moiety on the amine group, which becomes poorly nucleophilic and unreactive towards these reagents. The precipitated pyridinium salts

obtained were purified by treatment with concentrated ammonia solution (which also converts the eventually unreacted pyrylium salt to the corresponding pyridine which is soluble in acidic medium), reprecipitation with perchloric acid and recrystallization from water with 2-5 % HClO_4 .

5 Purification of Catalytic Domain of CA IX

[0120] The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al. [72]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene).
 10 The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2 % Triton X-100, as described by Wingo et al. [116]. The homogenate thus obtained was extensively centrifuged in order to remove soluble and membrane associated proteins as well as other cellular debris. The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution
 15 of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM ZnCl_2 , 2 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione. Active hCA IX was extensively dialysed into a solution of 10mM Hepes (pH 7.5), 10mM Tris HCl, 100mM Na_2SO_4 and 1 mM ZnCl_2 . The amount of protein was determined by spectrophotometric measurements and its activity by stopped-flow measurements, with CO_2 as substrate [44]. Optionally, the protein was further purified by sulfonamide affinity chromatography [44], the amount of enzyme was determined by spectrophotometric measurements and its activity
 20 by stopped-flow measurements, with CO_2 as substrate [44].

CA I, II and IV purification

[0121] Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group [54]. Cell growth conditions were those described in ref. [12], and enzymes were purified by affinity chromatography according to the method of Khalifah et al. [45]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of $49 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for CA I and $54 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for CA II, respectively, based on $M_r = 28.85 \text{ kDa}$ for CA I, and 29.3 kDa for CA II, respectively [53, 84]. CA IV was isolated from bovine lung microsomes as described by Maren et al, and its concentration has been determined by
 30 titration with ethoxzolamide [59].

Enzyme assays

CA CO_2 Hydrase Activity Assay

[0122] An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA CO_2 hydration activity assays [44]. A stopped flow variant of the Poker and Stone spectrophotometric method [76] has been employed, using an SX.18MV-R Applied Photophysics stopped flow instrument, as described previously [43]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM
 40 Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s. Saturated CO_2 solutions in water at 20°C were used as substrate [44]. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the
 45 formation of the E-I complex. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results.

CA Esterase Activity Assay

[0123] Initial rates of 4-nitrophenylacetate hydrolysis catalysed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [76]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between $2 \cdot 10^{-2}$ and $1 \cdot 10^{-6} \text{ M}$, working at 25°C . A molar absorption coefficient ϵ of $18,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature [76]. Non-enzymatic hydrolysis rates were always
 50 subtracted from the observed rates. Triplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1-3 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together

for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constant K_i was determined as described in references [44, 76].

Membrane Permeance Assay: Ex vivo Penetration through Red Blood Cells

[0124] An amount of 10 mL of freshly isolated human red cells thoroughly washed several times with Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min were treated with 25 mL of a 2 mM solution of sulfonamide inhibitor. Incubation has been done at 37 °C with gentle stirring, for periods of 30 - 120 min. After the incubation times of 30, 60 and 120 min., respectively, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 10 mL of the above mentioned buffer, in order to eliminate all unbound inhibitor [81, 96, 98]. The cells were then lysed in 25 mL of distilled water, centrifuged for eliminating membranes and other insoluble impurities. The obtained solution was heated at 100 °C for 5 minutes (in order to denature CA-s) and sulfonamides possibly present have been assayed in each sample by three methods: a HPLC method [36]; spectrophotometrically [4] and enzymatically [76].

[0125] HPLC: A variant of the methods of Gomaa [36] has been developed by us, as follows: a commercially available 5 μ m Bondapak C-18 column was used for the separation, with a mobile phase made of acetonitrile - methanol - phosphate buffer (pH 7.4) 10:2:88 (v/v/v), at a flow rate of 3 mL/min, with 0.3 mg/mL sulphadiazine (Sigma) as internal standard. The retention times were: 12.69 min for acetazolamide; 4.55 min for sulphadiazine; 10.54 min for benzolamide; 12.32 min for aminobenzolamide; 3.15 min for **71**; 4.41 min for **76**; 3.54 min for **89**; and 4.24 min for **91**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide, and wavelength in the range of 270 - 310 nm in the case of the other sulfonamides).

[0126] Spectrophotometrically: A variant of the pH-induced spectrophotometric assay of Abdine et al. [4] has been used, working for instance at 260 and 292 nm, respectively, for acetazolamide; at 225 and 265 nm, respectively, for sulfanilamide, etc. Standardized solutions of each inhibitor have been prepared in the same buffer as the one used for the membrane penetrability experiments.

[0127] Enzymatically: the amount of sulfonamide present in the lysate has been evaluated based on hCA II inhibition measured with the esterase method, as described above [76]. Standard inhibition curves have been obtained previously for each sulfonamide, using the pure compound, which were used thereafter for determining the amount of inhibitor present in the lysate. Mention should be made that the three methods presented above led to results in good agreement, within the limits of the experimental errors.

[0128] Statistical analysis: Values are expressed $\pm$ standard error of measurement. Statistical significance was determined using an unpaired t-test with $p < 0.05$ considered significant.

[0129] The following examples are for purposes of illustration only and are not meant to limit the invention in any way.

Example 1

Inhibition of the tumor-associated isozyme IX with aromatic and heterocyclic sulfonamides

[0130] The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme has been investigated with a series of aromatic and heterocyclic sulfonamides, including the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison.

[0131] **Chemistry**. Sulfonamides investigated for the inhibition of the tumor-associated isozyme CA IX, of types **1-26** are shown in Figure 4A-B. Compounds **1-6**, **11-12**, **20** and **26** are commercially available, whereas **7-10** [43], **13-19** [24, 79, 90, 97] and **21-25** [79] were prepared as reported earlier. The six clinically used compounds were also assayed, since no such data are available in the literature.

[0132] **CA inhibition data**. Inhibition data against four CA isozymes, CA I, II, IV and IX [44, 72, 116], with the above mentioned compounds **1-26** and the six clinically used inhibitors, are shown in Table 1. Compounds not claimed are recited for comparative reasons only.

Table 1: CA I, II, IV and IX inhibition data with sulfonamides **1-26** and clinically used inhibitors.

Inhibitor	hAC I ^a	K_i^* (nM) hCA II ^a	bCA IV ^b	hCA IX ^c
1	45400	295	1310	33
2	25000	240	2200	238
3	28000	300	3000	294
4	78500	320	3215	305

(continued)

Inhibitor	hAC I ^a	K _I [*] (nM) hCA II ^a	bCA IV ^b	hCA IX ^c
5	25000	170	2800	103
6	21000	160	2450	33
7	8300	60	180	245
8	9800	110	320	264
9	6500	40	66	269
10	6000	70	125	285
11	5800	63	154	24
12	8400	75	160	39
13	8600	60	540	41
14	9300	19	355	30
15	6	2	5	38
16	164	46	129	34
17	185	50	144	20
18	109	33	72	31
19	95	30	72	24
20	690	12	154	16
21	55	8	17	14
22	21000	125	415	32
(Table 1, continued)				
23	23000	133	438	30
24	24000	125	560	21
25	18000	110	450	22
26	135	40	86	26
AAZ	250	12	70	25
MZA	50	14	36	27
EZA	25	8	13	34
DCP	1200	38	380	50
DZA	50000	9	43	52
BRZ	-	3	45	37

^a Human cloned isozymes, esterase assay method [76];^b Isolated from bovine lung microsomes, esterase assay method [76];^c Human cloned isozyme, CO₂ hydrase assay method [44, 72, 116].

[0133] We report here the first inhibition study of the tumor-associated, transmembrane isozyme CA IX with a series of aromatic and heterocyclic sulfonamides, including also the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) are also provided for comparison. Very interesting inhibition profile against CA IX with these sulfonamides has been detected, which is a promising discovery for the potential design of CA IX-specific inhibitors, with applications as antitumor agents. Several nanomolar CA IX inhibitors have been detected, both among the aromatic (such as orthanilamide, homosulfanilamide, 4-carboxy-benzenesulfonamide, 1-naphthalene-sulfonamide and 1,3-benzenedisulfonamide derivatives) as well as the heterocyclic (such as 1,3,4-thiadiazole-2-sulfonamide, benzothiazole-2-sulfonamide, etc.) sulfonamides investigated.

Example 2

The first selective, membrane-impermeant inhibitors targeting the tumor-associated isozyme IX

[0134] Up to now no CA IX inhibition studies with this type of membrane-impermeant CAIs have been reported. Thus, we decided to explore some of the pyridinium derivatives of general formula (A) for their interaction with the catalytic domain of tumor-associated isozyme IX, recently cloned and purified by the inventors [33, 43, 114, 115, 117], as well

as the cytosolic, physiologically relevant isozymes CA I, II and the membrane-anchored isozyme CA IV [88, 96].

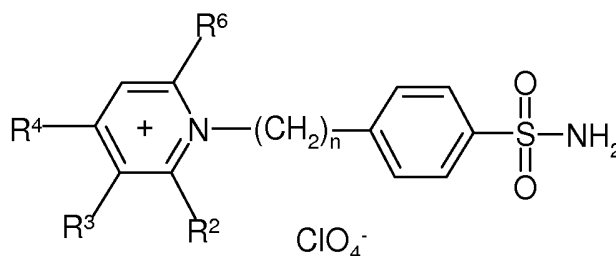
[0135] The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme has been investigated with a series of positively-charged, pyridinium derivatives of sulfanilamide, homosulfanilamide and 4-aminoethyl-benzenesulfonamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison. This is the first report of inhibitors that may selectively target CA IX, due to their membrane-impermeability and high affinity for this clinically relevant isozyme.

CA inhibition.

[0136] Data of Table 2 clearly show that most of the compounds **27-70** act as efficient CA IX inhibitors, and that their affinity for this isozyme differs considerably as compared to affinities for the cytosolic isozymes CA I and II, and the other membrane-associated isozyme investigated, CA IV. Compounds not claimed are recited for comparative reasons only.

[0137] In a series of substituted-pyridinium derived sulfanilamides, homosulfanilamides and *p*-aminoethylbenzenesulfonamides, a large number of effective hCA IX inhibitors were detected. Some low nanomolar CA IX inhibitors were reported for the first time. Since these compounds are membrane-impermeant due to their salt-like character, and as hCA IX is present on the extracellular side of many tumors with poor clinical prognosis, compounds of this type target specifically this tumor-associated CA isozyme without affecting the cytosolic CAs known to play important physiological functions. Thus, compounds of this type may constitute the basis of new anticancer therapies based on CA inhibitors.

Table 2: Inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX with the pyridinium salts **27-70**.



A

Compound	R ²	R ³	R ⁴	R ⁶	hCA Ia (μM)	hCA IIa (nM)	K _I [*] bCA IV ^b (nM)	hCA IX ^c (nM)
27	Me	H	Me	Me	10	150	290	165
28	Me	H	Ph	Me	7	60	211	48
29	Et	H	Ph	Et	6	60	182	43
30	<i>n</i> -Pr	H	Ph	<i>n</i> -Pr	10	120	194	178
31	<i>i</i> -Pr	H	Ph	<i>i</i> -Pr	5	50	90	160
32	Me	H	Ph	Ph	40	210	852	280
33	Et	H	Ph	Ph	43	400	1300	450
34	<i>n</i> -Pr	H	Ph	Ph	140	580	1483	>500
35	<i>i</i> -Pr	H	Ph	Ph	125	440	2102	>500
36	<i>n</i> -Bu	H	Ph	Ph	305	620	2155	>500
37	Ph	H	Ph	Ph	290	510	2500	>500
38	Me	Me	Me	Me	5	40	61	72
39	Me	H	Me	Me	7	50	92	38
40	<i>i</i> -Pr	H	Me	Me	6	50	80	42
41	<i>i</i> -Pr	H	Me	<i>i</i> -Pr	11	80	144	54
42	Me	H	Ph	Me	4	20	70	26
43	Et	H	Ph	Et	2	21	52	29
44	<i>n</i> -Pr	H	Ph	<i>n</i> -Pr	24	90	163	230
45	<i>i</i> -Pr	H	Ph	<i>i</i> -Pr	12	61	101	100
46	Me	H	Ph	Ph	32	121	161	64
47	Et	H	Ph	Ph	42	314	983	79

(continued)

Compound	R ²	R ³	R ⁴	R ⁶	hCA I ^a (μ M)	hCA II ^a (nM)	K _I [*] bCA IV ^b (nM)	hCA IX ^c (nM)
5 48	<i>n</i> -Pr	H	Ph	Ph	130	390	1260	85
49	<i>i</i> -Pr	H	Ph	Ph	112	370	1214	80
50	<i>n</i> -Bu	H	Ph	Ph	300	595	2104	135
51	<i>t</i> -Bu	H	Ph	Ph	110	321	1070	>500
10 52	Ph	H	Ph	Ph	280	472	1956	120
53	Ph	H	H	Ph	280	493	1954	106
54	Me	Me	Me	Me	3	30	51	35
55	Me	H	Me	Me	4	21	60	14
15 56	<i>i</i> -Pr	H	Me	Me	2	15	32	31
57	<i>i</i> -Pr	H	Me	<i>i</i> -Pr	3	20	70	49
58	Me	H	Ph	Me	1	8	20	6
59	Et	H	Ph	Et	1	9	21	8
60	<i>n</i> -Pr	H	Ph	<i>n</i> -Pr	7	42	82	205
20 61	<i>i</i> -Pr	H	Ph	<i>i</i> -Pr	6	21	70	89
62	Me	H	Ph	Ph	18	103	144	37
63	Et	H	Ph	Ph	40	220	761	70
64	<i>n</i> -Pr	H	Ph	Ph	112	270	1055	84
25 65	<i>i</i> -Pr	H	Ph	Ph	94	350	864	78
66	<i>n</i> -Bu	H	Ph	Ph	290	544	2008	120
67	<i>t</i> -Bu	H	Ph	Ph	92	275	1000	>500
68	Ph	H	Ph	Ph	270	419	1830	95
69	Ph	H	H	Ph	265	420	1905	81
30 70	Me	Me	Me	Me	2	10	21	8
acetazolamide					0.25	12	70	25
methazolamide					0.05	14	36	27
dichlorophenamide					1.2	38	380	50
35 indisulam					0.03	15	65	24

^a Human (cloned) isozymes; ^b From bovine lung microsomes; ^c Catalytic domain of the human, cloned isozyme.

* errors in the range of ± 10 % of the reported value, from three different determinations.

For compounds 27-38: *n* = 0; 39-54: *n* = 1; 55-70: *n* = 2

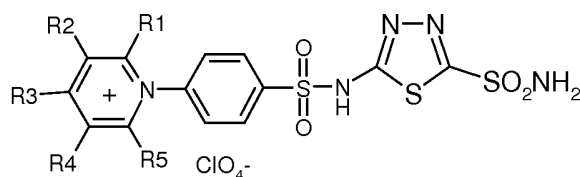
Reference Example 3

Design of selective, membrane-impermeant heterocyclic sulphonamide inhibitors targeting the human tumor-associated isozyme IX

[0138] A series of positively-charged sulfonamides were obtained by reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) with tri-/tetra-substituted pyrilium salts possessing alkyl-, aryl- or combinations of alkyl and aryl groups at the pyridinium ring. These new compounds are membrane-impermeant due to their salt-like character and were assayed for the inhibition of four physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic hCA I and II, the membrane-anchored bCA IV and the membrane-bound, tumor associated isozyme hCA IX. The high affinity of these new derivatives for the tumor-associated isozyme CA IX and their membrane impermeability, make this type of CA inhibitors interesting candidates for the selective inhibition of only the tumor associated isozyme and not the cytosolic ones, for which they also show high potency.

Results

[0139] CA inhibition. Inhibition data against isozymes I, II, IV and IX with compounds **71-91** reported here are shown in Table 3.

Table 3: Inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX with the pyridinium salts **71-91**.**B**

	R ¹	R ²	R ³	R ⁴	R ⁵	K _i [*] (nM)			
						hCA I ^a	hCA II ^a	bCA IV ^b	hCA IX ^c
71	Me	H	Me	H	Me	4	0.26	2.1	3
72	<i>i</i> -Pr	H	Me	H	Me	4	0.39	3.0	5
73	<i>i</i> -Pr	H	Me	H	<i>i</i> -Pr	7	1.54	4.7	16
74	<i>t</i> -Bu	H	Me	H	<i>t</i> -Bu	11	3.13	9.4	34
75	Me	H	Ph	H	Me	3	0.20	2.0	6
76	Et	H	Ph	H	Et	4	0.21	2.3	9
77	<i>n</i> -Pr	H	Ph	H	<i>n</i> -Pr	9	3.45	8.1	35
78	<i>n</i> -Bu	H	Ph	H	<i>n</i> -Bu	10	4.62	10.3	40
79	<i>i</i> -Pr	H	Ph	H	<i>i</i> -Pr	5	1.61	4.1	30
80	Me	H	Ph	H	Ph	4	1.21	3.0	24
81	Et	H	Ph	H	Ph	5	1.14	3.8	29
82	<i>n</i> -Pr	H	Ph	H	Ph	8	3.90	6.0	40
83	<i>i</i> -Pr	H	Ph	H	Ph	6	3.74	4.5	32
84	<i>n</i> -Bu	H	Ph	H	Ph	8	4.95	8.4	45
85	<i>t</i> -Bu	H	Ph	H	Ph	12	4.11	7.0	43
86	Ph	H	Me	H	Ph	6	4.78	5.8	12
87	Ph	H	Ph	H	Ph	5	5.96	5.6	12
88	Ph	H	H	H	Ph	5	4.93	5.4	16
89	Me	Me	Me	H	Me	3	0.30	2.4	5
90	Me	Me	Ph	H	Me	3	1.24	5.2	15
91	Me	R ³ ,R ⁵ = (CH ₂) ₉ ; R ⁴ =Me			Me	3	1.37	4.6	12
aminobenzolamide						6	2.04	5.1	38
acetazolamide						250	12	70	25
methazolamide						50	14	36	27
dichlorophenamide						1200	38	380	50
indisulam						30	15	65	24

^a Human (cloned) isozymes, esterase assay method [76].^b From bovine lung microsomes, esterase assay method [76].^c Catalytic domain of the human, cloned isozyme, CO₂ hydrase assay method [44].* Errors in the range of $\pm 10\%$ of the reported value, from three different determinations.

[0140] Ex vivo penetration through red blood cells. Levels of sulfonamides in red blood cells after incubation of human erythrocytes with millimolar solutions of inhibitor for 30-60 min (both classical as well as positively-charged sulfonamides were used in such experiments) are shown in Table 4 [4, 12, 36, 45, 53, 54, 58, 59, 84, 116, 118].

Table 4: Levels of sulfonamide CA inhibitors (μM) in red blood cells at 30 and 60 min, after exposure of 10 mL of blood to solutions of sulfonamide (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4). The concentrations of sulfonamide has been determined by three methods: HPLC; electronic spectroscopy (ES) and the enzymatic method (EI) - see Experimental for details.

Inhibitor	[sulfonamide], μM^*					
	HPLC ^a	t = 30 min ES ^b	EI ^c	HPLC ^a	t = 60 min ES ^b	EI ^c
AAZ	136	139	140	160	167	163
MZA	170	169	165	168	168	167
Benzolamide	110	108	112	148	146	149
Aminobenzolamide	125	127	122	154	156	158
71	0.3	0.5	0.5	0.4	0.5	0.3
76	1.0	1.1	1.0	1.1	1.2	1.1
89	0.3	0.2	0.5	0.3	0.6	0.4
91	0.4	0.3	0.5	0.3	0.6	0.5

* Standard error (from 3 determinations) < 5 % by : ^a the HPLC method [36]; ^b the electronic spectroscopic method [4]; ^c the enzymatic method [76].

[0141] The new compounds reported in the present work were characterized by standard chemical and physical methods (elemental analysis, within ± 0.4 % of the theoretical values; IR and NMR spectroscopy) that confirmed their structure (see Materials and Methods and Table 5 below for details) and were assayed for the inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX.

