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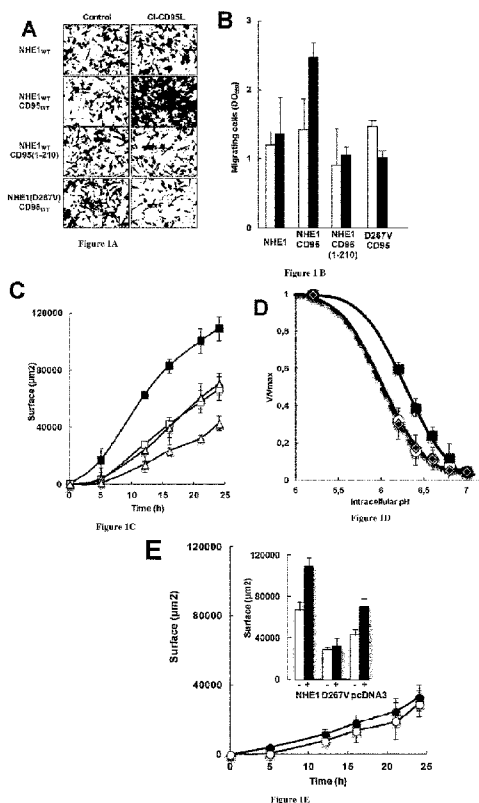
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(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR REDUCING CD95-MEDIATED CELL MOTILITY



(57) Abstract: The present invention relates to methods and pharmaceutical compositions for reducing CD95-mediated cell motility. The present invention relates to a method for reducing CD95-mediated cell motility in a subject comprising the steps consisting of i) determining the level of soluble CD95L in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of at least one compound selected from the group consisting of NHE1 inhibitors and ROCK inhibitors when the level determined at step i) is higher than the predetermined reference value.



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METHODS AND PHARMACEUTICAL COMPOSITIONS FOR REDUCING CD95-MEDIATED CELL MOTILITY

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for reducing CD95-mediated cell motility.

BACKGROUND OF THE INVENTION:

CD95 ligand (CD95L, also known as FasL) belongs to the TNF (Tumor Necrosis Factor) family and is the ligand for the “death receptor” CD95 (Fas/APO1). CD95L is a transmembrane “cytokine” whose extracellular domain can be cleaved by metalloproteases, to produce a soluble ligand. This soluble form was initially described as an inert ligand that competes with its membrane-bound counterpart for binding to CD95, thus acting as an antagonist of the death signal. More recent findings have shown that metalloprotease-cleaved-CD95L (cl-CD95L) can actively participate in aggravating inflammation in chronic inflammatory disorders, such as systemic lupus erythematosus and may exert pro-oncogenic functions by promoting the survival of ovarian and liver cancers and chemotherapy resistance of lung cancers. Binding of transmembrane CD95L to CD95 leads to the recruitment of the adaptor protein Fas-associated death domain protein (FADD) to the intracellular region of CD95 called the death domain (DD). In turn, FADD binds to caspases 8 and 10. This CD95/FADD/caspase complex is known as the Death-Inducing Signaling Complex (DISC) and plays a pivotal role in the initiation of the apoptotic signal. By contrast, cl-CD95L fails to induce DISC formation and instead promotes the formation of an atypical receptosome that we have designated Motility-Inducing Signaling Complex (MISC) (Tauzin S, Chaigne-Delalande B, Selva E, Khadra N, Daburon S, Contin-Bordes C, et al. The naturally processed CD95L elicits a c-yes/calcium/PI3K-driven cell migration pathway. PLoS Biol. 2011;9:e1001090.). Accordingly, compound that are able to reduce the reducing CD95-mediated cell motility is highly desirable.

The Na⁺/H⁺ exchanger NHE1 is a ubiquitous plasma membrane transporter, which regulates intracellular pH, cell volume and is involved in cell fate programs such as proliferation or apoptosis (for review see (Boedtkjer et al, 2012). This exchanger is cooperatively activated by intracellular H⁺ ions (Lacroix et al, 2004). A large set of stimuli such as hormonal and growth factor signals (Boedtkjer et al, 2012), or changes in membrane

shape and tension modulate NHE1 activity by modifying this cooperative response to protons (Lacroix et al, 2008). Pioneer studies have shown that NHE1 is also a crucial effector in cell spreading and migration (Denker & Barber, 2002; Denker et al, 2000). This transporter serves as a cytoskeletal anchoring platform and its activity maintains an asymmetrical gradient of protons along the axis of cell migration (Stock & Schwab, 2006). NHE1 is also known to promote cell migration by affecting different cellular processes such actin anchoring (Denker et al, 2000) and pH-mediated structural change of protein as cofilin (Frantz et al, 2008).

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for reducing CD95-mediated cell motility.

DETAILED DESCRIPTION OF THE INVENTION:

Transmembrane CD95L can be processed by metalloprotease to release a soluble ligand, cl-CD95L, contributing to chronic inflammation and cancer cell dissemination. The motility signaling pathway triggered by cl-CD95L remains poorly defined. Herein, the inventors demonstrate that cl-CD95L stimulates the Na^+/H^+ exchanger NHE1, whose activity is instrumental in CD95-mediated cell migration. Using pharmacological and genetic approaches, they observe that activation of Akt and RhoA signaling pathways allosterically activates NHE1 to implement the CD95-mediated cell migration. In summary, NHE1 represents a novel therapeutic target whose inhibition may antagonize the pro-inflammatory and pro-metastatic functions of cl-CD95L.

Thus one aspect of the invention relates to a method for reducing CD95-mediated cell motility in a subject comprising the steps consisting of i) determining the level of soluble CD95L in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of at least one compound selected from the group consisting of NHE1 inhibitors and ROCK inhibitors when the level determined at step i) is higher than the predetermined reference value.

In some embodiments, the method of the invention is particularly suitable for reducing CD95-mediated cancer cell motility. In some embodiments, the method of the present invention is particularly suitable for the treatment of cancer in a subject in need thereof.

As used herein, the term "cancer" has its general meaning in the art and includes, but is not limited to, solid tumors and blood borne tumors. The term cancer includes diseases of the skin, tissues, organs, bone, cartilage, blood and vessels. The term "cancer" further encompasses both primary and metastatic cancers. Examples of cancers that may be treated by methods and compositions of the invention include, but are not limited to, cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous; adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and robblastoma, malignant; Sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal

rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant;
 mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma,
 malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma;
 mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma
 5 ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma;
 hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant;
 lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma;
 chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone;
 ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma;
 10 ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma;
 glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary
 astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma;
 primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma;
 retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma;
 15 neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's
 disease; Hodgkin's lymphoma; paraganuloma; malignant lymphoma, small lymphocytic;
 malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides;
 other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast
 cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia;
 20 plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia;
 basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia;
 megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

In some embodiments, the subject suffers from a cancer selected from the group
 25 consisting of breast cancer, colon cancer, lung cancer, prostate cancer, testicular cancer, brain
 cancer, skin cancer, rectal cancer, gastric cancer, esophageal cancer, sarcomas, tracheal
 cancer, head and neck cancer, pancreatic cancer, liver cancer, ovarian cancer, lymphoid
 cancer, cervical cancer, vulvar cancer, melanoma, mesothelioma, renal cancer, bladder cancer,
 thyroid cancer, bone cancers, carcinomas, sarcomas, and soft tissue cancers.

30

In some embodiments, the subject suffers from a triple negative breast cancer. As used
 herein the expression "Triple negative breast cancer" has its general meaning in the art and
 means that said breast cancer lacks receptors for the hormones estrogen (ER-negative) and
 progesterone (PR-negative), and for the protein HER2.