Table 5: Elemental analysis data for the compounds described in Example 3

No	Formula	Elemental analysis data (calc./found)		
		% C	% H	% N
71	$\text{C}_{16}\text{H}_{18}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	35.59/35.32	3.36/3.62	12.97/12.93
72	$\text{C}_{18}\text{H}_{22}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	38.06/37.95	3.90/4.16	12.33/12.18
73	$\text{C}_{20}\text{H}_{26}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	40.30/39.99	4.40/4.54	11.75/11.63
74	$\text{C}_{22}\text{H}_{30}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	42.34/42.56	4.84/4.76	11.22/11.03
75	$\text{C}_{21}\text{H}_{20}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	41.89/42.02	3.35/3.03	11.63/11.48
76	$\text{C}_{23}\text{H}_{24}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	43.84/43.88	3.84/3.62	11.11/10.95
77	$\text{C}_{25}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	45.62/45.60	4.29/4.36	10.64/10.50
78	$\text{C}_{27}\text{H}_{32}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	47.26/47.45	4.70/4.89	10.21/10.14
79	$\text{C}_{25}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	45.62/45.49	4.29/4.18	10.64/10.61
80	$\text{C}_{26}\text{H}_{22}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	47.02/46.79	3.34/3.33	10.55/10.23
81	$\text{C}_{27}\text{H}_{24}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	47.82/47.73	3.57/3.73	10.33/10.40
82	$\text{C}_{28}\text{H}_{26}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	48.59/48.83	3.79/3.91	10.12/10.24
83	$\text{C}_{28}\text{H}_{26}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	48.59/48.27	3.79/3.82	10.12/10.05
84	$\text{C}_{29}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	49.32/49.59	4.00/4.23	9.92/9.67
85	$\text{C}_{29}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	49.32/49.16	4.00/3.94	9.92/9.71
86	$\text{C}_{26}\text{H}_{22}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	47.02/47.25	3.34/3.18	10.55/10.46
87	$\text{C}_{31}\text{H}_{24}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	51.27/51.50	3.33/3.60	9.64/9.67
88	$\text{C}_{25}\text{H}_{20}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	46.19/46.28	3.10/2.95	10.77/10.67
89	$\text{C}_{17}\text{H}_{20}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	36.86/36.72	3.64/3.53	12.64/12.45
90	$\text{C}_{22}\text{H}_{22}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	42.89/42.70	3.60/3.84	11.37/11.15
91	$\text{C}_{24}\text{H}_{32}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$	44.34/44.57	4.96/4.99	10.77/10.51

Conclusions

[0142] We report here a general approach for the preparation of positively-charged, membrane-impermeant sulfonamide CA inhibitors with high affinity for the cytosolic isozymes CA I and CA II, as well as for the membrane-bound ones CA IV and CA IX. They were obtained by attaching substituted-pyridinium moieties to aminobenzolamide, a very potent CA inhibitor itself. Ex vivo studies showed the new class of inhibitors reported here to discriminate for the membrane-bound versus the cytosolic isozymes. Correlated with the low nanomolar affinity of some of these compounds for the tumor-associated isozyme CA IX, this report constitutes the basis of selectively inhibiting only the target, tumor-associated CA IX in vivo, whereas the cytosolic isozymes would remain unaffected.

Characterization of Compounds 71-91 (For preparation, see Materials and Methods Section)

[0143] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-trimethyl-pyridinium perchlorate **71**: white crystals, mp >300 °C; IR (KBr), cm⁻¹ (bands in italics are due to the anion): 595, 625, 664, 787, 803, 884, 915, 1100, 1150, 1190, 1200, 1285, 1360, 1495, 1604, 3065; ¹H-NMR (D₂O), δ, ppm: 3.08 (s, 6H, 2,6-Me₂); 3.11 (s, 3H, 4-Me), 7.30 - 8.06 (m, AA'BB', 4H, ArH from phenylene); 9.05 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal C₁₆H₁₈N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0144] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-iso-propyl-4,6-dimethylpyridinium perchlorate **72**, colorless crystals, mp 290-1 °C; IR (KBr), cm⁻¹: 625, 680, 720, 1100, 1165, 1330, 1640, 3020, 3235; ¹H-NMR (TFA), δ, ppm: 1.50 (d, 6H, 2Me from *i*-Pr); 2.80 (s, 3H, 6-Me); 2.90 (s, 3H, 4-Me); 3.49 (heptet, 1 H, CH from *i*-Pr); 7.25 - 8.43 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.98 (s, 2H, ArH, 3,5-H from pyridinium). Anal C₁₈H₂₂N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0145] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-iso-propyl-4-methylpyridinium perchlorate **73**, tan crystals, mp 278-9 °C; IR (KBr), cm⁻¹: 625, 685, 820, 1100, 1165, 1340, 1635, 3030, 3250; ¹H-NMR (TFA), δ, ppm: 1.51 (d, 12H, 4Me from 2 *i*-Pr); 2.83 (s, 3H, 4-Me); 3.42 (heptet, 2H, 2CH from 2 *i*-Pr); 7.31 - 8.51 (m, AA'BB', 4H, ArH from 1,4-phenylene); 8.05 (s, 2H, ArH, 3,5-H from pyridinium). Anal C₂₀H₂₆N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0146] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-dimethyl-4-phenylpyridinium perchlorate **75**, white crystals, mp > 300 °C; IR (KBr), cm⁻¹: 625, 690, 770, 1100, 1170, 1330, 1635, 3030, 3260, 3330; ¹H-NMR (TFA), δ, ppm: 2.62 (s, 6H, 2,6-(Me)₂); 8.10 - 9.12 (m, 11 H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal C₂₁H₂₀N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0147] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diethyl-4-phenylpyridinium perchlorate **76**, tan crystals, mp 267-8 °C; IR (KBr), cm⁻¹: 625, 695, 765, 1100, 1180, 1340, 1630, 3040, 3270, 3360; ¹H-NMR (TFA), δ, ppm: 1.43 (t, 6H, 2 Me from ethyl); 2.82 (q, 4H, 2 CH₂ from Et); 7.68 - 8.87 (m, 11 H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal C₂₃H₂₄N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0148] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-*n*-propyl-4-phenylpyridinium perchlorate **77**, colorless crystals, mp 235-7 °C; IR (KBr), cm⁻¹: 625, 695, 770, 1100, 1180, 1340, 1630, 3050, 3220, 3315; ¹H-NMR (TFA), δ, ppm: 1.06 (t, 6H, 2 Me from propyl); 1.73 (sextet, 4H, 2CH₂ (β) from *n*-Pr); 2.84 (t, 4H, 2 CH₂ (α) from *n*-Pr); 7.55 - 8.71 (m, 11 H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal C₂₅H₂₈N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0149] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-isopropyl-4-phenylpyridinium perchlorate **79**, white crystals, mp 278-9 °C; IR (KBr), cm⁻¹: 625, 690, 765, 1100, 1180, 1340, 1625, 3040, 3270, 3315; ¹H-NMR (TFA), δ, ppm: 1.45 (d, 12H, 4 Me from *i*-Pr); 2.95 (heptet, 2H, 2 CH from *i*-Pr); 7.92 - 8.97 (m, 11 H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal C₂₅H₂₈N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0150] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-methyl-4,6-diphenylpyridinium perchlorate **80**, white crystals, mp 298-99 °C; IR (KBr), cm⁻¹: 625, 710, 770, 1100, 1170, 1345, 1625, 3040, 3245, 3350; ¹H-NMR (TFA), δ, ppm: 2.75 (s, 3H, 2-Me); 7.53 - 8.70 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal C₂₆H₂₂N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0151] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-ethyl-4,6-diphenylpyridinium perchlorate **81**, white crystals, mp 254-5 °C; IR (KBr), cm⁻¹: 625, 700, 770, 1100, 1180, 1340, 1620, 3040, 3250, 3350; ¹H-NMR (TFA), δ, ppm: 1.52 (t, 3H, Me from ethyl); 2.97 (q, 2H, CH₂); 7.40 - 8.57 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal C₂₇H₂₄N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0152] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-*n*-propyl-4,6-diphenylpyridinium perchlorate **82**, white crystals, mp 214-5 °C; IR (KBr), cm⁻¹: 625, 700, 770, 1100, 1180, 1340, 1620, 3030, 3270, 3350; ¹H-NMR (TFA), δ, ppm: 1.03 (t, 3H, Me from propyl); 1.95 (sextet, 2H, β-CH₂ from *n*-Pr); 2.88 (t, 2H, α-CH₂ from *n*-Pr); 7.39 - 8.55 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal C₂₈H₂₆N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0153] 1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-iso-propyl-4,6-diphenylpyridinium perchlorate **83**, white crystals, mp 186-8 °C; IR (KBr), cm⁻¹: 625, 700, 770, 1100, 1170, 1340, 1620, 3040, 3250, 3360; ¹H-NMR (TFA), δ, ppm: 1.51 (d, 6H, 2 Me from *i*-propyl); 2.50 - 3.27 (m, 1 H, CH from *i*-Pr); 7.32 - 8.54 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal C₂₈H₂₆N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

[0154] 1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-*n*-butyl-4,6-diphenylpyridinium perchlorate **84**, white crystals, mp 241-3 °C; IR (KBr), cm^{-1} : 625, 710, 770, 1100, 1180, 1335, 1625, 3040, 3260, 3345; $^1\text{H-NMR}$ (TFA), δ , ppm: 0.93 (t, 3H, Me from butyl); 1.12 - 2.14 (m, 4H, $\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-CH}_2$ from *n*-Bu); 2.96 (t, 2H, $\alpha\text{-CH}_2$ from *n*-Bu); 7.21 - 8.50 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal $\text{C}_{29}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

[0155] 1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-*tert*-butyl-4,6-diphenylpyridinium perchlorate **85**, white crystals, mp 203-5 °C; IR (KBr), cm^{-1} : 625, 705, 765, 1100, 1160, 1310, 1620, 3060, 3270; $^1\text{H-NMR}$ (TFA), δ , ppm: 1.91 (s, 9H, *t*-Bu); 6.80 - 8.74 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph₂ and 3,5-H from pyridinium). Anal $\text{C}_{29}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

[0156] 1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-triphenylpyridinium perchlorate **87**: pale yellow crystals, mp > 300 °C; IR (KBr), cm^{-1} (bands in italics are due to the anion): 625, 635, 703, 785, 896, *1100*, 1150, 1204, 1355, 1410, 1520, 1600, 3065; $^1\text{H-NMR}$ (D_2O), δ , ppm: 7.50-8.60 (m, 19H, ArH, 3Ph + C₆H₄); 9.27 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal $\text{C}_{31}\text{H}_{24}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

[0157] 1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diphenylpyridinium perchlorate **88**, yellow crystals, mp 218-20 °C; IR (KBr), cm^{-1} : 625, 705, 765, 1100, 1160, 1335, 1615, 3050, 3260; $^1\text{H-NMR}$ (TFA), δ , ppm: 6.75 - 8.43 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph₂ and 3,4,5-H from pyridinium). Anal $\text{C}_{25}\text{H}_{20}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

[0158] 1-*N*-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,3,4,6-tetramethylpyridinium perchlorate **89**, tan crystals, mp > 300 °C; IR (KBr), cm^{-1} : 625, 800, 1100, 1165, 1330, 1630, 3030, 3305; $^1\text{H-NMR}$ (TFA), δ , ppm: 2.62 (s, 3H, 4-Me); 2.74 (s, 3H, 3-Me); 2.88 (s, 6H, 2,6-(Me)₂); 7.21 - 8.50 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.93 (s, 1 H, ArH, 5-H from pyridinium). Anal $\text{C}_{17}\text{H}_{20}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

[0159] The description of the foregoing embodiments of the invention have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to explain the principles of the invention and its practical application to enable thereby others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

<110> Institute of Virology Supuran, Claudiu Scozzafava, Andrea

<120> CA-IX-SPECIFIC INHIBITORS

<130> P30395-EP01

<140> 12179990.2 <141> 2003-11-26

<150> 60/429,089 <151> 2002-11-26

<150> 60/489,473 <151> 2003-07-22

<150> 60/515,140 <151> 2003-10-28

<160> 10

<170> PatentIn version 3.4

<210> 1

<211> 1522

<212> DNA

<213> Homo sapiens

<220>

<221> CDS

<222> (13)..(1389)

<220>

<221> mat_peptide

<222> (124)..(1389)

EP 2 594 265 B1

<400> 1

5	acagtcagcc gc atg gct ccc ctg tgc ccc agc ccc tgg ctc cct ctg ttg	51
	Met Ala Pro Leu Cys Pro Ser Pro Trp Leu Pro Leu Leu	
	-35 -30 -25	
10	atc ccg gcc cct gct cca ggc ctc act gtg caa ctg ctg ctg tca ctg	99
	Ile Pro Ala Pro Ala Pro Gly Leu Thr Val Gln Leu Leu Leu Ser Leu	
	-20 -15 -10	
15	ctg ctt ctg atg cct gtc cat ccc cag agg ttg ccc cgg atg cag gag	147
	Leu Leu Leu Met Pro Val His Pro Gln Arg Leu Pro Arg Met Gln Glu	
	-5 -1 1 5	
20	gat tcc ccc ttg gga gga ggc tct tct ggg gaa gat gac cca ctg ggc	195
	Asp Ser Pro Leu Gly Gly Gly Ser Ser Gly Glu Asp Asp Pro Leu Gly	
	10 15 20	
25	gag gag gat ctg ccc agt gaa gag gat tca ccc aga gag gag gat cca	243
	Glu Glu Asp Leu Pro Ser Glu Glu Asp Ser Pro Arg Glu Glu Asp Pro	
	25 30 35 40	
30	ccc gga gag gag gat cta cct gga gag gag gat cta cct gga gag gag	291
	Pro Gly Glu Glu Asp Leu Pro Gly Glu Glu Asp Leu Pro Gly Glu Glu	
	45 50 55	
35		
40		
45		
50		
55		

EP 2 594 265 B1

	gat	cta	cct	gaa	gtt	aag	cct	aaa	tca	gaa	gaa	gag	ggc	tcc	ctg	aag	339
	Asp	Leu	Pro	Glu	Val	Lys	Pro	Lys	Ser	Glu	Glu	Glu	Gly	Ser	Leu	Lys	
				60					65					70			
5	tta	gag	gat	cta	cct	act	gtt	gag	gct	cct	gga	gat	cct	caa	gaa	ccc	387
	Leu	Glu	Asp	Leu	Pro	Thr	Val	Glu	Ala	Pro	Gly	Asp	Pro	Gln	Glu	Pro	
			75					80					85				
	cag	aat	aat	gcc	cac	agg	gac	aaa	gaa	ggg	gat	gac	cag	agt	cat	tgg	435
10	Gln	Asn	Asn	Ala	His	Arg	Asp	Lys	Glu	Gly	Asp	Asp	Gln	Ser	His	Trp	
		90					95					100					
	cgc	tat	gga	ggc	gac	ccg	ccc	tgg	ccc	cgg	gtg	tcc	cca	gcc	tgc	gcg	483
	Arg	Tyr	Gly	Gly	Asp	Pro	Pro	Trp	Pro	Arg	Val	Ser	Pro	Ala	Cys	Ala	
	105					110					115					120	
15	ggc	cgc	ttc	cag	tcc	ccg	gtg	gat	atc	cgc	ccc	cag	ctc	gcc	gcc	ttc	531
	Gly	Arg	Phe	Gln	Ser	Pro	Val	Asp	Ile	Arg	Pro	Gln	Leu	Ala	Ala	Phe	
				125						130					135		
	tgc	ccg	gcc	ctg	cgc	ccc	ctg	gaa	ctc	ctg	ggc	ttc	cag	ctc	ccg	ccg	579
20	Cys	Pro	Ala	Leu	Arg	Pro	Leu	Glu	Leu	Leu	Gly	Phe	Gln	Leu	Pro	Pro	
				140					145					150			
	ctc	cca	gaa	ctg	cgc	ctg	cgc	aac	aat	ggc	cac	agt	gtg	caa	ctg	acc	627
25	Leu	Pro	Glu	Leu	Arg	Leu	Arg	Asn	Asn	Gly	His	Ser	Val	Gln	Leu	Thr	
			155					160					165				
	ctg	cct	cct	ggg	cta	gag	atg	gct	ctg	ggg	ccc	ggg	cgg	gag	tac	cgg	675
	Leu	Pro	Pro	Gly	Leu	Glu	Met	Ala	Leu	Gly	Pro	Gly	Arg	Glu	Tyr	Arg	
		170					175					180					
30	gct	ctg	cag	ctg	cat	ctg	cac	tgg	ggg	gct	gca	ggg	cgt	ccg	ggc	tcg	723
	Ala	Leu	Gln	Leu	His	Leu	His	Trp	Gly	Ala	Ala	Gly	Arg	Pro	Gly	Ser	
	185					190					195					200	
	gag	cac	act	gtg	gaa	ggc	cac	cgt	ttc	cct	gcc	gag	atc	cac	gtg	gtt	771
35	Glu	His	Thr	Val	Glu	Gly	His	Arg	Phe	Pro	Ala	Glu	Ile	His	Val	Val	
				205						210					215		
	cac	ctc	agc	acc	gcc	ttt	gcc	aga	gtt	gac	gag	gcc	ttg	ggg	cgc	ccg	819
	His	Leu	Ser	Thr	Ala	Phe	Ala	Arg	Val	Asp	Glu	Ala	Leu	Gly	Arg	Pro	
				220					225					230			
40	gga	ggc	ctg	gcc	gtg	ttg	gcc	gcc	ttt	ctg	gag	gag	ggc	ccg	gaa	gaa	867
	Gly	Gly	Leu	Ala	Val	Leu	Ala	Ala	Phe	Leu	Glu	Glu	Gly	Pro	Glu	Glu	
			235					240					245				
	aac	agt	gcc	tat	gag	cag	ttg	ctg	tct	cgc	ttg	gaa	gaa	atc	gct	gag	915
45	Asn	Ser	Ala	Tyr	Glu	Gln	Leu	Leu	Ser	Arg	Leu	Glu	Glu	Ile	Ala	Glu	
		250					255					260					
	gaa	ggc	tca	gag	act	cag	gtc	cca	gga	ctg	gac	ata	tct	gca	ctc	ctg	963
50	Glu	Gly	Ser	Glu	Thr	Gln	Val	Pro	Gly	Leu	Asp	Ile	Ser	Ala	Leu	Leu	
	265					270					275					280	
	ccc	tct	gac	ttc	agc	cgc	tac	ttc	caa	tat	gag	ggg	tct	ctg	act	aca	1011
	Pro	Ser	Asp	Phe	Ser	Arg	Tyr	Phe	Gln	Tyr	Glu	Gly	Ser	Leu	Thr	Thr	
				285						290					295		
55	ccg	ccc	tgt	gcc	cag	ggg	gtc	atc	tgg	act	gtg	ttt	aac	cag	aca	gtg	1059
	Pro	Pro	Cys	Ala	Gln	Gly	Val	Ile	Trp	Thr	Val	Phe	Asn	Gln	Thr	Val	
				300					305					310			

EP 2 594 265 B1

	atg ctg agt gct aag cag ctc cac acc ctc tct gac acc ctg tgg gga	1107
	Met Leu Ser Ala Lys Gln Leu His Thr Leu Ser Asp Thr Leu Trp Gly	
	315 320 325	
5	cct ggt gac tct cgg cta cag ctg aac ttc cga gcg acg cag cct ttg	1155
	Pro Gly Asp Ser Arg Leu Gln Leu Asn Phe Arg Ala Thr Gln Pro Leu	
	330 335 340	
10	aat ggg cga gtg att gag gcc tcc ttc cct gct gga gtg gac agc agt	1203
	Asn Gly Arg Val Ile Glu Ala Ser Phe Pro Ala Gly Val Asp Ser Ser	
	345 350 355 360	
15	cct cgg gct gct gag cca gtc cag ctg aat tcc tgc ctg gct gct ggt	1251
	Pro Arg Ala Ala Glu Pro Val Gln Leu Asn Ser Cys Leu Ala Ala Gly	
	365 370 375	
	gac atc cta gcc ctg gtt ttt ggc ctc ctt ttt gct gtc acc agc gtc	1299
	Asp Ile Leu Ala Leu Val Phe Gly Leu Leu Phe Ala Val Thr Ser Val	
	380 385 390	
20	gcg ttc ctt gtg cag atg aga agg cag cac aga agg gga acc aaa ggg	1347
	Ala Phe Leu Val Gln Met Arg Arg Gln His Arg Arg Gly Thr Lys Gly	
	395 400 405	
25	ggt gtg agc tac cgc cca gca gag gta gcc gag act gga gcc	1389
	Gly Val Ser Tyr Arg Pro Ala Glu Val Ala Glu Thr Gly Ala	
	410 415 420	
	tagaggctgg atcttgaga atgtgagaag ccagccagag gcatctgagg gggagccggt	1449
30	aactgtcctg tcctgctcat tatgccactt ccttttaact gccaagaaat tttttaaaat	1509
	aaatatattat aat	1522
	<210> 2	
	<211> 459	
35	<212> PRT	
	<213> Homo sapiens	
	<400> 2	
40		
45		
50		
55		