In some embodiments, the method of the present invention is particularly suitable for reducing CD95-mediated lymphocyte (*e.g.*, T cell) motility. In some embodiments, the method of the present invention is particularly suitable for the treatment of an auto-immune disease.

As used herein, an "autoimmune disease" is a disease or disorder arising from and directed at an individual's own tissues. Examples of autoimmune diseases include, but are not limited to Addison's Disease, Allergy, Alopecia Areata, Alzheimer's disease, Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, Ankylosing Spondylitis, Antiphospholipid Syndrome (Hughes Syndrome), arthritis, Asthma, Atherosclerosis, Atherosclerotic plaque, autoimmune disease (*e.g.*, lupus, RA, MS, Graves' disease, etc.), Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Autoimmune inner ear disease, Autoimmune Lymphoproliferative syndrome, Autoimmune Myocarditis, Autoimmune Oophoritis, Autoimmune Orchitis, Azoospermia, Behcet's Disease, Berger's Disease, Bullous Pemphigoid, Cardiomyopathy, Cardiovascular disease, Celiac Sprue/Coeliac disease, Chronic Fatigue Immune Dysfunction Syndrome (CFIDS), Chronic idiopathic polyneuritis, Chronic Inflammatory Demyelinating, Polyradicalneuropathy (CIPD), Chronic relapsing polyneuropathy (Guillain-Barré syndrome), Churg-Strauss Syndrome (CSS), Cicatricial Pemphigoid, Cold Agglutinin Disease (CAD), chronic obstructive pulmonary disease (COPD), CREST syndrome, Crohn's disease, Dermatitis, Herpetiformis, Dermatomyositis, diabetes, Discoid Lupus, Eczema, Epidermolysis bullosa acquisita, Essential Mixed Cryoglobulinemia, Evan's Syndrome, Exophthalmos, Fibromyalgia, Goodpasture's Syndrome, Hashimoto's Thyroiditis, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia Purpura (ITP), IgA Nephropathy, immunoproliferative disease or disorder (*e.g.*, psoriasis), Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, Insulin Dependent Diabetes Mellitus (IDDM), Interstitial lung disease, juvenile diabetes, Juvenile Arthritis, juvenile idiopathic arthritis (JIA), Kawasaki's Disease, Lambert-Eaton Myasthenic Syndrome, Lichen Planus, lupus, Lupus Nephritis, Lymphocytic Lymphitis, Ménière's Disease, Miller Fish Syndrome/acute disseminated encephalomyeloradiculopathy, Mixed Connective Tissue Disease, Multiple Sclerosis (MS), muscular rheumatism, Myalgic encephalomyelitis (ME), Myasthenia Gravis, Ocular Inflammation, Pemphigus Foliaceus, Pemphigus Vulgaris, Pernicious Anaemia, Polyarteritis Nodosa, Polychondritis, Polyglandular Syndromes (Whitaker's syndrome), Polymyalgia Rheumatica, Polymyositis,

Primary Agammaglobulinemia, Primary Biliary Cirrhosis/Autoimmune cholangiopathy, Psoriasis, Psoriatic arthritis, Raynaud's Phenomenon, Reiter's Syndrome/Reactive arthritis, Restenosis, Rheumatic Fever, rheumatic disease, Rheumatoid Arthritis, Sarcoidosis, Schmidt's syndrome, Scleroderma, Sjörger's Syndrome, Stiff-Man Syndrome, Systemic
5 Lupus Erythematosus (SLE), systemic scleroderma, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Thyroiditis, Type 1 diabetes, Type 2 diabetes, Ulcerative colitis, Uveitis, Vasculitis, Vitiligo, and Wegener's Granulomatosis.

10 In some embodiments, the method of the present invention is particularly suitable for the treatment of systemic lupus erythematosus.

In some embodiments, method of the present invention is particularly suitable for the treatment of an inflammatory condition.

15 The term "inflammatory condition" as used herein refers to acute or chronic localized or systemic responses to harmful stimuli, such as pathogens, damaged cells, physical injury or irritants, that are mediated in part by the activity of cytokines, chemokines, or inflammatory cells (e.g., neutrophils, monocytes, lymphocytes, macrophages) and is characterized in most instances by pain, redness, swelling, and impairment of tissue function. The inflammatory
20 condition may be selected from the group consisting of: sepsis, septicemia, pneumonia, septic shock, systemic inflammatory response syndrome (SIRS), Acute Respiratory Distress Syndrome (ARDS), acute lung injury, aspiration pneumonitis, infection, pancreatitis, bacteremia, peritonitis, abdominal abscess, inflammation due to trauma, inflammation due to surgery, chronic inflammatory disease, ischemia, ischemia-reperfusion injury of an organ or
25 tissue, tissue damage due to disease, tissue damage due to chemotherapy or radiotherapy, and reactions to ingested, inhaled, infused, injected, or delivered substances, glomerulonephritis, bowel infection, opportunistic infections, and for subjects undergoing major surgery or dialysis, subjects who are immunocompromised, subjects on immunosuppressive agents, subjects with HIV/AIDS, subjects with suspected endocarditis, subjects with fever, subjects
30 with fever of unknown origin, subjects with cystic fibrosis, subjects with diabetes mellitus, subjects with chronic renal failure, subjects with bronchiectasis, subjects with chronic obstructive lung disease, chronic bronchitis, emphysema, or asthma, subjects with febrile neutropenia, subjects with meningitis, subjects with septic arthritis, subjects with urinary tract infection, subjects with necrotizing fasciitis, subjects with other suspected Group A

streptococcus infection, subjects who have had a splenectomy, subjects with recurrent or suspected enterococcus infection, other medical and surgical conditions associated with increased risk of infection, Gram positive sepsis, Gram negative sepsis, culture negative sepsis, fungal sepsis, meningococemia, post-pump syndrome, cardiac stun syndrome, stroke, congestive heart failure, hepatitis, epiglottitis, E. coli 0157:H7, malaria, gas gangrene, toxic shock syndrome, pre-eclampsia, eclampsia, HELP syndrome, mycobacterial tuberculosis, Pneumocystic carinii, pneumonia, Leishmaniasis, hemolytic uremic syndrome/thrombotic thrombocytopenic purpura, Dengue hemorrhagic fever, pelvic inflammatory disease, Legionella, Lyme disease, Influenza A, Epstein-Barr virus, encephalitis, inflammatory diseases and autoimmunity including Rheumatoid arthritis, osteoarthritis, progressive systemic sclerosis, systemic lupus erythematosus, inflammatory bowel disease, idiopathic pulmonary fibrosis, sarcoidosis, hypersensitivity pneumonitis, systemic vasculitis, Wegener's granulomatosis, transplants including heart, liver, lung kidney bone marrow, graft-versus-host disease, transplant rejection, sickle cell anemia, nephrotic syndrome, toxicity of agents such as OKT3, cytokine therapy, and cirrhosis.

In some embodiments, the sample is a blood sample. By "blood sample" is meant a volume of whole blood or fraction thereof, eg, serum, plasma, etc.

As used herein, the term "CD95" has its general meaning in the art and refers to CD95 to the receptor present on the surface of mammalian cells, which has been originally shown to have the capacity to induce apoptosis upon binding of the trimeric form of its cognate ligand, CD95L (Krammer, P.H. (2000). CD95's deadly mission in the immune system. Nature 407, 789-795). CD95 is also known as Fas or Apo-1. As used herein the term "CD95L" has its general meaning in the art and refers to the cognate ligand of CD95 that is a transmembrane protein. As used herein the term "soluble CD95L" has its general meaning in the art and refers to the soluble ligand produced by the cleavage of the transmembrane CD95L (also known as FasL) (Matsuno et al., 2001; Vargo-Gogola et al., 2002; Kiaei et al., 2007; Kirkin et al., 2007; or Schulte et al., 2007). The term "serum CD95L", "soluble CD95L", "metalloprotease-cleaved CD95L" and "cl-CD95L" have the same meaning along the specification.