EP 2 594 265 B1

	Met	Ala	Pro	Leu	Cys	Pro	Ser	Pro	Trp	Leu	Pro	Leu	Leu	Ile	Pro	Ala
			-35					-30					-25			
5	Pro	Ala	Pro	Gly	Leu	Thr	Val	Gln	Leu	Leu	Leu	Ser	Leu	Leu	Leu	Leu
		-20					-15					-10				
10	Met	Pro	Val	His	Pro	Gln	Arg	Leu	Pro	Arg	Met	Gln	Glu	Asp	Ser	Pro
	-5				-1	1				5					10	
	Leu	Gly	Gly	Gly	Ser	Ser	Gly	Glu	Asp	Asp	Pro	Leu	Gly	Glu	Glu	Asp
				15					20					25		
15	Leu	Pro	Ser	Glu	Glu	Asp	Ser	Pro	Arg	Glu	Glu	Asp	Pro	Pro	Gly	Glu
			30					35					40			
20	Glu	Asp	Leu	Pro	Gly	Glu	Glu	Asp	Leu	Pro	Gly	Glu	Glu	Asp	Leu	Pro
		45					50					55				
25																
30																
35																
40																
45																
50																
55																

EP 2 594 265 B1

	Glu	Val	Lys	Pro	Lys	Ser	Glu	Glu	Glu	Gly	Ser	Leu	Lys	Leu	Glu	Asp	60	65	70	75
5	Leu	Pro	Thr	Val	Glu	Ala	Pro	Gly	Asp	Pro	Gln	Glu	Pro	Gln	Asn	Asn	80	85	90	
10	Ala	His	Arg	Asp	Lys	Glu	Gly	Asp	Asp	Gln	Ser	His	Trp	Arg	Tyr	Gly	95	100	105	
	Gly	Asp	Pro	Pro	Trp	Pro	Arg	Val	Ser	Pro	Ala	Cys	Ala	Gly	Arg	Phe	110	115	120	
15	Gln	Ser	Pro	Val	Asp	Ile	Arg	Pro	Gln	Leu	Ala	Ala	Phe	Cys	Pro	Ala	125	130	135	
20	Leu	Arg	Pro	Leu	Glu	Leu	Leu	Gly	Phe	Gln	Leu	Pro	Pro	Leu	Pro	Glu	140	145	150	155
25	Leu	Arg	Leu	Arg	Asn	Asn	Gly	His	Ser	Val	Gln	Leu	Thr	Leu	Pro	Pro	160	165	170	
	Gly	Leu	Glu	Met	Ala	Leu	Gly	Pro	Gly	Arg	Glu	Tyr	Arg	Ala	Leu	Gln	175	180	185	
30	Leu	His	Leu	His	Trp	Gly	Ala	Ala	Gly	Arg	Pro	Gly	Ser	Glu	His	Thr	190	195	200	
35	Val	Glu	Gly	His	Arg	Phe	Pro	Ala	Glu	Ile	His	Val	Val	His	Leu	Ser	205	210	215	
40	Thr	Ala	Phe	Ala	Arg	Val	Asp	Glu	Ala	Leu	Gly	Arg	Pro	Gly	Gly	Leu	220	225	230	235
	Ala	Val	Leu	Ala	Ala	Phe	Leu	Glu	Glu	Gly	Pro	Glu	Glu	Asn	Ser	Ala	240	245	250	
45	Tyr	Glu	Gln	Leu	Leu	Ser	Arg	Leu	Glu	Glu	Ile	Ala	Glu	Glu	Gly	Ser	255	260	265	
50	Glu	Thr	Gln	Val	Pro	Gly	Leu	Asp	Ile	Ser	Ala	Leu	Leu	Pro	Ser	Asp	270	275	280	
	Phe	Ser	Arg	Tyr	Phe	Gln	Tyr	Glu	Gly	Ser	Leu	Thr	Thr	Pro	Pro	Cys	285	290	295	
55	Ala	Gln	Gly	Val	Ile	Trp	Thr	Val	Phe	Asn	Gln	Thr	Val	Met	Leu	Ser	300	305	310	315

EP 2 594 265 B1

	Ala	Lys	Gln	Leu	His	Thr	Leu	Ser	Asp	Thr	Leu	Trp	Gly	Pro	Gly	Asp	
					320					325					330		
5	Ser	Arg	Leu	Gln	Leu	Asn	Phe	Arg	Ala	Thr	Gln	Pro	Leu	Asn	Gly	Arg	
				335					340					345			
10	Val	Ile	Glu	Ala	Ser	Phe	Pro	Ala	Gly	Val	Asp	Ser	Ser	Pro	Arg	Ala	
			350					355					360				
15	Ala	Glu	Pro	Val	Gln	Leu	Asn	Ser	Cys	Leu	Ala	Ala	Gly	Asp	Ile	Leu	
		365					370					375					
20	Ala	Leu	Val	Phe	Gly	Leu	Leu	Phe	Ala	Val	Thr	Ser	Val	Ala	Phe	Leu	
		380				385					390					395	
25	Val	Gln	Met	Arg	Arg	Gln	His	Arg	Arg	Gly	Thr	Lys	Gly	Gly	Val	Ser	
					400					405					410		
30	Tyr	Arg	Pro	Ala	Glu	Val	Ala	Glu	Thr	Gly	Ala						
				415					420								
35	<210> 3																
	<211> 10898																
	<212> DNA																
40	<213> Homo sapiens																
45	<220>																
	<221> gene																
	<222> (1)..(10898)																
50	<220>																
	<221> misc_feature																
	<222> (1974)..(1974)																
	<223> n is a, c, g, or t																
55	<400> 3																

EP 2 594 265 B1

	ggatcctggt gactcgtgac cttaccccca accctgtgct ctctgaaaca tgagctgtgt	60
	ccactcaggg tttaatggat taagggcggg gcaagatgtg ctttggttaa cagatgcttg	120
5	aaggcagcat gctcgttaag agtcatcacc aatcccta ctcaagtaat cagggacaca	180
	aacactgagg aaggccgag ggtcctctgc ctaggaaaac cagagacctt tgttcacttg	240
	tttatctgac cttccctcca ctattgtcca tgaccctgcc aaatccccct ctgtgagaaa	300
10	cacccaagaa ttatcaataa aaaaataaat ttaaaaaaaaa aatacaaaaa aaaaaaaaaa	360
	aaaaaaaaa gacttacgaa tagttattga taaatgaata gctattggta aagccaagta	420
15	aatgatcata ttcaaaacca gacggccatc atcacagctc aagtctacct gatttgatct	480
	ctttatcatt gtcattcttt ggattcacta gattagtcac catcctcaaa attctcccc	540
	aagttctaata tacgttccaa acatttaggg gttacatgaa gcttgaacct actaccttct	600
20	ttgcttttga gccatgagtt gtaggaatga tgagtttaca ccttacatgc tggggattaa	660

25

30

35

40

45

50

55

EP 2 594 265 B1

	tttaaacttt acctctaagt cagttgggta gcctttggct tatttttcta gctaattttg	720
	tagttaatgg atgcactgtg aatcttgcta tgatagtttt cctccacact ttgccactag	780
5	gggtaggtag gtactcagtt ttcagtaatt gcttacctaa gaccctaagc cctatttctc	840
	ttgtactggc ctttatctgt aatatgggca tatttaatac aatataattt ttggagtttt	900
	tttgtttgtt tgtttgtttg tttttttgag acggagtctt gcatctgtca tgcccaggct	960
10	ggagtagcag tggtgccatc tcggctcact gcaagctcca cctcccgagt tcacgccatt	1020
	ttcctgcctc agcctcccg aatagctggga ctacaggcgc ccgccaccat gcccggttaa	1080
	ttttttgtat ttttggtaga gacgggggtt caccgtgtta gccagaatgg tctcgatctc	1140
15	ctgacttcgt gatccaccgc cctcggcctc ccaaagttct gggattacag gtgtgagcca	1200
	ccgcacctgg ccaatttttt gagtctttta aagtaaaaat atgtcttgta agctggtaac	1260
	tatggtacat ttccttttat taatgtggtg ctgacgggtca tataggttct tttgagtttg	1320
20	gcatgcatat gctacttttt gcagtccttt cattacattt ttctctcttc atttgaagag	1380
	catgttatat ctttttagctt cacttggctt aaaaggttct ctcatagcc taacacagtg	1440
	tcattgttgg taccacttgg atcataagtg gaaaaacagt caagaaattg cacagtaata	1500
25	cttgtttcta agagggatga ttcagggtga tctgacacta agaaactccc ctacctgagg	1560
	tctgagattc ctctgacatt gctgtatata ggcttttctt ttgacagcct gtgactgcgg	1620
	actatttttc ttaagcaaga tatgctaaag ttttgtgagc ctttttccag agagaggtct	1680
30	catacttgca tcaagtgaga acatataatg tctgcatgtt tccatatttc aggaatgttt	1740
	gcttgtgttt tatgctttta tatagacagg gaaacttggt cctcagtgc ccaaaagagg	1800
35	tgggaattgt tattggatat catcattggc ccacgcttct tgacctgga aacaattaag	1860
	ggttcataat ctcaattctg tcagaattgg tacaagaaat agctgctatg tttcttgaca	1920
	ttccacttgg taggaaataa gaatgtgaaa ctcttcagtt ggtgtgtgtc cctngttttt	1980
40	ttgcaatttc cttcttactg tgttaaaaaa aagtatgatc ttgctctgag aggtgaggca	2040
	ttcttaataca tgatctttaa agatcaataa tataatcctt tcaaggatta tgtctttatt	2100
	ataataaaga taatttgtct ttaacagaat caataatata atcccttaa ggattatatt	2160
45	tttgctgggc gcagtggctc acacctgtaa tcccagcact ttgggtggcc aagggtgaag	2220
	gatcaaattt gcctacttct atattatctt ctaaagcaga attcatctct cttccctcaa	2280
	tatgatgata ttgacagggt ttgccctcac tcaactagatt gtgagctcct gctcagggca	2340
50	ggtagcgttt tttgtttttg tttttgtttt tcttttttga gacaggtctt tgctctgtca	2400
	cccaggccag agtgcaatgg tacagtctca gctcactgca gcctcaaccg cctcggctca	2460
	aaccatcatc ccatttcagc ctctgagta gctgggacta caggcacatg ccattacacc	2520
55	tggtcaattt ttttgtattt ctagtagaga cagggtttgg ccatgttgcc cgggctggtc	2580

EP 2 594 265 B1

	tcgaactcct	ggactcaagc	aatccaccca	cctcagcctc	ccaaaatgag	ggaccgtgtc	2640
	ttatttcattt	ccatgtccct	agtccatagc	ccagtgtctg	acctatggta	gtactaaata	2700
5	aatatittgtt	gaatgcaata	gtaaatagca	tttcagggag	caagaactag	attaacaaag	2760
	gtggtaaaag	gtttggagaa	aaaaataata	gtttaatttg	gctagagtat	gagggagagt	2820
	agtaggagac	aagatggaaa	ggtctcttgg	gcaaggtttt	gaaggaagtt	ggaagtcaga	2880
10	agtacacaat	gtgcatatcg	tggcaggcag	tggggagcca	atgaaggctt	ttgagcagga	2940
	gagtaatgtg	ttgaaaaata	aatatagggt	aaacctatca	gagccctctc	gacacataca	3000
	cttgcttttc	attcaagctc	aagtttgtct	cccacatacc	cattacttaa	ctcaccctcg	3060
15	ggctccccta	gcagcctgcc	ctacctcttt	acctgcttcc	tgggtggagt	agggatgtat	3120
	acatgagctg	ctttccctct	cagccagagg	acatgggggg	cccagctcc	cctgcctttc	3180
	cccttctgtg	cctggagctg	ggaagcaggc	caggggttagc	tgaggctggc	tggcaagcag	3240
20	ctgggtggtg	ccagggagag	cctgcatagt	gccagggtgt	gccttgggtt	ccaagctagt	3300
	ccatggcccc	gataaccttc	tgctgtgca	cacacctgcc	cctcactcca	ccccatcct	3360
	agcttttggt	tgggggagag	ggcacagggc	cagacaaacc	tgtgagactt	tggctccatc	3420
25	tctgcaaaag	ggcgtctgt	gagtcagcct	gctcccctcc	aggcttgctc	ctccccacc	3480
	cagctctcgt	ttccaatgca	cgtacagccc	gtacacaccg	tgtgctggga	caccccacag	3540
	tcagccgcat	ggctcccctg	tgccccagcc	cctggctccc	tctgttgatc	ccggcccctg	3600
30	ctccaggcct	cactgtgcaa	ctgctgctgt	cactgctgct	tctggtgcct	gtccatcccc	3660
	agaggttgcc	ccggatgcag	gaggattccc	ccttgggagg	aggctcttct	ggggaagatg	3720
	accactggg	cgaggaggat	ctgcccagtg	aagaggattc	accagagag	gaggatccac	3780
35	ccggagagga	ggatctacct	ggagaggagg	atctacctgg	agaggaggat	ctacctgaag	3840
	ttaagcctaa	atcagaagaa	gagggctccc	tgaagttaga	ggatctacct	actgttgagg	3900
40	ctcctggaga	tcctcaagaa	ccccagaata	atgccacag	ggacaaagaa	ggtaagtgg	3960
	catcaatctc	caaatccagg	ttccaggagg	ttcatgactc	ccctcccata	ccccagccta	4020
	ggctctgttc	actcagggaa	ggaggggaga	ctgtactccc	cacagaagcc	cttccagagg	4080
45	tcccatacca	atatcccat	ccccactctc	ggaggtagaa	agggacagat	gtggagagaa	4140
	aataaaaagg	gtgcaaaagg	agagaggtga	gctggatgag	atgggagaga	agggggaggc	4200
	tggagaagag	aaagggatga	gaactgcaga	tgagagaaaa	aatgtgcaga	cagaggaaaa	4260
50	aaataggtgg	agaaggagag	tcagagagtt	tgaggggaag	agaaaaggaa	agcttgggag	4320
	gtgaagtggg	taccagagac	aagcaagaag	agctggtaga	agtcactctc	tcttaggcta	4380
	caatgaggaa	ttgagacct	ggaagaagg	acacagcagg	tagagaaacg	tggcttcttg	4440
55	actccaagc	caggaatttg	gggaaagggg	ttggagacca	tacaaggcag	agggatgagt	4500
	ggggagaaga	aagaagggag	aaaggaaaga	tgggtgtact	actcatttgg	gactcaggac	4560

EP 2 594 265 B1

	tgaagtgcc	actcactttt	tttttttttt	tttttgagac	aaactttcac	ttttgttgcc	4620
	caggctggag	tgcaatggcg	cgatctcggc	tcactgcaac	ctccacctcc	cgggttcaag	4680
5	tgattctcct	gcctcagcct	ctagccaagt	agctgcgatt	acaggcatgc	gccaccacgc	4740
	ccggctaatt	tttgtatttt	tagtagagac	ggggtttcgc	catgttggtc	aggctggctc	4800
	cgaactcctg	atctcaggtg	atccaaccac	cctggcctcc	caaagtgctg	ggattatagg	4860
10	cgtgagccac	agcgcctggc	ctgaagcagc	cactcacttt	tacagaccct	aagacaatga	4920
	ttgcaagctg	gtaggattgc	tgtttggccc	accagctgc	ggtgttgagt	ttgggtgcgg	4980
	tctcctgtgc	tttgcacctg	gcccgcctaa	ggcatttggt	acccgtaatg	ctcctgtaag	5040
15	gcctctgcgt	ttgtgacatc	gttttggtcg	ccaggaagg	attggggctc	taagcttgag	5100
	cggttcatcc	ttttcattta	tacaggggat	gaccagagtc	attggcgcta	tggaggtgag	5160
	acaccacccc	gctgcacaga	cccaatctgg	gaaccacagc	ctgtggatct	cccctacagc	5220
20	cgtccctgaa	cactgggtccc	gggcgtccca	cccgcgcgcc	accgtcccac	cccctcacct	5280
	tttctaccog	ggttccctaa	gttcttgacc	taggcgtcag	acttctctac	tatactctcc	5340
	caccccaggc	gacccgccct	ggccccgggt	gtccccagcc	tgcgcgggcc	gcttccagtc	5400
25	cccgttgat	atccgcccc	agctcgccgc	cttctgccc	gccctgcgcc	ccctggaact	5460
	cctgggcttc	cagctcccg	cgctcccaga	actgcgcctg	cgcaacaatg	gccacagtgg	5520
30	tgagggggtc	tccccgcga	gacttgggga	tggggcgggg	cgcagggaag	ggaaccgtcg	5580
	cgcagtgcct	gcccgggggt	tgggctggcc	ctaccggg	gggcccgtc	acttgcctct	5640
	ccctacgcag	tgcaactgac	cctgcctcct	gggctagaga	tggctctggg	tcccgggcgg	5700
35	gagtaccggg	ctctgcagct	gcctctgcac	tggggggctg	caggtcgtcc	gggctcggag	5760
	cacactgtgg	aaggccaccg	tttccctgcc	gaggtgagcg	cggactggcc	gagaaggggc	5820
	aaaggagcgg	ggcggacggg	ggccagagac	gtggccctct	cctaccctcg	tgtccttttc	5880
40	agatccacgt	ggttcacctc	agcaccgcct	ttgccagagt	tgacgaggcc	ttggggcgcc	5940
	cgggaggcct	ggccgtgttg	gccgcctttc	tggaggtacc	agatcctgga	caccccctac	6000
	tccccgcttt	cccatcccat	gtcctcccg	gactctatcg	tggagccaga	gaccccatcc	6060
45	cagcaagctc	actcaggccc	ctggctgaca	aactcattca	cgcactgttt	gttcatttaa	6120
	caccactgt	gaaccaggca	ccagccccc	acaaggattc	tgaagctgta	ggtccttgcc	6180
	tctaaggagc	ccacagccag	tgggggaggc	tgacatgaca	gacacatagg	aaggacatag	6240
50	taaagatggt	ggtcacagag	gaggtgacac	ttaaagcctt	cactggtaga	aaagaaaagg	6300
	aggtgttcat	tgcagaggaa	acagaatgtg	caaagactca	gaatatggcc	tatttaggga	6360
	atggctacat	acaccatgat	tagaggaggc	ccagtaaagg	gaagggatgg	tgagatgcct	6420
55	gctaggttca	ctcactcact	tttatttatt	tatttatttt	tttgacagtc	tctctgtcgc	6480

EP 2 594 265 B1

	ccaggctgga	gtgcagtggt	gtgatcttgg	gtcactgcaa	cttccgcctc	ccgggttcaa	6540
	gggattotcc	tgctcagct	tcctgagtag	ctgggggttac	aggtgtgtgc	caccatgccc	6600
5	agctaatttt	tttttgtatt	tttagtagac	agggtttcac	catgttggtc	aggctggctc	6660
	caaactcctg	gcctcaagtg	atccgcctga	ctcagcctac	caaagtgctg	attacaagtg	6720
	tgagccaccg	tgcccagcca	cactcactga	ttctttaatg	ccagccacac	agcacaaagt	6780
10	tcagagaaat	gcctccatca	tagcatgtca	atatgttcat	actcttaggt	tcattgatgtt	6840
	cttaacatta	gggtcataag	caaaataaga	aaaaagaata	ataaataaaa	gaagtggcat	6900
	gtcaggacct	cacctgaaaa	gccaaacaca	gaatcatgaa	ggatgaatgca	gaggtgacac	6960
15	caacacaaag	gtgtatatat	ggtttcctgt	ggggagtatg	tacggaggca	gcagtgagtg	7020
	agactgcaaa	cgtcagaagg	gcacgggtca	ctgagagcct	agtatcctag	taaagtgggc	7080
	tctctccctc	tctctccagc	ttgtcattga	aaaccagtcc	accaagcttg	ttggttcgca	7140
20	cagcaagagt	acatagagtt	tgaataata	cataggattt	taagagggag	acactgtctc	7200
	taaaaaaaa	aacaacagca	acaacaaaa	gcaacaacca	ttacaatttt	atgttccctc	7260
	agcattctca	gagctgagga	atgggagagg	actatgggaa	cccccttcat	gttccggcct	7320
25	tcagccatgg	ccctggatac	atgcactcat	ctgtcttaca	atgtcattcc	cccaggaggg	7380
	cccgaagaa	aacagtgcct	atgagcagtt	gctgtctcgc	ttggaagaaa	tcgctgagga	7440
	aggtcagttt	gttggctctg	ccactaatct	ctgtggccta	gttcataaag	aatcacccctt	7500
30	tggagcttca	ggtctgaggc	tggagatggg	ctccctccag	tgacaggagg	attgaagcat	7560
	gagccagcgc	tcattcttgat	aataaccatg	aagctgacag	acacagttac	ccgcaaacgg	7620
	ctgcctacag	attgaaaacc	aagcaaaaac	cgccggggcac	ggtggctcac	gcctgtaatc	7680
35	ccagcacttt	gggaggccaa	ggcaggtgga	tcacgaggtc	aagagatcaa	gaccatcctg	7740
	gccaaacatg	tgaaccccca	tctctactaa	aaatacgaaa	aaatagccag	gcgtgggtggc	7800
	gggtgcctgt	aatcccagct	actcgggagg	ctgaggcagg	agaatggcat	gaacccggga	7860
40	ggcagaagtt	gcagtgagcc	gagatcgtgc	cactgcactc	cagcctgggc	aacagagcga	7920
	gactcttgtc	tcaaaaaaaaa	aaaaaaaaaa	gaaaaccaag	caaaaaccaa	aatgagacaa	7980
	aaaaaacaag	acaaaaaaat	ggtgtttgga	aattgtcaag	gtcaagtctg	gagagctaaa	8040
45	ctttttctga	gaactgttta	tctttaataa	gcatacaata	ttttaacttt	gtaaataactt	8100
	ttgttgga	tcgttctctt	cttagtcact	cttgggtcat	tttaaatctc	acttactcta	8160
	ctagaccttt	taggtttctg	ctagactagg	tagaactctg	cctttgcatt	tcttgtgtct	8220
50	gttttgtata	gttatcaata	ttcatattta	tttacaagtt	attcagatca	ttttttcttt	8280
	tctttttttt	tttttttttt	ttttttacat	ctttagtaga	gacagggttt	caccatattg	8340
	gccaggctgc	tctcaaactc	ctgaccttgt	gatccaccag	cctcggcctc	ccaaagtgct	8400
55	gggattcatt	ttttcttttt	aatttgctct	gggcttaaac	ttgtggccca	gcactttatg	8460

EP 2 594 265 B1

	atggtacaca gagttaagag tgtagactca gacggtcttt cttctttcct tctcttcctt	8520
	cctcccttcc ctccacctt cccttctctc cttcctttct ttcttcctct cttgcttcct	8580
5	caggcctctt ccagttgctc caaagccctg tacttttttt tgagttaacg tcttatggga	8640
	agggcctgca cttagtgaag aagtggctct agagttgagt taccttggct tctgggaggt	8700
	gaaactgtat ccctataccc tgaagcttta agggggtgca atgtagatga gacccaaca	8760
10	tagatcctct tcacaggctc agagactcag gtcccaggac tggacatatc tgcactcctg	8820
	ccctctgact tcagccgcta cttccaatat gaggggtctc tgactacacc gccctgtgcc	8880
	cagggtgtca tctggactgt gtttaaccag acagtgatgc tgagtgctaa gcaggtgggc	8940
15	ctgggggtg tgtaggacaca gtgggtgagg gggaaagagg atgtaagatg agatgagaaa	9000
	caggagaaga aagaaatcaa ggctgggctc tgtggcttac gcctataatc ccaccacgtt	9060
	gggaggctga ggtgggagaa tggtttgagc ccaggagttc aagacaaggc ggggcaacat	9120
20	agtgtgacct catctctacc aaaaaaaccc caacaaaacc aaaaatagcc gggcatggtg	9180
	gtatgcggcc tagtcccagc tactcaagga ggctgaggtg ggaagatcgc ttgattccag	9240
	gagtttgaga ctgcagttag ctatgatccc accactgcct accatcttta ggatacattt	9300
25	atttatattat aaaagaaatc aagaggctgg atggggaata caggagctgg aggggtggagc	9360
	cctgaggtgc tggttgtgag ctggcctggg acccttgttt cctgtcatgc catgaacca	9420
	cccacactgt cactgacct ccctagctcc acaccctctc tgacaccctg tggggacctg	9480
30	gtgactctcg gctacagctg aacttccgag cgacgcagcc tttgaatggg cgagtgattg	9540
	aggcctcctt ccctgctgga gtggacagca gtccctcggg tgctgagcca ggtacagctt	9600
35	tgtctggttt cccccagcc agtagtcctt taccctcca tgtgtgtgcc agtgtctgtc	9660
	attggtggtc acagcccgcc tctcacatct cttttttctc tccagtccag ctgaattcct	9720
	gcctggctgc tggtagtct gcccctcctc ttggtcctga tgccaggaga ctctcagca	9780
40	ccattcagcc ccagggtgc tcaggaccgc ctctgtctcc tctccttttc tgcagaacag	9840
	acccaaccc caatattaga gaggcagatc atggtgggga ttccccatt gtccccagag	9900
	gctaattgat tagaatgaag cttgagaaat ctcccagcat ccctctcgca aaagaatccc	9960
45	ccccctttt tttaaagata gggctctact ctgtttgccc caggctgggg tgttgtggca	10020
	cgatcatagc tactgcagc ctogaactcc taggtcagg caatcctttc accttagctt	10080
	ctcaaagcac tgggactgta ggcatgagcc actgtgcctg gcccacaaac gcccttttac	10140
50	ttggctttta ggaagcaaaa acggtgctta tcttaccctt tctcgtgtat ccaccctcat	10200
	cccttggtg gcctcttctg gagactgagg cactatgggg ctgcctgaga actcggggca	10260
	ggggtggtgg agtgactga ggcagggtgt gaggaactct gcagaccctt cttccttccc	10320
55	aaagcagccc tctctgctct ccatcgcagg tgacatccta gccctggttt ttggcctcct	10380

EP 2 594 265 B1

5
 10
 15
 20
 25
 30
 35
 40
 45
 50
 55

ttttgctgtc accagcgtcg cgttccttgt gcagatgaga aggcagcaca ggtattacac 10440
 tgaccctttc ttcaggcaca agcttcccc acccttgtgg agtcacttca tgcaaagcgc 10500
 atgcaaatga gctgctcctg ggccagtttt ctgattagcc tttcctgttg tgtacacaca 10560
 gaaggggaac caaaggggggt gtgagctacc gccagcaga ggtagccgag actggagcct 10620
 agaggctgga tcttgagaga tgtgagaagc cagccagagg catctgaggg ggagccggta 10680
 actgtcctgt cctgctcatt atgccacttc cttttaactg ccaagaaatt ttttaaata 10740
 aatatttata ataaaatatg tgtagtcac ctttgttccc caaatcagaa ggaggtat 10800
 gaatttccta ttactgttat tagcaccaat ttagtggtaa tgcatttatt ctattacagt 10860
 tcggcctcct tccacacatc actccaatgt gttgctcc 10898

<210> 4
 <211> 59
 <212> PRT
 <213> Homo sapiens

<400> 4

Ser Ser Gly Glu Asp Asp Pro Leu Gly Glu Glu Asp Leu Pro Ser Glu
 1 5 10 15

Glu Asp Ser Pro Arg Glu Glu Asp Pro Pro Gly Glu Glu Asp Leu Pro
 20 25 30

Gly Glu Glu Asp Leu Pro Gly Glu Glu Asp Leu Pro Glu Val Lys Pro
 35 40 45

Lys Ser Glu Glu Glu Gly Ser Leu Lys Leu Glu
 50 55

<210> 5
 <211> 257
 <212> PRT
 <213> Homo sapiens

<400> 5

EP 2 594 265 B1

Gly Asp Asp Gln Ser His Trp Arg Tyr Gly Gly Asp Pro Pro Trp Pro
1 5 10 15

5 Arg Val Ser Pro Ala Cys Ala Gly Arg Phe Gln Ser Pro Val Asp Ile
20 25 30

10 Arg Pro Gln Leu Ala Ala Phe Cys Pro Ala Leu Arg Pro Leu Glu Leu
35 40 45

15 Leu Gly Phe Gln Leu Pro Pro Leu Pro Glu Leu Arg Leu Arg Asn Asn
50 55 60

15

20

25

30

35

40

45

50

55

EP 2 594 265 B1

	Gly	His	Ser	Val	Gln	Leu	Thr	Leu	Pro	Pro	Gly	Leu	Glu	Met	Ala	Leu	
	65					70					75					80	
5	Gly	Pro	Gly	Arg	Glu	Tyr	Arg	Ala	Leu	Gln	Leu	His	Leu	His	Trp	Gly	
					85					90					95		
10	Ala	Ala	Gly	Arg	Pro	Gly	Ser	Glu	His	Thr	Val	Glu	Gly	His	Arg	Phe	
				100					105					110			
15	Pro	Ala	Glu	Ile	His	Val	Val	His	Leu	Ser	Thr	Ala	Phe	Ala	Arg	Val	
			115					120					125				
20	Asp	Glu	Ala	Leu	Gly	Arg	Pro	Gly	Gly	Leu	Ala	Val	Leu	Ala	Ala	Phe	
	130						135					140					
25	Leu	Glu	Glu	Gly	Pro	Glu	Glu	Asn	Ser	Ala	Tyr	Glu	Gln	Leu	Leu	Ser	
	145					150					155					160	
30	Arg	Leu	Glu	Glu	Ile	Ala	Glu	Glu	Gly	Ser	Glu	Thr	Gln	Val	Pro	Gly	
					165					170					175		
35	Leu	Asp	Ile	Ser	Ala	Leu	Leu	Pro	Ser	Asp	Phe	Ser	Arg	Tyr	Phe	Gln	
				180					185					190			
40	Tyr	Glu	Gly	Ser	Leu	Thr	Thr	Pro	Pro	Cys	Ala	Gln	Gly	Val	Ile	Trp	
			195					200					205				
45	Thr	Val	Phe	Asn	Gln	Thr	Val	Met	Leu	Ser	Ala	Lys	Gln	Leu	His	Thr	
		210					215					220					
50	Leu	Ser	Asp	Thr	Leu	Trp	Gly	Pro	Gly	Asp	Ser	Arg	Leu	Gln	Leu	Asn	
	225					230				235						240	
55	Phe	Arg	Ala	Thr	Gln	Pro	Leu	Asn	Gly	Arg	Val	Ile	Glu	Ala	Ser	Phe	
					245					250					255		
	Pro																
	<210> 6																
	<211> 37																
	<212> PRT																
	<213> Homo sapiens																
	<400> 6																

EP 2 594 265 B1

Met Ala Pro Leu Cys Pro Ser Pro Trp Leu Pro Leu Leu Ile Pro Ala
 1 5 10 15

5 Pro Ala Pro Gly Leu Thr Val Gln Leu Leu Leu Ser Leu Leu Leu Leu
 20 25 30

Met Pro Val His Pro
 35

10

<210> 7

<211> 377

<212> PRT

<213> Homo sapiens

15

<400> 7

20

25

30

35

40

45

50

55

EP 2 594 265 B1

	Gln	Arg	Leu	Pro	Arg	Met	Gln	Glu	Asp	Ser	Pro	Leu	Gly	Gly	Gly	Ser	
	1				5					10					15		
5	Ser	Gly	Glu	Asp	Asp	Pro	Leu	Gly	Glu	Glu	Asp	Leu	Pro	Ser	Glu	Glu	
				20					25					30			
10	Asp	Ser	Pro	Arg	Glu	Glu	Asp	Pro	Pro	Gly	Glu	Glu	Asp	Leu	Pro	Gly	
			35					40					45				
	Glu	Glu	Asp	Leu	Pro	Gly	Glu	Glu	Asp	Leu	Pro	Glu	Val	Lys	Pro	Lys	
		50					55					60					
15	Ser	Glu	Glu	Glu	Gly	Ser	Leu	Lys	Leu	Glu	Asp	Leu	Pro	Thr	Val	Glu	
	65					70					75					80	
20	Ala	Pro	Gly	Asp	Pro	Gln	Glu	Pro	Gln	Asn	Asn	Ala	His	Arg	Asp	Lys	
					85					90					95		
25	Glu	Gly	Asp	Asp	Gln	Ser	His	Trp	Arg	Tyr	Gly	Gly	Asp	Pro	Pro	Trp	
				100					105					110			
30	Pro	Arg	Val	Ser	Pro	Ala	Cys	Ala	Gly	Arg	Phe	Gln	Ser	Pro	Val	Asp	
			115					120					125				
	Ile	Arg	Pro	Gln	Leu	Ala	Ala	Phe	Cys	Pro	Ala	Leu	Arg	Pro	Leu	Glu	
		130					135					140					
35	Leu	Leu	Gly	Phe	Gln	Leu	Pro	Pro	Leu	Pro	Glu	Leu	Arg	Leu	Arg	Asn	
	145					150					155					160	
40	Asn	Gly	His	Ser	Val	Gln	Leu	Thr	Leu	Pro	Pro	Gly	Leu	Glu	Met	Ala	
					165					170					175		
	Leu	Gly	Pro	Gly	Arg	Glu	Tyr	Arg	Ala	Leu	Gln	Leu	His	Leu	His	Trp	
				180					185					190			
45	Gly	Ala	Ala	Gly	Arg	Pro	Gly	Ser	Glu	His	Thr	Val	Glu	Gly	His	Arg	
			195					200					205				

50

55

EP 2 594 265 B1

	Phe	Pro	Ala	Glu	Ile	His	Val	Val	His	Leu	Ser	Thr	Ala	Phe	Ala	Arg	
	210						215					220					
5	Val	Asp	Glu	Ala	Leu	Gly	Arg	Pro	Gly	Gly	Leu	Ala	Val	Leu	Ala	Ala	
	225					230					235					240	
10	Phe	Leu	Glu	Glu	Gly	Pro	Glu	Glu	Asn	Ser	Ala	Tyr	Glu	Gln	Leu	Leu	
					245					250					255		
15	Ser	Arg	Leu	Glu	Glu	Ile	Ala	Glu	Glu	Gly	Ser	Glu	Thr	Gln	Val	Pro	
				260					265					270			
20	Gly	Leu	Asp	Ile	Ser	Ala	Leu	Leu	Pro	Ser	Asp	Phe	Ser	Arg	Tyr	Phe	
			275					280					285				
25	Gln	Tyr	Glu	Gly	Ser	Leu	Thr	Thr	Pro	Pro	Cys	Ala	Gln	Gly	Val	Ile	
		290					295					300					
30	Trp	Thr	Val	Phe	Asn	Gln	Thr	Val	Met	Leu	Ser	Ala	Lys	Gln	Leu	His	
	305				310						315					320	
35	Thr	Leu	Ser	Asp	Thr	Leu	Trp	Gly	Pro	Gly	Asp	Ser	Arg	Leu	Gln	Leu	
				325						330					335		
40	Asn	Phe	Arg	Ala	Thr	Gln	Pro	Leu	Asn	Gly	Arg	Val	Ile	Glu	Ala	Ser	
				340					345					350			
45	Phe	Pro	Ala	Gly	Val	Asp	Ser	Ser	Pro	Arg	Ala	Ala	Glu	Pro	Val	Gln	
			355					360					365				
50	Leu	Asn	Ser	Cys	Leu	Ala	Ala	Gly	Asp								
	370						375										
55	Ile	Leu	Ala	Leu	Val	Phe	Gly	Leu	Leu	Phe	Ala	Val	Thr	Ser	Val	Ala	
	1				5					10					15		
	Phe	Leu	Val	Gln													
				20													
	<210> 8																
	<211> 20																
	<212> PRT																
	<213> Homo sapiens																
	<400> 8																
	Ile	Leu	Ala	Leu	Val	Phe	Gly	Leu	Leu	Phe	Ala	Val	Thr	Ser	Val	Ala	
	1				5					10					15		
	Phe	Leu	Val	Gln													
				20													
	<210> 9																
	<211> 25																
	<212> PRT																

<213> Homo sapiens

<400> 9

5 Met Arg Arg Gln His Arg Arg Gly Thr Lys Gly Gly Val Ser Tyr Arg
1 5 10 15

10 Pro Ala Glu Val Ala Glu Thr Gly Ala
20 25

<210> 10

<211> 59

<212> PRT

15 <213> homo sapiens

<400> 1

20 Ser Ala Ser Glu Glu Pro Ser Pro Ser Glu Val Pro Phe Pro Ser Glu
1 5 10 15

25 Glu Pro Ser Pro Ser Glu Glu Pro Phe Pro Ser Val Arg Pro Phe Pro
20 25 30

30 Ser Val Val Leu Phe Pro Ser Glu Glu Pro Phe Pro Ser Lys Glu Pro
35 40 45

50 Ser Pro Ser Glu Glu Pro Ser Ala Ser Glu Glu
55

35 Claims

1. A pharmaceutical composition for use in a method of treating a preneoplastic and/or neoplastic disease associated with MN/CA IX overexpression in a patient, said method comprising:

40 (a) determining if MN/CA IX is overexpressed in a sample taken from said patient, at a level above that for a control sample; and
(b) if said determining step (a) indicates that MN/CA IX is overexpressed in said sample administering to said patient a composition comprising a specific inhibitor of activated MN/CA IX,

45 wherein said inhibitor of activated MN/CA IX is an MN/CA IX-specific sulfonamide selected from aromatic sulfonamides and heterocyclic sulfonamides selected from the group consisting of compounds 1, 6, 11 - 13, 16 - 19, 22 - 26, 28, 29, 39, 40, 55, 58, 59, 62 and 70,

and wherein said MN/CA IX-specific sulfonamide is **determined to be** a potent and specific inhibitor of MN/CA IX; wherein said MN/CA IX-specific sulfonamide is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay comprising determining the inhibition constant K_i of said sulfonamide, wherein if said inhibition constant K_i is determined to be less than about 50 nanomolar, said MN/CA IX-specific sulfonamide is determined to be a potent inhibitor of MN/CA IX enzymatic activity; and

50 wherein said sulfonamide is determined to be a specific inhibitor of MN/CA IX if said sulfonamide is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of at least one of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV;

55 and wherein said administration of said composition comprising a specific inhibitor of activated MN/CA IX treats the preneoplastic and/or neoplastic disease associated with MN/CA IX overexpression in a patient by inhibiting the activity of MN/CA IX.