A predetermined reference value can be relative to a number or value derived from population studies, including without limitation, subjects of the same or similar age range,

subjects in the same or similar ethnic group, and subjects having the same severity of the disease (e.g. cancer). Such predetermined reference values can be derived from statistical analyses and/or risk prediction data of populations obtained from mathematical algorithms and computed indices of the disease. In some embodiments, the predetermined reference values are derived from the level of soluble CD95L in a control sample derived from one or more subjects who were not subjected to the disease. Furthermore, retrospective measurement of the level of soluble CD95L in properly banked historical subject samples may be used in establishing these predetermined reference values.

In some embodiments, when the subject suffers from cancer, the predetermined reference value is correlated with the risk of relapse and distant metastasis, the duration of the disease-free survival (DFS) and/or the overall survival (OS). Accordingly, the predetermined reference value may be typically determined by carrying out a method comprising the steps of:

a) providing a collection of blood samples from subject suffering from the same cancer;

b) providing, for each blood sample provided at step a), information relating to the actual clinical outcome for the corresponding subject (i.e. risk of relapse and distant metastasis, the duration of the disease-free survival (DFS) and/or the overall survival (OS));

c) providing a serial of arbitrary quantification values;

d) determining the level of soluble CD95L for each blood sample contained in the collection provided at step a);

e) classifying said blood samples in two groups for one specific arbitrary quantification value provided at step c), respectively: (i) a first group comprising blood samples that exhibit a quantification value for level that is lower than the said arbitrary quantification value contained in the said serial of quantification values; (ii) a second group comprising blood samples that exhibit a quantification value for said level that is higher than the said arbitrary quantification value contained in the said serial of quantification values; whereby two groups of blood samples are obtained for the said specific quantification value, wherein the blood samples of each group are separately enumerated;

f) calculating the statistical significance between (i) the quantification value obtained at step e) and (ii) the actual clinical outcome of the patients from which blood samples contained in the first and second groups defined at step f) derive;

g) reiterating steps f) and g) until every arbitrary quantification value provided at step d) is tested;

h) setting the said predetermined reference value as consisting of the arbitrary quantification value for which the highest statistical significance (most significant) has been

5 calculated at step g).

For example the level of soluble CD95L has been assessed for 100 blood samples of 100 patients. The 100 samples are ranked according to the level of soluble CD95L. Sample 1 has the highest level and sample 100 has the lowest level. A first grouping provides two
10 subsets: on one side sample Nr 1 and on the other side the 99 other samples. The next grouping provides on one side samples 1 and 2 and on the other side the 98 remaining samples etc., until the last grouping: on one side samples 1 to 99 and on the other side sample Nr 100. According to the information relating to the actual clinical outcome for the corresponding cancer patient, Kaplan Meier curves are prepared for each of the 99 groups of
15 two subsets. Also for each of the 99 groups, the p value between both subsets was calculated. The predetermined reference value is then selected such as the discrimination based on the criterion of the minimum p value is the strongest. In other terms, the level of soluble CD95L corresponding to the boundary between both subsets for which the p value is minimum is considered as the predetermined reference value. It should be noted that the predetermined
20 reference value is not necessarily the median value of levels of soluble CD95L.

Thus in some embodiments, the predetermined reference value thus allows discrimination between an increased risk of relapse and distant metastasis and a decreased risk of relapse and distant metastasis (or a poor and a good prognosis with respect to DFS and
25 OS for a patient). Practically, high statistical significance values (e.g., low P values) are generally obtained for a range of successive arbitrary quantification values, and not only for a single arbitrary quantification value. Thus, in one alternative embodiment of the invention, instead of using a definite predetermined reference value, a range of values is provided. Therefore, a minimal statistical significance value (minimal threshold of significance, e.g.
30 maximal threshold P value) is arbitrarily set and a range of a plurality of arbitrary quantification values for which the statistical significance value calculated at step g) is higher (more significant, e.g. lower P value) are retained, so that a range of quantification values is provided. This range of quantification values includes a "cut-off" value as described above.