2. The pharmaceutical composition for use in a method of claim 1 wherein said MN/CA IX- specific sulfonamide is a membrane-impermeant pyridinium derivative of an aromatic sulfonamide or a membrane-impermeant pyridinium derivative of a heterocyclic sulfonamide; and wherein said MN/CA IX-specific sulfonamide is determined to be membrane-impermeant if it is tested in an exemplary membrane permeance assay comprising: (a) incubating a millimolar solution of said MN/CA IX-specific sulfonamide with human erythrocytes at 37° C for at least 30 minutes; (b) washing said human erythrocytes; (c) quantitating the levels of said MN/CA IX-specific sulfonamide present in said human erythrocytes; and (d) if said MN/CA IX-specific sulfonamide is present in said human erythrocytes at 1 micromolar or less, then said MN/CA IX-specific sulfonamide is determined to be membrane-impermeant.
3. The pharmaceutical composition for use in a method of claim 1 wherein said MN/CA IX-specific sulfonamide is selected from the group consisting of Compounds 6 and 39, optionally wherein said MN/CA IX-specific sulfonamide, that is a specific inhibitor of activated MN/CA IX, is conjugated to a gene that expresses a cytotoxic protein.
4. The pharmaceutical composition for use in a method of claim 1 wherein said MN/CA IX- specific sulfonamide, that is a specific inhibitor of activated MN/CA IX, is conjugated to a vector comprising a gene that expresses a cytotoxic protein, optionally wherein said vector further comprises a MN/CA9 promoter or MN/CA9 promoter fragment, and/or wherein said vector comprises one or more hypoxia response elements.
5. The pharmaceutical composition for use in a method of claim 1 wherein said MN/CA IX- specific sulfonamide, that is a specific inhibitor of activated MN/CA IX, is used to modulate the efficiency of chemotherapeutic drugs whose uptake or activity is pH- dependent.
6. The pharmaceutical composition for use in a method of claim 1, wherein if said sulfonamide is cell membrane-impermeant, said sulfonamide is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of CA IV; and wherein said MN/CA IX-specific sulfonamide is determined to be membrane-impermeant if it is tested in an exemplary membrane permeance assay comprising: (a) incubating a millimolar solution of said MN/CA IX-specific sulfonamide with human erythrocytes at 37° C for at least 30 minutes; (b) washing said human erythrocytes; (c) quantitating the levels of said MN/CA IX-specific sulfonamide present in said human erythrocytes; and (d) if said MN/CA IX-specific sulfonamide is present in said human erythrocytes at 1 micromolar or less, then said MN/CA IX-specific sulfonamide is determined to be membrane-impermeant or wherein if said sulfonamide is cell membrane-permeant, said sulfonamide is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of CA II; wherein said MN/CA IX-specific sulfonamide is determined to be membrane permeant if it is tested in an exemplary membrane permeance assay comprising: (a) incubating a millimolar solution of said MN/CA IX-specific sulfonamide with human erythrocytes at 37° C for at least 30 minutes; (b) washing said human erythrocytes; (c) quantitating the levels of said MN/CA IX-specific sulfonamide present in said human erythrocytes; and (d) if said MN/CA IX-specific sulfonamide is present in said human erythrocytes at more than 1 micromolar, then said MN/CA IX-specific sulfonamide is determined to be membrane permeant.
7. The pharmaceutical composition for use in a method of claim 1 wherein said sulfonamide is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of at least two of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV, or wherein said sulfonamide is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV.
8. The pharmaceutical composition for use in a method of claim 1 wherein said disease is a hypoxic preneoplastic and/or neoplastic disease.

Patentansprüche

1. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren zum Behandeln einer präneoplastischen und/oder neoplastischen Erkrankung, die mit MN/CA-IX-Überexpression in einem Patienten in Verbindung steht, wobei das Verfahren umfasst:

(a) Bestimmen, ob MN/CA IX in einer von dem Patienten genommenen Probe auf ein Niveau über jenem in einer Kontrollprobe überexprimiert ist; und

(b) wenn der Bestimmungsschritt (a) anzeigt, dass MN/CA IX in der Probe überexprimiert ist, Verabreichen einer Zusammensetzung, die einen spezifischen Inhibitor von aktiviertem MN/CA IX umfasst, an den Patienten,

wobei der Inhibitor von aktiviertem MN/CA IX ein MN/CA-IX-spezifisches Sulfonamid ausgewählt aus aromatischen Sulfonamiden und heterocyclischen Sulfonamiden ausgewählt aus der Gruppe bestehend aus den Verbindungen 1, 6, 11-13, 16-19, 22-26, 28, 29, 39, 40, 55, 58, 59, 62 und 70 ist,

und wobei von dem MN/CA-IX-spezifischen Sulfonamid bestimmt ist, dass es ein wirkungsstarker und spezifischer Inhibitor von MN/CA IX ist;

wobei von dem MN/CA-IX-spezifischen Sulfonamid in einem Screening-Assay umfassend das Bestimmen der Inhibitionskonstante K_i des Sulfonamids bestimmt ist, dass es ein wirkungsstarker Inhibitor der enzymatischen Aktivität von MN/CA IX ist, wobei, wenn die Inhibitionskonstante K_i als kleiner als etwa 50 nanomolar bestimmt ist, von dem MN/CA-IX-spezifischen Sulfonamid bestimmt ist, dass es ein wirkungsstarker Inhibitor der enzymatischen Aktivität von MN/CA IX ist; und

wobei von dem Sulfonamid bestimmt ist, dass es ein spezifischer Inhibitor von MN/CA IX ist, wenn das Sulfonamid ein wirkungsstärkerer Inhibitor der enzymatischen Aktivität von MN/CA IX als der enzymatischen Aktivität von wenigstens einer der Carboanhydrasen in der Gruppe bestehend aus CA I, CA II und CA IV ist;

und wobei die Verabreichung der Zusammensetzung, die einen spezifischen Inhibitor von aktiviertem MN/CA IX umfasst, die präneoplastische und/oder neoplastische Erkrankung, die mit MN/CA-IX-Überexpression in einem Patienten in Verbindung steht, durch Hemmen der Aktivität von MN/CA IX behandelt.

2. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren gemäß Anspruch 1, wobei das MN/CA-IX-spezifische Sulfonamid ein nicht membrangängiges Pyridiniumderivat eines aromatischen Sulfonamids oder ein nicht membrangängiges Pyridiniumderivat eines heterocyclischen Sulfonamids ist; und wobei von dem MN/CA-IX-spezifischen Sulfonamid bestimmt ist, dass es nicht membrangängig ist, wenn es in einem beispielhaften Membrangängigkeitstest geprüft wird, der umfasst: (a) wenigstens 30 Minuten Inkubieren einer millimolaren Lösung des MN/CA-IX-spezifischen Sulfonamids mit menschlichen Erythrozyten bei 37 °C; (b) Waschen der menschlichen Erythrozyten; (c) Quantifizieren der Mengen des in den menschlichen Erythrozyten vorhandenen MN/CA-IX-spezifischen Sulfonamids; und (d) wenn das MN/CA-IX-spezifische Sulfonamid in den menschlichen Erythrozyten 1 mikromolar oder weniger vorhanden ist, ist von dem MN/CA-IX-spezifischen Sulfonamid bestimmt, dass es nicht membrangängig ist.
3. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren gemäß Anspruch 1, wobei das MN/CA-IX-spezifische Sulfonamid ausgewählt ist aus der Gruppe bestehend aus den Verbindungen 6 und 39, gegebenenfalls wobei das MN/CA-IX-spezifische Sulfonamid, das ein spezifischer Inhibitor von aktiviertem MN/CA IX ist, an ein Gen konjugiert ist, das ein zytotoxisches Protein exprimiert.
4. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren gemäß Anspruch 1, wobei das MN/CA-IX-spezifische Sulfonamid, das ein spezifischer Inhibitor von aktiviertem MN/CA IX ist, an einen Vektor konjugiert ist, der ein Gen umfasst, das ein zytotoxisches Protein exprimiert, gegebenenfalls wobei der Vektor ferner einen MN/CA9-Promoter oder ein MN/CA9-Promoterfragment umfasst und/oder wobei der Vektor ein oder mehrere Hypoxie-Antwortelemente umfasst.
5. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren gemäß Anspruch 1, wobei das MN/CA-IX-spezifische Sulfonamid, das ein spezifischer Inhibitor von aktiviertem MN/CA IX ist, zum Modulieren der Wirksamkeit von chemotherapeutischen Arzneimitteln, deren Aufnahme oder Aktivität pH-Wert-abhängig ist, verwendet wird.
6. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren gemäß Anspruch 1, wobei, wenn das Sulfonamid nicht membrangängig ist, das Sulfonamid ein wirkungsstärkerer Inhibitor der enzymatischen Aktivität von MN/CA IX als der enzymatischen Aktivität von CA IV ist; und wobei von dem MN/CA-IX-spezifischen Sulfonamid bestimmt ist, dass es nicht membrangängig ist, wenn es in einem beispielhaften Membrangängigkeitstest geprüft wird, der umfasst: (a) wenigstens 30 Minuten Inkubieren einer millimolaren Lösung des MN/CA-IX-spezifischen Sulfonamids mit menschlichen Erythrozyten bei 37 °C; (b) Waschen der menschlichen Erythrozyten; (c) Quantifizieren der Mengen des in den menschlichen Erythrozyten vorhandenen MN/CA-IX-spezifischen Sulfonamids; und (d) wenn das MN/CA-IX-spezifische Sulfonamid in den menschlichen Erythrozyten 1 mikromolar oder weniger vorhanden ist, ist von dem MN/CA-IX-spezifischen Sulfonamid bestimmt, dass es nicht membrangängig ist, oder wobei, wenn das Sulfonamid membrangängig ist, das Sulfonamid ein wirkungsstärkerer Inhibitor der enzymatischen

Aktivität von MN/CA IX als der enzymatischen Aktivität von CA II ist; wobei von dem MN/CA-IX-spezifische Sulfonamid bestimmt ist, dass es membrangängig ist, wenn es in einem beispielhaften Membrangängigkeitstest geprüft wird, der umfasst: (a) wenigstens 30 Minuten Inkubieren einer millimolaren Lösung des MN/CA-IX-spezifischen Sulfonamids mit menschlichen Erythrozyten bei 37 °C; (b) Waschen der menschlichen Erythrozyten; (c) Quantifizieren der Mengen des in den menschlichen Erythrozyten vorhandenen MN/CA-IX-spezifischen Sulfonamids; und (d) wenn das MN/CA-IX-spezifische Sulfonamid in den menschlichen Erythrozyten mehr als 1 mikromolar vorhanden ist, ist von dem MN/CA-IX-spezifischen Sulfonamid bestimmt, dass es membrangängig ist.

7. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren gemäß Anspruch 1, wobei das Sulfonamid ein wirkungsstärkerer Inhibitor der enzymatischen Aktivität von MN/CA IX als der enzymatischen Aktivität von wenigstens zwei der Carboanhydrasen in der Gruppe bestehend aus CA I, CA II und CA IV ist, oder wobei das Sulfonamid ein wirkungsstärkerer Inhibitor der enzymatischen Aktivität von MN/CA IX als der enzymatischen Aktivität von jeder der Carboanhydrasen in der Gruppe bestehend aus CA I, CA II und CA IV ist.
8. Pharmazeutische Zusammensetzung für die Verwendung bei einem Verfahren gemäß Anspruch 1, wobei die Erkrankung eine hypoxische präneoplastische und/oder neoplastische Erkrankung ist.

Revendications

1. Composition pharmaceutique pour utilisation dans un procédé de traitement d'une maladie préneoplasique et/ou néoplasique associée à une surexpression de MN/CA IX chez un patient, ledit procédé comprenant :

- (a) la détermination du fait que MN/CA IX est surexprimé ou non dans un échantillon prélevé à partir dudit patient, à un taux supérieur à celui d'un échantillon témoin ; et
- (b) si ladite étape de détermination (a) indique que MN/CA IX est surexprimé dans ledit échantillon, administration audit patient d'une composition comprenant un inhibiteur spécifique de MN/CA IX activé,

où ledit inhibiteur de MN/CA IX activé est un sulfonamide spécifique pour MN/CA IX choisi parmi des sulfonamides aromatiques et des sulfonamides hétérocycliques choisis dans le groupe constitué de composés 1, 6, 11 à 13, 16 à 19, 22 à 26, 28, 29, 39, 40, 55, 58, 59, 62 et 70, et où ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un inhibiteur puissant et spécifique de MN/CA IX ;

où ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un inhibiteur puissant de l'activité enzymatique de MN/CA IX dans un essai de criblage comprenant la détermination de la constante d'inhibition K_i dudit sulfonamide, où, si ladite constante d'inhibition K_i est déterminée comme étant inférieure à environ 50 nanomolaires, ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un inhibiteur puissant de l'activité enzymatique de MN/CA IX ; et

où ledit sulfonamide est déterminé comme étant un inhibiteur spécifique de MN/CA IX si ledit sulfonamide est un inhibiteur plus puissant de l'activité enzymatique de MN/CA IX que de l'activité enzymatique d'au moins une des anhydrases carboniques dans le groupe constitué de CA I, CA II et CA IV ;

et où ladite administration de ladite composition comprenant un inhibiteur spécifique de MN/CA IX activé traite la maladie préneoplasique et/ou néoplasique associée à la surexpression de MN/CA IX chez un patient par inhibition de l'activité de MN/CA DC.

2. Composition pharmaceutique pour utilisation dans un procédé de la revendication 1 dans laquelle ledit sulfonamide spécifique pour MN/CA IX est un dérivé pyridinium d'un sulfonamide aromatique imperméant membranaire ou un dérivé pyridinium d'un sulfonamide hétérocyclique imperméant membranaire ; et où ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un imperméant membranaire s'il est testé dans un essai de perméance membranaire exemplaire comprenant : (a) l'incubation d'une solution millimolaire dudit sulfonamide spécifique pour MN/CA IX avec des érythrocytes humains à 37 °C pendant au moins 30 minutes ; (b) lavage desdits érythrocytes humains ; (c) quantification des taux dudit sulfonamide spécifique pour MN/CA IX présent dans lesdits érythrocytes humains ; et (d) si ledit sulfonamide spécifique pour MN/CA IX est présent dans lesdits érythrocytes humains à 1 micromolaire ou moins, alors ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un imperméant membranaire.
3. Composition pharmaceutique pour utilisation dans un procédé de la revendication 1 dans laquelle ledit sulfonamide spécifique pour MN/CA IX est choisi dans le groupe constitué des composés 6 et

39, facultativement

où ledit sulfonamide spécifique pour MN/CA IX, qui est un inhibiteur spécifique de MN/CA IX activé, est conjugué à un gène qui exprime une protéine cytotoxique.

- 5 4. Composition pharmaceutique pour utilisation dans un procédé de la revendication 1 dans laquelle un sulfonamide spécifique pour MN/CA IX, qui est un inhibiteur spécifique de MN/CA IX activé, est conjugué à un vecteur comprenant un gène qui exprime une protéine cytotoxique, facultativement
où ledit vecteur comprend en outre un promoteur de MN/CA9 ou un fragment de promoteur de MN/CA9, et/ou où ledit vecteur comprend un ou plusieurs éléments de réponse à une hypoxie.
- 10 5. Composition pharmaceutique pour utilisation dans un procédé de la revendication 1 dans laquelle ledit sulfonamide spécifique pour MN/CA IX, qui est un inhibiteur spécifique de MN/CA IX activé, est utilisé pour moduler l'efficacité de médicaments chimiothérapeutiques dont l'absorption ou l'activité est dépendante du pH.
- 15 6. Composition pharmaceutique pour utilisation dans un procédé de la revendication 1, où, si ledit sulfonamide est un imperméant de membrane cellulaire, ledit sulfonamide est un inhibiteur plus puissant de l'activité enzymatique MN/CA IX que de l'activité enzymatique de CA IV ; et où ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un imperméant membranaire s'il est testé dans un essai de perméance membranaire exemplaire comprenant : (a) l'incubation d'une solution millimolaire dudit sulfonamide spécifique pour MN/CA IX avec des érythrocytes humains à 37 °C pendant au moins 30 minutes ; (b) lavage desdits érythrocytes humains ; (c) quantification des taux dudit sulfonamide spécifique pour MN/CA IX présent dans lesdits érythrocytes humains ; et (d) si ledit sulfonamide spécifique pour MN/CA IX est présent dans lesdits érythrocytes humains à 1 micromolaire ou moins, alors ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un imperméant membranaire ou
20 où, si ledit sulfonamide est un perméant de membrane cellulaire, ledit sulfonamide est un inhibiteur plus puissant de l'activité enzymatique MN/CA IX que de l'activité enzymatique de CA II ; où ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un perméant membranaire s'il est testé dans un essai de perméance membranaire exemplaire comprenant : (a) l'incubation d'une solution millimolaire dudit sulfonamide spécifique pour MN/CA IX avec des érythrocytes humains à 37 °C pendant au moins 30 minutes ; (b) lavage desdits érythrocytes humains ; (c) quantification des taux dudit sulfonamide spécifique pour MN/CA IX présent dans lesdits érythrocytes humains ; et (d) si ledit sulfonamide spécifique pour MN/CA IX est présent dans lesdits érythrocytes humains à plus
25 de 1 micromolaire, alors ledit sulfonamide spécifique pour MN/CA IX est déterminé comme étant un perméant membranaire.
- 30 7. Composition pharmaceutique pour utilisation dans un procédé de la revendication 1 où ledit sulfonamide est un inhibiteur plus puissant de l'activité enzymatique MN/CA IX que de l'activité enzymatique d'au moins deux des anhydrases carboniques dans le groupe constitué de CA I, CA II et CA IV, ou
35 où ledit sulfonamide est un inhibiteur plus puissant de l'activité enzymatique MN/CA IX que de l'activité enzymatique de chacune des anhydrases carboniques dans le groupe constitué de CA I, CA II et CA IV.
- 40 8. Composition pharmaceutique pour utilisation dans un procédé de la revendication 1 où ladite maladie est une maladie préneoplasique et/ou néoplasique hypoxique.

45

50

55

```

1      1      ACA GTC AGC CGC ATG GCT CCC CTG TGC CCC AGC CCC TGG CTC CCT CTG L 12
1      48
13     13     L I P A P A P G L T V Q L L L S 28
49     49     TTG ATC CCG GCC CCT GCT CCA GGC CTC ACT GTG CAA CTG CTG TCA 96

29     29     L L L L M P V H P Q R L P R M Q 44
97     97     CTG CTG CTT CTG ATG CCT GTC CAT CCC CAG AGG TTG CCC CGG ATG CAG 144

45     45     E D S P L G G G S S G E D D P L 60
145    145    GAG GAT TCC CCC TTG GGA GGA GGC TCT TCT GGG GAA GAT GAC CCA CTG 192

61     61     G E E D L P S S E E D S P R E E D 76
193    193    GGC GAG GAG GAT CTG CCC AGT GAA GAG GAT TCA CCC AGA GAG GAG GAT 240

77     77     P P G E E D L P G E E D L P G E 92
241    241    CCA CCC GGA GAG GAG GAT CTA CCT GGA GAG GAT CTA CCT GGA GAG 288

93     93     E D L P E V K P K S E E E G S L 108
289    289    GAG GAT CTA CCT GAA GTT AAG CCT AAA TCA GAA GAA GAG GGC TCC CTG 336

109    109    K L E D L P T V E A P G D P Q E 124
337    337    AAG TTA GAG GAT CTA CCT ACT ACT GTT GAG GCT CCT GGA GAT CCT CAA GAA 384

125    125    P Q N N A H R D K E G D D Q S H 140
385    385    CCC CAG AAT AAT GCC CAC AGG GAC AAA GAA GGG GAT GAC CAG AGT CAT 432

141    141    W R Y G G G G C C G C C C T G G C C C C G G T G T C C A G C C T G C 156
433    433    TGG CGC TAT GGA GGC GAC CCG CCC TGG CCC CGG GTG TCC CCA GCC TGC 480

157    157    A G R F Q S P V D I R P Q L A A 172
481    481    GCG GGC CGC TTC CAG TCC CCG GTG GAT ATC CGC CCC CAG CTC GCC GCC 528

```

FIG. 1A

173	F	C	P	A	L	R	P	L	E	L	L	G	F	Q	L	P	188
529	TTC	TGC	CCG	GCC	CTG	CGC	CCC	CTG	GAA	CTC	CTG	GGC	TTC	CAG	CTC	CCG	576
189	P	L	P	E	L	R	L	R	N	N	G	H	S	V	Q	L	204
577	CCG	CTC	CCA	GAA	CTG	CGC	CTG	CGC	AAC	AAT	GGC	CAC	AGT	GTG	CAA	CTG	624
205	T	L	P	P	G	L	E	M	A	L	G	P	G	R	E	Y	220
625	ACC	CTG	CCT	CCT	GGG	CTA	GAG	ATG	GCT	CTG	GGT	CCC	GGG	CGG	GAG	TAC	672
221	R	A	L	Q	L	H	L	H	W	G	A	A	G	R	P	G	236
673	CGG	GCT	CTG	CAG	CTG	CAT	CTG	CAC	TGG	GGG	GCT	GCA	GGT	CGT	CCG	GGC	720
237	S	E	H	T	V	E	G	H	R	F	P	A	E	I	H	V	252
721	TCG	GAG	CAC	ACT	GTG	GAA	GGC	CAC	CGT	TTC	CCT	GCC	GAG	ATC	CAC	GTG	768
253	V	H	L	S	T	A	F	A	R	V	D	E	A	L	G	R	268
769	GTT	CAC	CTC	AGC	ACC	GCC	TTT	GCC	AGA	GTT	GAC	GAG	GCC	TTG	GGG	CGC	816
269	P	G	G	L	A	V	L	A	A	F	L	E	E	G	P	E	284
817	CCG	GGA	GGC	CTG	GCC	GTG	TTG	GCC	GCC	TTT	CTG	GAG	GAG	GGC	CCG	GAA	864
285	E	N	S	A	Y	E	Q	L	L	S	R	L	E	E	I	A	300
865	GAA	AAC	AGT	GCC	TAT	GAG	CAG	TTG	CTG	TCT	CGC	TTG	GAA	GAA	ATC	GCT	912
301	E	E	G	S	E	T	Q	V	P	G	L	D	I	S	A	L	316
913	GAG	GAA	GGC	TCA	GAG	ACT	CAG	GTC	CCA	GGA	CTG	GAC	ATA	TCT	GCA	CTC	960
317	L	P	S	D	F	S	R	Y	F	Q	Y	E	G	S	L	T	332
961	CTG	CCC	TCT	GAC	TTC	AGC	CGC	TAC	TTC	CAA	TAT	GAG	GGG	TCT	CTG	ACT	1008
333	T	P	P	C	A	Q	G	V	I	W	T	V	F	N	Q	T	348
1009	ACA	CCG	CCC	TGT	GCC	CAG	GGT	GTC	ATC	TGG	ACT	GTG	TTT	AAC	CAG	ACA	1056

FIG._1B

349 V M L S A K Q L H T L S D T L W 364
 1057 GTG ATG CTG AGT GCT AAG CAG CAG CTC CAC ACC CTC TCT GAC ACC CTG TGG 1104

 365 G P G D S R L Q L N F R A T Q P 380
 1105 GGA CCT GGT GAC TCT CGG CTA CAG CTG AAC TTC CGA GCG ACG CAG CCT 1152

 381 L N G R V I E A S F P A G V D S 396
 1153 TTG AAT GGG CGA GTG ATT GAG GCC TCC TTC CCT GCT GGA GTG GAC AGC 1200

 397 S P R A A E P V Q L N S C L A A 412
 1201 AGT CCT CGG GCT GCT GAG CCA GTC CAG CTG AAT TCC TGC CTG GCT GCT 1248

 413 G D I L A L V F G L L F A V T S 428
 1249 GGT GAC ATC CTA GCC CTG CTG GTT TTT GGC CTC CTT TTT GCT GTC ACC AGC 1296

 429 V A F L L V Q M R R Q H R R G T K 444
 1297 GTC GCG TTC CTT GTG CAG ATG AGA AGG CAG CAC AGA AGG GGA ACC AAA 1344

 445 G G V S Y R P A E V A E T G A * 460
 1345 GGG GGT GTG AGC TAC CGC CCA GCA GAG GTA GCC GAG ACT GGA GCC TAG 1392

 1393 AGG CTG GAT CTT GGA GAA TGT GAG AAG CCA GCC AGA GGC ATC TGA GGG 1440

 1441 GGA GCC GGT AAC TGT CCT GTC CTG CTC ATT ATG CCA CTT CCT TTT AAC 1488

 1489 TGC CAA GAA ATT TTT TAA AAT AAA TAT TTA TAA T 1522

FIG._1C

FIG._1A

FIG._1B

FIG._1C

FIG._1

1 ggatccctgtt gactcgtgac cttaccccca accctgtgct ctctgaaca tgagctgtgt
 61 ccactcaggg ttaaatggat taaggcggt gcaagatgtg ctttgttaa cagatgcttg
 121 aaggcagcat gctcgttaag agtcatcacc aatccctaata taggaaaac cagagacctt tgttcacttg
 181 aacactgcgg aaggccgcag gtcctctgc ctattgtcca tgaacctgcc aaatccccct ctgtgagaaa
 241 ttatctgac ctccctcca ctattgtcca ttaaaaaaa taaatgaata gctattggtg aagccaagta
 301 cacccaagaa ttatcaataa gacttacgaa tagttattga gacggccatc atcacagctc aagctacctt gatttgatct
 361 aaaaaaaaaa gacttacgaa tagttattga gacggccatc atcacagctc aagctacctt gatttgatct
 421 aatgatacata ttcaaaacca gacggccatc atcacagctc aagctacctt gatttgatct
 481 cttatcattt gtcattctttt gatttcacta gattagtcac gctcctcaaa attctcccc
 541 aagttctaat tacgttccaa acatttaggg gttacatgaa gcttgaacct actaccttct
 601 ttgcttttga gccatgagtt gtaggaatga tgagtttaca gcttacctaa cctacatgc tgggatttaa
 661 tttaaaactt acctctaagt cagttgggta gcctttggct tatttttcta gctaattttg
 721 tagttaatgg atgcactgtg aatcttgcta tgatagtttt cctccacctt tggccactag
 781 gggtaggtag gtactcagtt ttcagtaatt gcttacctaa gacctaaag cctatttctc
 841 ttgtactggc cttatctgt aatatgggca tattaatac acggagctctt gcactctgtc tggagtttt
 901 tttgtttgtt tgtttgtttg tttttttgag acggagctctt gcaagctcca cctcccgagt tcacgccatt
 961 ggagtagcag tggtgccatc tagctggga gacggggttt caccgtgtta gccagaatgg tctcgatctc
 1021 ttcctgcctc agcctcccga gtagctggga gacggggttt caccgtgtta gccagaatgg tctcgatctc
 1081 ttttttgtat ttttggtaga gacggggttt caccgtgtta gccagaatgg tctcgatctc
 1141 ctgacttcgt gatccacccg cctcggcctc gactcttcta aagtaaaaat atgtcttcta agctggtaac
 1201 ccgcacctgg ccaatttttt taatgtggtg gcagtccttt cacttggttctt aagtaaaaat atgtcttcta agctggtaac
 1261 tatggtacat ttccttttat gactcttttt cacttggttctt aagtaaaaat atgtcttcta agctggtaac
 1321 gcatgcataat gctacttttt cacttggttctt aagtaaaaat atgtcttcta agctggtaac
 1381 catgttatat cttttagctt cacttggttctt aagtaaaaat atgtcttcta agctggtaac
 1441 tcattgtttg taccacttgg atcataagtg ttcagggtgaa gctgtatata gctgtataa gctgtataa
 1501 cttgttttga agaggatga ttcagggtgaa gctgtataa gctgtataa gctgtataa
 1561 tctgagattc ccttgacatt ttaagcaaga tatgctaaa gctgtataa gctgtataa
 1621 actatttttc ttaagcaaga tatgctaaa gctgtataa gctgtataa gctgtataa
 1681 catatctgca tcaagtgaga acatataatg tatgctaaa gctgtataa gctgtataa
 1741 gcttgtgttt tatgctttta tatagacagg gaaactgttt tccatatttc agaatgttt
 1801 tgggaattgt tatggatat catcatgggc ccacgctttc tacaagaaat agctgctatg tttcttgaca
 1861 ggttcataat ctcaattctg tcagaattgg cacttggttctt tacaagaaat agctgctatg tttcttgaca
 1921 ttcacttgg taggaaataa gaatgtgaaa ctcttcagtt ggtgtgtgtc cct?gttttt

FIG.-2A

1981 ttgcaatttc cttcttactg ttgtaaaaaa aagtatgac ttgctctgag aggtgaggca
 2041 ttcttaatca tgatctttaa agatcaataa tataatcctt tcaaggatta tgtctttatt
 2101 ataataaaga taatttgtct ttaacagaat caataatata atcccttaa ggattatatac
 2161 ttgctgggc gcagtggtc acacctgtaa tccagcact ttgggtggcc aaggtggaag
 2221 gatcaaat ttgactactt atattatctt ctacagcaga atcatctct ctccctcaa
 2281 tatgatgata ttgacaggt ttgacctcac tcactagatt gtgagctcct gctcagggca
 2341 ggtagcgtt ttgtttttg ttttgtttt tctttttga gacagggtct tgtctgtca
 2401 ccaggccag agtgcaatg tacagtctca gctcactgca gcctcaacc cctcggtca
 2461 aaccatcatc ccatttcagc ctctgagta gctgggacta caggcacatg ccattacac
 2521 tggctaattt ttttgtattt ctagttaga cagggtttgg ccatgttgc cgggctggtc
 2581 tcgaactcct gactcaagc aatccacca cctcagcctc ccaaatgag ggaccgtgtc
 2641 ttattcattt ccatgtcctt agtccatagc cctcaggtg acctatggtataactaaata
 2701 aatatattgtt gaatgcaata gtaaatagca ttccaggag caagaactag ataaacaaag
 2761 gtggtaaaag gtttgagaa ggtctcttg gcaaggtttt gaaggaagt gagggagagt
 2821 agtaggagac agatggaaa ttgagagcag tggggagcca atgaaggctt ttagcagga
 2881 agtacacaat gtgcatactg ttgaaaaata aatataggtt aaacctatac cattacttaa ctaccctcg
 2941 gagtaatgtg ttgaaaaata attcaagctc ctacctctt acctgcttcc tgggtgagtc agggatgtat
 3001 cttgcttttc attcaagctc gcagcctgcc ctacctctt cagccagagg acatggggg cccagctcc tggcctttc
 3061 ggctcccta gcagcctgcc ctacctctt cagccagagg acatggggg cccagctcc tggcctttc
 3121 acatgagctg ctttccctct cagccagagg acatggggg cccagctcc tggcctttc
 3181 ccttctgtg cctggagctg ggaagcaggc cctgcatagt gccaggtggt gccttgggtt ccaagctagt
 3241 ctgggtggtg ccaggagag gataaccttc tgcctgtgca cacacctgcc cctcactcca ccccatcct
 3301 ccattgggtg tgggggagag ggcagcctgt gactcagcct gctccctcc aggttgtc ctccccacc
 3361 agctttggtg tgggggagag ggcagcctgt gactcagcct gctccctcc aggttgtc ctccccacc
 3421 tctgcaaaag ggcgctctgt gactcagcct gctccctcc aggttgtc ctccccacc
 3481 cagctctgt tccaatgca cgtacagccc gtacacaccg tgtgtggga caccacacag
 3541 TCAGCCGCAT GGCTCCCCTG TGCCCCAGCC CTTGGCTCCC CTTGGTGTATC CCGCCCCCTG
 3601 CTCCAGGCCT CACTGTGCAA CTGCTGCTGT CACTGCTGCT TCTGGTGCTT GTCCATCCCC
 3661 AGAGGTTGCC CCGGATGCC GAGGATTCCC CTTGGGAGG AGGCTCTTCT GGGGAAGATG
 3721 ACCCACTGGG CGAGGAGGAT CTGCCAGTG AGAGGATTG ACCAGAGAG GAGGATCCAC
 3781 CCGGAGAGGA GGATCTACCT GGAGAGGAGG ATCTACCTGG AGAGGAGGAT CTACCTGAAG
 3841 TTAAGCCTAA ATCAGAAGAA GAGGCTCCC TGAAGTTAGA GGATCTACCT ACTGTTGAGG
 3901 CTCTGGAGA TCCTCAAGAA CCCCAGAATA ATGCCACAG GGACAAAGAA Ggtaagtgtt

FIG.--2B

3961 catcaatctc caaatccagg ttccaggagg ttcatgactc ccctcccata cccagccta
 4021 ggctctgttc actcagggaa ggaggggaga ctgtactccc cacagaagcc ctccagagg
 4081 tcccatacca atatcccat cccactctc ggaggtagaa agggacagat gtggagagaa
 4141 aataaaaagg gtgcaaaaagg agagaggtga gctggatgag atgggagaga agggggaggc
 4201 tggagaaagg aaaggatga gaaactgcaga tgagagaaaa atgtgcaga cagaggaaaa
 4261 aatataggtgg agaaggagag taccagagac agcaagaag tgaggggaa agcttggag
 4321 gtgaagtggg caatgaggaa ttgagacctt ggaagaagg acacagcagg tagagaaacg tcttaggcta
 4381 caatgaggaa actcccaagc caggaatttg ggaagaagg ttggagacca tacaaggcag agggatgagt
 4441 ggggagaaga tgaagtggc actcactttt tttttttttt cgatctcggc tcaactgcaac ctccacctcc cgggttcaag
 4501 ggggagaaga tgaagtggc actcactttt tttttttttt aaaggaaga agctgcgatt acaggcattgc gccaccacgc
 4561 tgaagtggc actcactttt tttttttttt tagtagagac ggggttttcgc cctggcctcc cactcacttt
 4621 caggctggag tgcaatggcg gctcagcct ctagccaagt agctgcgatt agctgcgatt acaggcattgc gccaccacgc
 4681 tgatttctct cctcagcct tttgtatttt atccaaccac ctgaagcagc cactcacttt
 4741 ccggctaatt tttgtatttt tagtagagac ggggttttcgc cctggcctcc cactcacttt
 4801 cgaactcctg atctcaggtg agcgcctggc ctgaagcagc cactcacttt
 4861 cgtgagccac agcgcctggc ctgaagcagc cactcacttt
 4921 ttgcaagctg taggattgc ttgttggtc gcccgttaa ggcatttgtt acccgtaatg ctccgttaag
 4981 tctcctgtgc ttgtgacatc ttgttggtc gcccgttaa ggcatttgtt acccgtaatg ctccgttaag
 5041 gcatctgcgt ttgtgacatc ttgttggtc gcccgttaa ggcatttgtt acccgtaatg ctccgttaag
 5101 cggttcatcc ttttcattta tacaggggat gaccagagtc attggggctc taagcttgag
 5161 acaccaccc gctgcacaga cccaatctgg gaaccagct ctgtggatct cccctacagc
 5221 cgtccctgaa cactggtccc gggcggtccc cccgcccgc accgtcccac cccctcacct
 5281 ttcttaccg ggttccctaa gttcctgacc taggcgtcag acttccctcac tatactctcc
 5341 caccacagc GACCCGCCCT GACCCGCCCT GACCCGCCCT GTCCCCAGCC TGCGCGGGCC GCTTCCAGTC
 5401 CCCGGTGGAT ATCCGCCCCC AGCTCGCCCG CTCTGCCCCG ACTGCGCCCTG CGCAACAATG GCCACAGTGG
 5461 CCTGGGCTTC CAGCTCCCCG CAGCTCCCCG CAGCTCCCCG CAGCTCCCCG CAGCTCCCCG CAGCTCCCCG
 5521 tgaaggggtc tccccgcga gacttgggga tggggcgggg cgaggggaa ggaaccgtcg
 5581 cgcagtgcct gcccgggggt tgggctggcc ctaccgggag gggccggctc acttgcctct
 5641 ccctacgcag TGCAACTGAC CCTGCCTCCT GGGCTAGAGA TGGCTCTGGG TCCCGGGCGG
 5701 GAGTACCGGG CTCTGCAGCT GCATCTGCAC TGGGGGGCTG CAGCTCGTCC GGGCTCGGAG
 5761 CACACTGTGG AAGGCCACCG TTTCCTCTGCC GAGgtgagcg cggactggcc gagaaggggc
 5821 aaaggagcgg ggcggacggg ggcagagac gtggccctct cctaccctcg tgccttttc
 5881 agATCCACGT GGTTCACCTC AGCACCGCCT TTGCCAGAGT TGACGAGGCG TTGGGGCGCC

FIG.-2C

5941 CGGAGGGCCCT GCGCGTGTTG GCGCGCTTTC TGGAGGtacc agatcctgga caccocctac
 6001 tccccgcttt cccatcccat gctcctcccg gactctatcg tggagccaga gaccccatcc
 6061 cagcaagctc actcaggccc gaaccaggca ccagccccc acaaggattc tgacatgaca gacacatagg
 6121 caccactgt tctaaggagc ggtcacagag gaggtagacac ttaagcctt cactggtaga aagaaaaagg
 6181 taaagatggg aggtgttcat tgcagaggaa acaccatgat tttatttatt tttgacagtc tctctgtcgc
 6241 taaagatggg aggtgttcat tgcagaggaa acaccatgat tttatttatt tttgacagtc tctctgtcgc
 6301 aggtgttcat tgcagaggaa acaccatgat tttatttatt tttgacagtc tctctgtcgc
 6361 atggctacat ctcaactcact gtgcagtggg tgcctcagct ttttgtatt gctcaagtg
 6421 gctaggttca ccaggctgga gggattctcc caaactcctg tgagccaccg
 6481 ccaggctgga gggattctcc caaactcctg tgagccaccg tgagccaccg
 6541 gggattctcc caaactcctg tgagccaccg tgagccaccg tgagccaccg
 6601 agctaatttt caaactcctg tgagccaccg tgagccaccg tgagccaccg
 6661 caaactcctg tgagccaccg tgagccaccg tgagccaccg tgagccaccg
 6721 tgagccaccg tgagccaccg tgagccaccg tgagccaccg tgagccaccg
 6781 tcagagaaat gcttaacatta gttcaggaact cactcactga tctttaaag
 6841 cttacacatta gttcaggaact cactcactga tctttaaag tctttaaag
 6901 gtcaggaccct cactcactga tctttaaag tctttaaag tctttaaag
 6961 caacacaaag gttcaggaact cactcactga tctttaaag tctttaaag
 7021 agactgcata tcttccctc cactcactga tctttaaag tctttaaag
 7081 tcttccctc cactcactga tctttaaag tctttaaag tctttaaag
 7141 cagcaagagt acatagagt tgaataataa caaaccagtc cataggattt
 7201 taaaaaaaaa acaaacagca acaacaaaaa gcaacaaacca
 7261 agcatcttca gagctgagga atgggagagg actatgggaa ccccttcat
 7321 tcagccatgg cctggatcac atgcactcat ctgtcttaca atgtcattcc
 7381 CCCGGAAGAA AACAGTGCCT ATGAGCAGTT GCTGTCTCGC TTGGAAGAAA TCGCTGAGGA
 7441 AGgtcagttt gttggtcttg ccactaatct ctgtggccta gttcataaaag
 7501 tggagcttca ggtctgaggc tggagatggg ctccctccag tgcaggaggg
 7561 gagccagcgc tcatcttgat aataaccatg aagctgacag acacagttac
 7621 ctgcttacag attgaaaaac aagcaaaaac cgccggggcac ggtggctcac
 7681 ccagcacttt gggaggccaa ggcaggtgga tccagaggtc aagagatcaa
 7741 gccaacatgg tgaaccccca tctctactaa aaatacgaaa aatatagccag
 7801 ggggtgcctgt aatcccagct actcgggagg ctgagggcagg agaattggcat
 7861 ggcagaagtt gcagtgagcc gagatcgtgc cactgcactc cagcctgggc

FIG. 2D

7921 gactcttgct tcaaaaaaa aaaaaaaaaa gaaaccaaag caaaaaccaa aatgagacaa
 7981 aaaaaaag accaaaaaat ggtgtttgga aattgtcaag gtcaagtctg gagagctaaa
 8041 ctttttctga gaactgttta tcttaataa gcatcaata ttttaacttt gtaaatactt
 8101 ttgttggaat tcgttctctt cttagtcact ctgggtcat tttaaatctc acttactcta
 8161 ctgacacctt taggtttctg cttagactag tagaactctg cctttgcatt tcttgtgtct
 8221 gttttgtata gttatcaata tttttttttt ttatttacct ttacaaagt ttatttcttt
 8281 tctttttttt tctcaaacct ctgaccttgt gatccaccag gacagggttt caccatattg
 8341 gccaggctgc tctcaaacct ctgaccttgt gatccaccag gacagggttt caccatattg
 8401 gggattcatt ttttcttttt aatttgctct gggcttaaac ttgtggccca gcactttatg
 8461 atggtacaca ggttaagag tgtagactca gacggtcttt ctcttctctt tcttctctt
 8521 cctcccttcc cctccacctt ccttctctc caaagccctg tacttttttt tgagttaacg tcttatggga
 8581 caggcctctt ccagtgtct cctagtgtct aagtgtctc agagttgagt taccttggct tctgggaggt
 8641 agggcctgca cttagtgaag aagtgtctc tgaagcttta aggggtgca atgtagatga gacccaaca
 8701 gaaactgtat ccctataccc tcacagGCTC AGAGACTCAG GTCCCAGGAC TGGACATATC TGCACCTCTG
 8761 tagatcctct TCAGCCGCTA CTTCCAATAT GAGGGGTCTC TGACTACACC GCCCTGTGCC
 8821 CCTCTGACT TCTGGACTGT GTTTAACAG ACAGTGATGC TGAGTGCTAA GCAGgtgggc
 8881 CAGGGTGCTA TGTGGACACA GTGGgtgcgg gggaaagagg atgtaagatg agatgagaaa
 8941 ctgggggtgt gttggacaca aagaaatcaa ggctgggctc tgtggcttac gcctataatc ccaccagtt
 9001 caggagaaga ggtggagaa tggtttgagc caaagagttc ccagacaaggc ggggcaacat
 9061 gggaggctga catctctacc tactcaagga ggtgaggtg ggaagatcgc ttgattccag
 9121 agtgtgacct tagtcccagc ctatgatccc accactgcct accatcttta ggatacatct
 9181 gtatgcggcc ctgcagtgag aagaggctgg atggggaata caggagctgg aggtggagc
 9241 ggttttgaga ttggttgtag ctggcctggg acccttgttt ACACCTCTC TGACACCTCTG TGGGACCTG
 9301 attatttat aaaagaaatc aagaggctgg atggggaata caggagctgg aggtggagc
 9361 cctgaggtgc ccactgacct ccctagCTC AACTTCCGAG CGACGCAGCC TTTGAATGGG CGAGTGATG
 9421 ccacacctgt GCTACAGCTG CCTGCTGGA GTGGACAGCA GTCCTCGGGC TGCTGAGCCA Ggtacagctt
 9481 GTGACTCTCG CCTGCTGGA GTGGACAGCA GTCCTCGGGC TGCTGAGCCA Ggtacagctt
 9541 AGGCTCTCTT cctccagcc agtagtccct tatcctccca tcttctcttc tcttctcttc
 9601 tgtctggttt cctccagcc agtagtccct tatcctccca tcttctcttc tcttctcttc
 9661 attggtggtc acagcccgcc tctcacatct cctttttctc ttggtcctga tgcctcagca
 9721 GCCTGGCTGC TGgtgagctt gcccctctc ctctgctccc atggtgggga tccccatt
 9781 ccattcagcc ccagggtgc caatataga gaggcagatc atggtgggga tccccatt
 9841 accccaacc caatataga gaggcagatc atggtgggga tccccatt gtccccagag

FIG. 2E

9901 gctaattgat tagaatgaag cttgagaaat ctccagcat ccctctcgca aaagaatccc
 9961 cccccctttt ttaaaagata gggctctcact ctgtttgccc caggctgggg tggtgtggca
 10021 cgatcatagc tcaactgcagc ctcgaaactcc taggctcagg caatcctttc accttagctt
 10081 ctcaaagcac tgggactgta ggcattgagcc actgtgacctg gcccacaaag gcccttttac
 10141 ttggctttta ggaagcaaaa acggtgctta tcttaccctt tctcgtgtat ccaccctcat
 10201 cccttggctg gcctcttctg gagactgagg cactatgggg ctgcctgaga actcggggca
 10261 ggggtggagg agtgcaactga ggcagggtgtt gaggaactct gcagaccctt cttccttccc
 10321 aaagcagccc tctctgctct ccatcgagc TGACATCCTA GCCCTGGTTT TTGGCCCTCCT
 10381 TTTTGCTGTC ACCAGCGTCG CGTTCCCTTGT GCAGATGAGA AGGCAGCACA Ggtattacac
 10441 tgacccttcc ttcaggcaca agcttccccc acccttgttg agtcacttca tgcaaaagcgc
 10501 atgcaaatga gctgctcctg ggccagtctt ctgattagcc ttctctgttg tgtacacaca
 10561 gAAGGGGAAC CAAAGGGGT GTGAGCTACC GCCCAGCAGA GGTAGCCGAG ACTGGAGCCT
 10621 AGAGGCTGGA TCTTGGAGAA TGTGAGAAAGC CAGCCAGAGG CATCTGAGGG GGAGCCGGTA
 10681 ACTGTCCTGT CCTGCTCAT TATGCCACTTC CTTTTAACTG CCAAGAAATT TTTTAAATA
 10741 AATATTTATA ATaaaatatg tgttagtcac ctttgttccc caaatcagaa ggaggatat
 10801 gaatttccta ttactgttat tagcaccaat ttagtggtaa tgcatttatt ctattacagt
 10861 tcggcctcct tccacacatc actccaatgt gttgctcc

FIG._2F

FIG._2A

FIG._2B

FIG._2C

FIG._2D

FIG._2E

FIG._2F

FIG._2

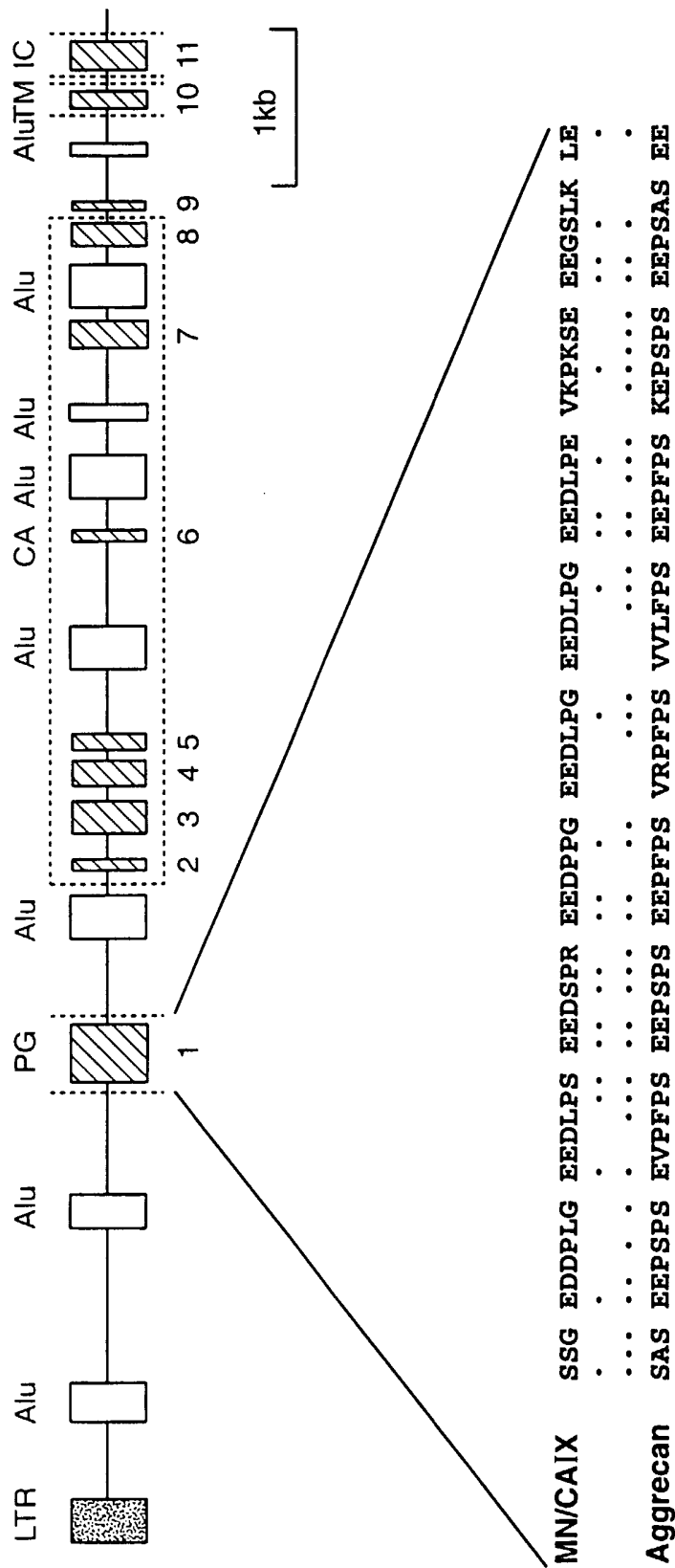
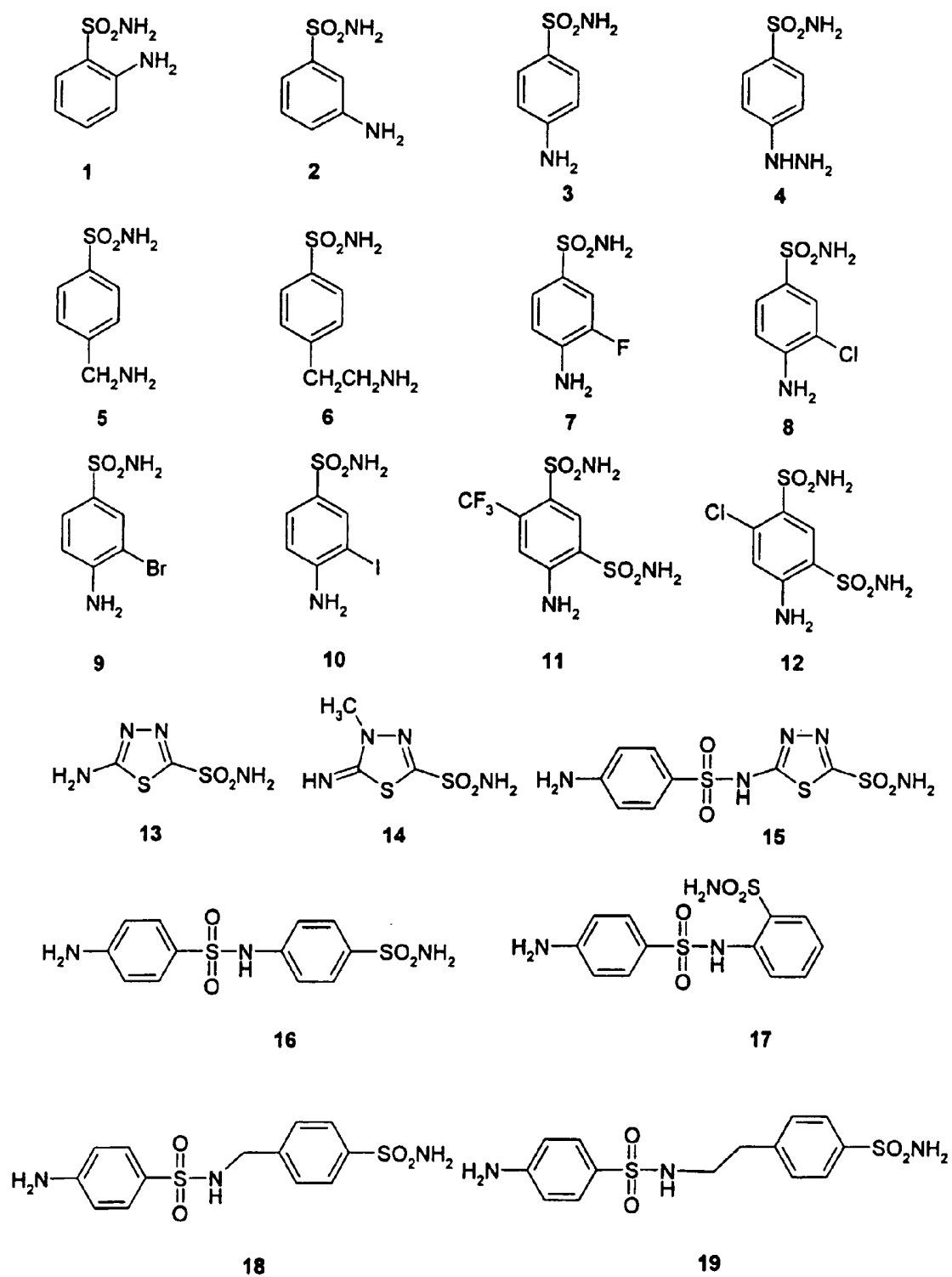


FIG._3

**FIG. 4A**

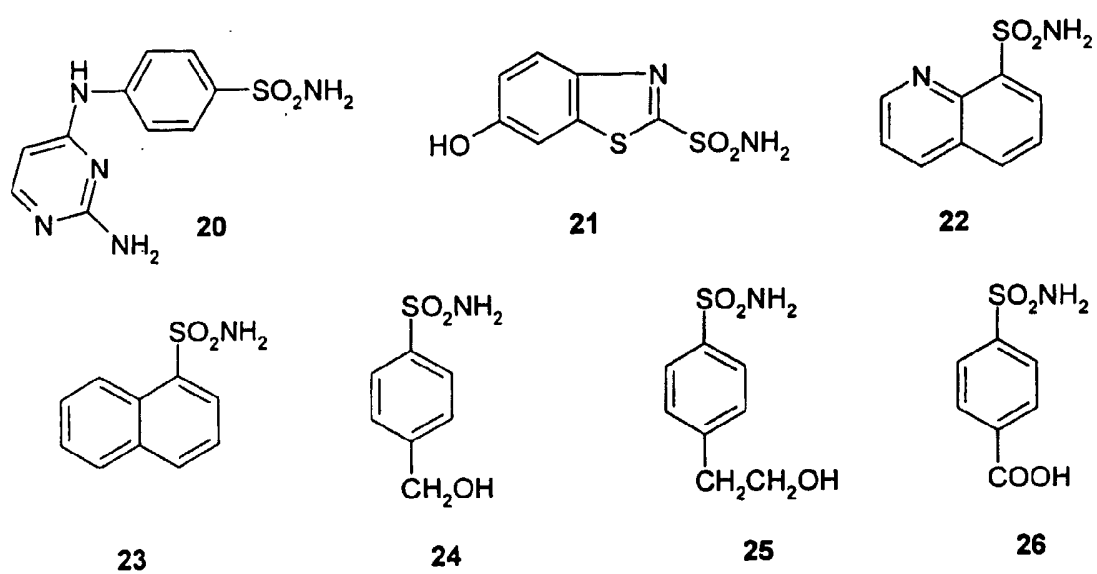


FIG._4B

Scheme 1

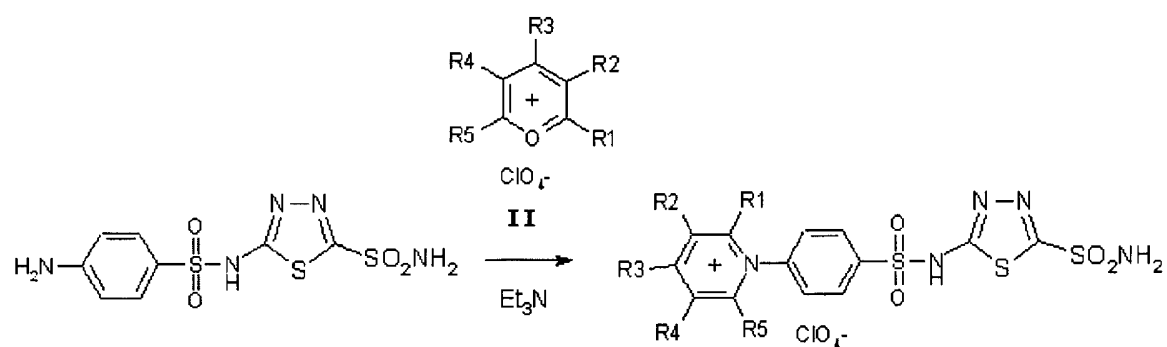


FIG._5

Scheme 2

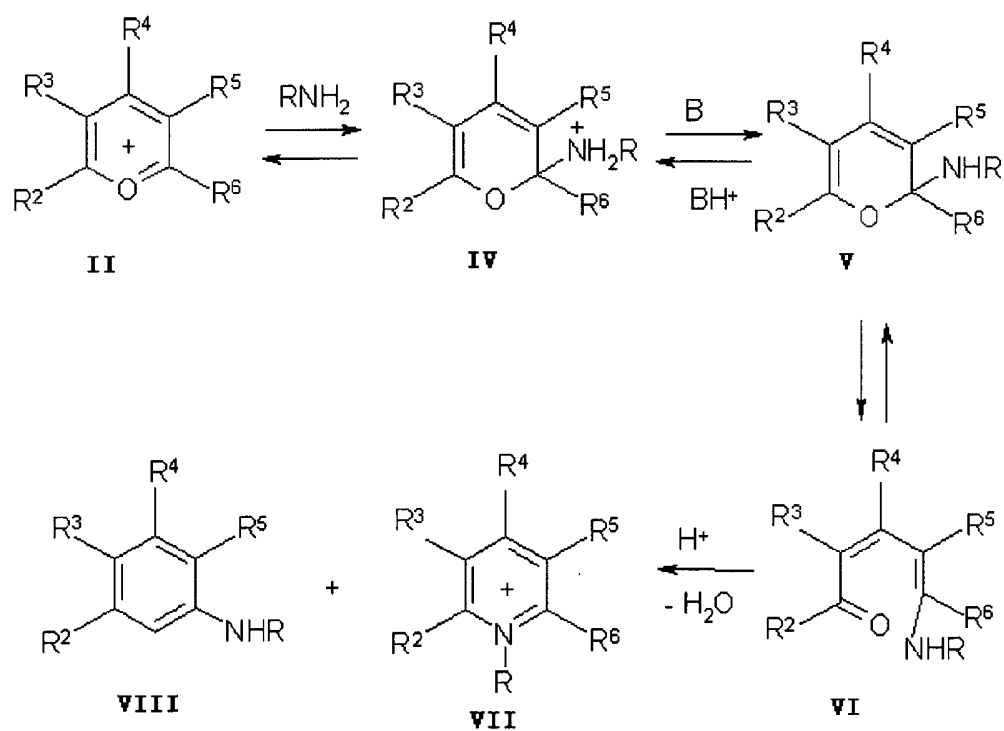
(For R⁶ or R² Me)

FIG._6

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 5387676 A, Zavada [0002] [0004]
- WO 9318152 A, Zavada [0002] [0096]
- WO 9534650 A, Zavada [0002] [0096] [0103]
- WO 0024913 A, Zavada [0002]
- US 5942434 A, Ratcliffe [0056]
- US 6265390 B [0056]
- WO 12179990 A [0159]
- US 60429089 B [0159]
- US 60489473 B [0159]
- US 60515140 B [0159]

Non-patent literature cited in the description

- **ABBATE et al.** *J. Med. Chem.*, 2002, vol. 45, 3583-3587 [0072]
- **ABBATE et al.** *J. Enz. Inhib. Med. Chem.*, 2003, vol. 18, 303-308 [0072]
- **ABBATE et al.** *Bioorg. Med. Chem. Lett.* In Press, 2003, vol. 13 [0072]
- **ABDINE et al.** *J. Assoc. Off. Anal. Chem.*, 1978, vol. 61, 695-701 [0072]
- **ALDRED et al.** *Biochemistry*, 1991, vol. 30, 569-575 [0072]
- Pyrylium Salts: Syntheses, Reactions and Physical Properties. **BALABAN et al.** *Advances in Heterocyclic Chemistry*. Academic Press, 1982, 8-360 [0072]
- **BARLOW et al.** *Nucl. Acids Res.*, 1987, vol. 15, 2386 [0072]
- **BARTOSOVA et al.** *J. Pathol.*, 2002, vol. 197, 314-321 [0072]
- **BAYER, A.** *Ber. Dtsch. Chem. Ges.*, 1910, vol. 43, 2337-2349 [0072]
- **BAYER ; PICCARD.** *Liebigs Ann. Chem.*, 1911, vol. 384, 208-223 [0072]
- **BEASLEY et al.** *Cancer Res.*, 2001, vol. 61, 5262-5267 [0072]
- **BEHRAMAN et al.** *Eur. J. Biochem.*, 1990, vol. 190, 351-357 [0072]
- **BORRAS et al.** *Bioorg. Med. Chem.*, 1999, vol. 7, 2397-2406 [0072]
- **BRIGANTI et al.** *Biochemistry*, 1997, vol. 36, 10384-10392 [0072]
- **BRIGANTI et al.** *Inorg. Chim. Acta*, 1998, 275-276, 295-300 [0072]
- **BROWN, J.M.** Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. *Molecular Medicine Today*, April 2000, vol. 6, 157-162 [0072]
- **CASINI et al.** *J. Med. Chem.*, 2000, vol. 43, 4884-4892 [0072]
- **CASINI et al.** *Curr. Cancer Drug Targets*, 2002, vol. 2, 55-75 [0072]
- **CASINI et al.** *Bioorg. Med. Chem. Lett.*, 2003, vol. 13, 841-845 [0072]
- **CASINI et al.** *Bioorg. Med. Chem. Lett.*, 2003, vol. 13, 2763-2769 [0072]
- The Roles of carbonic anhydrase isozymes in cancer. **CHEGWIDDEN et al.** *Gene Families: Studies of DNA, RNA, Enzymes and Proteins*. World Scientific, 2001, 157-169 [0072]
- **CHIA et al.** *J. Clin. Oncol.*, 2001, vol. 19, 3660-3668 [0072]
- **CHIRICA et al.** *Biochim. Biophys. Acta*, 2001, vol. 1544, 55-63 [0072]
- **CLARE ; SUPURAN.** *Eur. J. Med. Chem.*, 1997, vol. 32, 311-319 [0072]
- **CLARE ; SUPURAN.** *Eur. J. Med. Chem.*, 1999, vol. 34, 463-474 [0072]
- **CUTHBERT et al.** *J. Physiol.*, 2003, vol. 551, 79-92 [0072]
- **DINCULESCU ; BALABAN.** *Rev. Roum. Chem.*, 1980, vol. 25, 1505-1528 [0072]
- **DODGSON et al.** *The Carbonic Anhydrases*. Plenum Press, 1991, 398 [0072]
- **DOEGE et al.** *J. Biol. Chem.*, 1991, vol. 266, 894-902 [0072]
- **ELLEBY et al.** *Eur. J. Biochem.*, 2001, vol. 268, 1613-1619 [0072]
- **FERRARONI et al.** *Biochemistry*, 2002, vol. 41, 6237-6244 [0072]
- **FERRARONI et al.** *Inorg. Chim. Acta*, 2002, vol. 339, 135-144 [0072]
- **FLEMING et al.** *J. Clin. Invest.*, 1995, vol. 96, 2907-2913 [0072]
- **FRANCHI et al.** *J. Enz. Inhib. Med. Chem.*, 2003, vol. 18, 333-338 [0072]
- **GEERS ; GROS.** *Physiol. Rev.*, 2000, vol. 80, 681-715 [0072]
- **GIATROMANOLAKI et al.** *Cancer Res.*, 2001, vol. 61, 7992-7998 [0072]
- **GOMAA, Z.S.** *Biomed. Chromatogr.*, 1993, vol. 7, 134-135 [0072]
- **GRUNEBERG et al.** *Angew. Chem. Int. Ed.*, 2001, vol. 40, 389-393 [0072]

- **HAKANSSON et al.** *J. Mol. Biol.*, 1992, vol. 227, 1192-1204 [0072]
- **HEMING et al.** *J. Appl. Physiol.*, 1986, vol. 61, 1849-1856 [0072]
- Evolution and distribution of the carbonic anhydrase gene families. **HEWETT-EMMETT, D. et al.** *The Carbonic Anhydrases - New Horizons*. Birkhauser Verlag, 2000, 29-78 [0072]
- **HÖCKEL ; VAUPEL.** *J. Natl. Cancer Inst.*, 2001, vol. 93, 266-276 [0072]
- **ILIES et al.** *Bioorg. Med. Chem.*, 2000, vol. 8, 2145-2155 [0072]
- **ILIES et al.** *J. Med. Chem.*, 2003, vol. 46, 2187-2196 [0072]
- **KHALIFAH, R.G.** *J. Biol. Chem.*, 1971, vol. 246, 2561-2573 [0072]
- **KHALIFAH et al.** *Biochemistry*, 1977, vol. 16, 2241-2247 [0072]
- **KIM et al.** *J. Am. Chem. Soc.*, 2000, vol. 122, 12125-12134 [0072]
- **KIM et al.** *J. Am. Chem. Soc.*, 2001, vol. 123, 9620-9627 [0072]
- **KOUKOURAKIS et al.** *Clin. Cancer Res.*, 2001, vol. 7, 3399-3403 [0072]
- **KRUNGKRAI et al.** *Int. J. Parasitol.*, 2001, vol. 31, 661-668 [0072]
- **LIAO et al.** *Am. J. Pathol.*, 1994, vol. 145, 598-609 [0072]
- **LIAO et al.** *Cancer Res.*, 1997, vol. 57, 2827-2831 [0072]
- **LIESKOVSKA et al.** *Neoplasma*, 1999, vol. 46, 17-24 [0072]
- **LINDSKOG ; COLEMAN.** *Proc. Natl. Acad. Sci. (USA)*, 1964, vol. 70, 2505-2508 [0072]
- Structure-function relations in human carbonic anhydrase II as studied by site-directed mutagenesis. **LINDSKOG et al.** *Carbonic anhydrase - From biochemistry and genetics to physiology and clinical medicine*. VCH, 1991, 1-13 [0072] [0074]
- **LLOYD et al.** *Genes. Dev.*, 1987, vol. 1, 594-602 [0072]
- **LONCASTER et al.** *Cancer Res.*, 2001, vol. 61, 6394-6399 [0072]
- **MAREN, T.H.** *Physiol. Rev.*, 1967, vol. 47, 595-781 [0072]
- Benzolamide - a renal carbonic anhydrase inhibitor. **MAREN, T.H.** *Orphan Drugs*. M. Dekker, 1982, 89-115 [0072]
- **MAREN et al.** *Mol. Pharmacol.*, 1993, vol. 44, 901-906 [0072]
- **MAREN et al.** *J. Pharmacol. Exp. Ther.*, 1997, vol. 280, 98-104 [0072]
- Growth Factors. **MENDELSON et al.** *Cancer: Principles and Practice of Oncology*. Lippincott, 1993, 114-133 [0072]
- **MINCIONE et al.** *Eur. J. Pharm. Sci.*, 1999, vol. 9, 185-199 [0072]
- **MONTGOMERY et al.** *Nucl. Acids. Res.*, 1987, vol. 15, 4687 [0072]
- **MORI et al.** *Gastroenterol.*, 1993, vol. 105, 820-826 [0072]
- **OKUYAMA et al.** *PNAS (USA)*, 1992, vol. 89, 1315-1319 [0072]
- **OPAVSKY et al.** *Genomics*, 1996, vol. 33, 480-487 [0072]
- **OWA ; NAGASU.** *Exp. Opin. Ther. Patents*, 2000, vol. 10, 1725-1740 [0072]
- **OWA et al.** *J. Med. Chem.*, 1999, vol. 42, 3789-3799 [0072]
- **PARKKILA et al.** *Gut*, 1994, vol. 35, 646-650 [0072]
- **PARKKILLA et al.** *Histochem. J.*, 1995, vol. 27, 133-138 [0072]
- **PARKKILA et al.** *Hepatology*, 1996, vol. 24, 104 [0072]
- **PASTOREK et al.** *Oncogene*, 1994, vol. 9, 2788-2888 [0072]
- **PASTOREKOVA et al.** *Virology*, 1992, vol. 187, 620-626 [0072]
- **PASTOREKOVA et al.** *Gastroenterology*, 1997, vol. 112, 398-408 [0072]
- **PEARSON ; LIPMAN.** *PNAS (USA)*, 1988, vol. 85, 2444 [0072]
- **POCKER ; STONE.** *Biochemistry*, 1967, vol. 6, 668-678 [0072]
- **SCOZZAFAVA ; SUPURAN.** *Bioorg. Med. Chem. Lett.*, 2000, vol. 10, 117-1120 [0072]
- **SCOZZAFAVA et al.** *Curr. Med. Chem.*, 2003, vol. 10, 925-953 [0072]
- **SCOZZAFAVA et al.** *J. Med. Chem.*, 1999, vol. 42, 2641-2650 [0072]
- **SCOZZAFAVA et al.** *J. Med. Chem.*, 1999, vol. 42, 3690-3700 [0072]
- **SCOZZAFAVA et al.** *J. Med. Chem.*, 2000, vol. 43, 292-300 [0072]
- **SLY ; HU.** *Annu. Rev. Biochem.*, 1995, vol. 64, 375-401 [0072]
- **SMITH ; FERRY.** *FEMS Microbiol. Rev.*, 2000, vol. 24, 335-366 [0072]
- **STEINER et al.** *Eur. J. Biochem.*, 1975, vol. 59, 253-259 [0072]
- **STERLING et al.** *Am. J. Physiol.-Cell Physiol.*, 2002, vol. 283, C1522-C1529 [0072]
- **STUBBS et al.** *Mol. Med. Toddy*, 2000, vol. 6, 15-19 [0072]
- **SUPURAN, C.T.** *Opin. Investig. Drugs*, 2003, vol. 12, 283-287 [0072]
- **SUPURAN ; CLARE.** *Eur. J. Med. Chem.*, 1995, vol. 30, 687-696 [0072]
- **SUPURAN ; CLARE.** *Eur. J. Med. Chem.*, 1998, vol. 33, 489-500 [0072]
- **SUPURAN ; CLARE.** *Eur. J. Med. Chem.*, 1999, vol. 34, 41-50 [0072]
- **SUPURAN ; SCOZZAFAVA.** *Enzyme Inhib.*, 2000, vol. 15, 597-610 [0072]

- **SUPURAN ; SCOZZAFAVA.** *Eur. J. Med. Chem.*, 2000, vol. 35, 867-874 [0072]
- **SUPURAN ; SCOZZAFAVA.** *Exp. Opin. Ther. Patents*, 2000, vol. 10, 575-600 [0072]
- **SUPURAN ; SCOZZAFAVA.** *Curr. Med. Chem.-Imm., Endoc. Metab. Agents*, 2001, vol. 1, 61-97 [0072]
- **SUPURAN ; SCOZZAFAVA.** *Exp. Opin. Ther. Patents*, 2002, vol. 12, 217-242 [0072]
- **SUPURAN et al.** *Eur. J. Med. Chem.*, 1998, vol. 33, 577-594 [0072]
- **SUPURAN et al.** *Eur. J. Med. Chem.*, 1998, vol. 33, 739-752 [0072]
- **SUPURAN et al.** *J. Enz. Inhib.*, 2000, vol. 15, 381-401 [0072]
- **SUPURAN et al.** *J. Med. Chem.*, 2000, vol. 35, 309-321 [0072]
- **SUPURAN et al.** *Bioorg. Med. Chem.*, 2001, vol. 9, 703-714 [0072]
- **SUPURAN et al.** *Curr. Med. Chem.-Imm., Endoc. Metab. Agents*, 2001, vol. 1, 61-97 [0072]
- **SUPURAN et al.** *Med. Res. Rev.*, 2003, vol. 23, 146-189 [0072]
- **SVASTOVA et al.** *Experimental Cell Research*, 2003, vol. 290, 332-345 [0072]
- **SYMINGTON.** *J. Biol. Chem.*, 1992, vol. 267, 25744 [0072]
- **TASHIAN, R. E.** *Adv. in Genetics*, 1992, vol. 30, 321-356 [0072]
- **TEICHER et al.** *Anticancer Research*, 1993, vol. 13, 1549-1556 [0072]
- **TINKER et al.** *J. Pharmacol. Exp. Ther.*, 1981, vol. 218, 600-607 [0072]
- **TOMA ; BALABAN.** *Tetrahedron*, 1996, vol. 7, 27-34 [0072]
- **TRINH et al.** *Cancer Res.*, 1995, vol. 55, 4808-4812 [0072]
- **TURNER et al.** *Hum. Pathol.*, 1997, vol. 28, 740-744 [0072]
- **TURNER et al.** *Br. J. Cancer*, 2002, vol. 86, 1276-1282 [0072]
- **UEMURA et al.** *J. Urology*, 16 April 1997, vol. 157 (4), 377 [0072]
- **VERMYLEN et al.** *Eur. Respir. J.*, 1999, vol. 14, 806-811 [0072]
- **VULLO et al.** *Bioorg. Med. Chem. Lett.*, 2003, vol. 13, 1005-1009 [0072]
- **VULLO et al.** *J. Enz. Inhib. Med. Chem.*, 2003, vol. 18, 403-406 [0072]
- **WINGO et al.** *Biochem. Biophys. Res. Comm.*, 2001, vol. 288, 666-669 [0072]
- **WINUM et al.** *J. Med. Chem.*, 2003, vol. 46, 2197-2204 [0072]
- Design of carbonic anhydrase inhibitors and the relationship between the pharmacodynamics and pharmacokinetics of acetazolamide. **WISTRAND ; LINDQVIST et al.** *Carbonic Anhydrase - From Biochemistry and Genetics to Physiology and Clinical Medicine*. VCH, 1991, 352-378 [0072]
- **WORKMAN ; STAFFORD.** *Cancer and Metastasis Reviews*, 1993, vol. 12, 73-82 [0072]
- **WU et al.** *J. Membr. Biol.*, 1998, vol. 162, 31-38 [0072]
- **WYKOFF et al.** *Cancer Research*, 2000, vol. 60, 7075-7083 [0072]
- **WYKOFF et al.** *Am. J. Pathol.*, 2001, vol. 158, 1011-1019 [0072]
- **ZAVADA et al.** *Int. J. Cancer*, 1993, vol. 54, 268-274 [0072]

Szabadalmi igénypontok

1. Gyógyászati készítmény MN/CA IX túltermelésével kapcsolatos preneoplasztikus és/vagy neoplasztikus betegség páciensben való kezelésében történő alkalmazásra, amely eljárás magában foglalja:

(a) annak meghatározását, hogy a MN/CA IX túltermelődik-e a páciensből vett mintában egy kontroll minta szintje feletti szinten; és

(b) ha az (a) lépésbeli meghatározás arra utal, hogy az MN/CA IX túltermelődik a mintában, aktivált MN/CA IX specifikus inhibitorát tartalmazó készítmény beadását a páciensnek,

ahol az aktivált MN/CA IX inhibitora MN/CA IX-specifikus szulfonamid a következő aromás szulfonamidok és heterociklusos szulfonamidok által alkotott csoportból választott: 1, 6, 11 - 13, 16 - 19, 22 - 26, 28, 29, 39, 40, 55, 58, 59, 62 és 70 számú vegyület,

és ahol az MN/CA IX-specifikus szulfonamid az MN/CA IX specifikus és hatásos inhibitorának van meghatározva;

ahol az MN/CA IX-specifikus szulfonamid a következő lépéseket magában foglaló szkrinelő vizsgálati eljárással lett az MN/CA IX enzimatisz aktivitása hatásos inhibitorának meghatározva: a szulfonamid K_i gátlási állandójának meghatározása, ahol ha a K_i gátlási állandó kevesebb mint 50 nanomoláris, a MN/CA IX-specifikus szulfonamid az MN/CA IX enzimatisz aktivitás hatásos inhibitora; és

ahol a szulfonamid az MN/CA IX specifikus inhibitorának van meghatározva, ha a szulfonamid az MN/CA IX enzimatisz aktivitásának hatásosabb inhibitora, mint a CA I, CA II és CA IV által alkotott csoportból kiválasztott legalább egyik szénsav-anhidráz enzimatisz aktivitásáé;

és ahol az aktivált MN/CA IX specifikus inhibitorát tartalmazó készítmény beadása az MN/CA IX aktivitás gátlásával kezeli az MN/CA IX túltermelésével kapcsolatos preneoplasztikus és/vagy neoplasztikus betegséget a páciensben.

2. Az 1. igénypont szerinti gyógyászati készítmény alkalmazásra, ahol az MN/CA IX-specifikus szulfonamid egy aromás szulfonamid membrán-impermeábilis piridinium-származéka vagy egy heterociklusos szulfonamid membrán-impermeábilis piridinium-származéka; és ahol a MN/CA IX-specifikus szulfonamid membrán-impermeábilisnak van meghatározva, ha szemléltető membrán-permeabilitási vizsgálati eljárásban van tesztelve, amely a következő lépéseket foglalja magában: (a) az MN/CA IX-specifikus szulfonamid millimoláris oldatának inkubálása humán vörösvértestekkel 37° C-on legalább 30 percen keresztül; (b) a humán vörösvértestek mosását; (c) a humán vörösvértestekben lévő MN/CA IX-specifikus szulfonamid jelenlétének meghatározását; és (d) ha a humán vörösvértestekben lévő MN/CA IX-specifikus szulfonamid szintje 1 mikromoláris vagy kisebb, akkor az MN/CA IX-specifikus szulfonamid membrán-impermeábilisnak van meghatározva.

3. Az 1. igénypont szerinti gyógyászati készítmény alkalmazásra, ahol az MN/CA IX-specifikus szulfonamid a 6 és 39 számú vegyület által alkotott csoportból van kiválasztva, adott esetben

ahol az MN/CA IX-specifikus szulfonamid, azaz az aktivált MN/CA IX specifikus inhibitora olyan génhez van konjugálva, amely citotoxikus fehérjét expresszál.

4. Az 1. igénypont szerinti gyógyászati készítmény alkalmazásra, ahol az MN/CA IX-specifikus szulfonamid, azaz az aktivált MN/CA IX specifikus inhibitora olyan vektorhoz van konjugálva, amely citotoxikus fehérjét expresszáló gént tartalmaz, adott esetben

ahol a vektor továbbá tartalmaz MN/CA9 promótert vagy MN/CA9 promóter-fragmenst is, és/vagy ahol a vektor egy vagy több hipoxia-válaszelemet tartalmaz.

5. Az 1. igénypont szerinti gyógyászati készítmény alkalmazására, ahol az MN/CA IX-specifikus szulfonamid, azaz az aktivált MN/CA IX specifikus inhibitora olyan kemoterápiás szerek hatásosságának modulálásra van alkalmazva, amelyek felvétele vagy aktivitása pH-függő.

6. Az 1. igénypont szerinti gyógyászati készítmény alkalmazására, ahol a szulfonamid sejtmembrán-impermeábilis, a szulfonamid az MN/CA IX enzimaktivitás hatásosabb inhibitora, mint a CA IV enzimaktivitása; és ahol a MN/CA IX-specifikus szulfonamid membrán-impermeábilisnak van meghatározva, ha szemléltető membrán-permeabilitási vizsgálati eljárásban van tesztelve, amely a következő lépéseket foglalja magában: (a) az MN/CA IX-specifikus szulfonamid millimoláris oldatának inkubálása humán vörösvértestekkel 37° C-on legalább 30 percen keresztül; (b) a humán vörösvértestek mosását; (c) a humán vörösvértestekben lévő MN/CA IX-specifikus szulfonamid jelenlétének meghatározását; és (d) ha a humán vörösvértestekben lévő MN/CA IX-specifikus szulfonamid szintje 1 mikromoláros vagy kisebb, akkor az MN/CA IX-specifikus szulfonamid membrán-impermeábilisnak van meghatározva vagy

ahol a szulfonamid sejtmembrán-permeábilis, a szulfonamid az MN/CA IX enzimaktivitás hatásosabb inhibitora, mint a CA II enzimaktivitása; ahol a MN/CA IX-specifikus szulfonamid membrán-permeábilisnak van meghatározva, ha szemléltető membrán-permeabilitási vizsgálati eljárásban van tesztelve, amely a következő lépéseket foglalja magában: (a) az MN/CA IX-specifikus szulfonamid millimoláris oldatának inkubálása humán vörösvértestekkel 37° C-on legalább 30 percen keresztül; (b) a humán vörösvértestek mosását; (c) a humán vörösvértestekben lévő MN/CA IX-specifikus szulfonamid jelenlétének meghatározását; és (d) ha a humán vörösvértestekben lévő MN/CA IX-specifikus szulfonamid szintje 1 mikromoláros vagy nagyobb, akkor az MN/CA IX-specifikus szulfonamid membrán-permeábilisnak van meghatározva.

7. Az 1. igénypont szerinti gyógyászati készítmény alkalmazására, ahol a szulfonamid az MN/CA IX hatásosabb inhibitora, mint a CA I, CA II és CA IV által alkotott csoportból kiválasztott legalább két szénsav-anhidráz enzimaktivitása vagy

ahol a szulfonamid az MN/CA IX enzimaktivitás hatásosabb inhibitora, mint a CA I, CA II és CA IV által alkotott csoportból kiválasztott mindegyik szénsav-anhidráz enzimaktivitása.

8. Az 1. igénypont szerinti gyógyászati készítmény alkalmazására, ahol a betegség hipoxiás preneoplasztikus és/vagy neoplasztikus betegség.