For example, according to this specific embodiment of a "cut-off" value, the outcome can be determined by comparing the level of soluble CD95L with the range of values which are identified. In certain embodiments, a cut-off value thus consists of a range of quantification values, e.g. centered on the quantification value for which the highest statistical significance value is found (e.g. generally the minimum p value which is found). For example, on a hypothetical scale of 1 to 10, if the ideal cut-off value (the value with the highest statistical significance) is 5, a suitable (exemplary) range may be from 4-6. Therefore, a patient may be assessed by comparing values obtained by measuring the level of soluble CD95L, where values greater than 5 reveal an increased risk of relapse and distant metastasis (or a poor prognosis) and values less than 5 reveal a decreased risk of relapse and distant metastasis (or a good prognosis). In some embodiments, a patient may be assessed by comparing values obtained by measuring the level of soluble CD95L and comparing the values on a scale, where values above the range of 4-6 indicate an increased risk of relapse and distant metastasis (or a poor prognosis) and values below the range of 4-6 indicate a decreased risk of relapse and distant metastasis (or a good prognosis), with values falling within the range of 4-6 indicating an intermediate occurrence (or prognosis).

Typically, when the subject suffers from triple negative breast cancer the predetermined reference value may be 80 pg/ml or 120 pg/ml for soluble CD95L (Malleter M, Tauzin S, Bessede A, Castellano R, Goubard A, Godey F, Levêque J, Jézéquel P, Campion L, Campone M, Ducret T, Macgrogan G, Debure L, Collette Y, Vacher P, Legembre P. CD95L cell surface cleavage triggers a prometastatic signaling pathway in triple-negative breast cancer. *Cancer Res.* 2013 Nov 15;73(22):6711-21.).

According to the invention, the measure of the level of soluble CD95L can be performed by a variety of techniques. Typically, the methods may comprise contacting the sample with a binding partner capable of selectively interacting with soluble CD95L in the sample. In some aspects, the binding partners are antibodies, such as, for example, monoclonal antibodies or even aptamers.

The aforementioned assays generally involve the binding of the partner (ie. antibody or aptamer) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e. g., in membrane or microtiter well form); polyvinylchloride (e. g., sheets or microtiter wells); polystyrene latex (e.g., beads or

microtiter plates); polyvinylidene fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

The level of soluble CD95L may be measured by using standard immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, agglutination tests; enzyme-labelled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation.

An exemplary biochemical test for identifying specific proteins employs a standardized test format, such as ELISA test, although the information provided herein may apply to the development of other biochemical or diagnostic tests and is not limited to the development of an ELISA test (see, e.g., Molecular Immunology: A Textbook, edited by Atassi et al. Marcel Dekker Inc., New York and Basel 1984, for a description of ELISA tests). It is understood that commercial assay enzyme-linked immunosorbant assay (ELISA) kits for various plasma constituents are available. Therefore ELISA method can be used, wherein the wells of a microtiter plate are coated with a set of antibodies which recognize soluble CD95L. A sample containing or suspected of containing soluble CD95L is then added to the coated wells. After a period of incubation sufficient to allow the formation of antibody-antigen complexes, the plate(s) can be washed to remove unbound moieties and a detectably labelled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample marker protein, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Measuring the level of soluble CD95L (with or without immunoassay-based methods) may also include separation of the compounds: centrifugation based on the compound's molecular weight; electrophoresis based on mass and charge; HPLC based on hydrophobicity; size exclusion chromatography based on size; and solid-phase affinity based on the compound's affinity for the particular solid-phase that is used. Once separated, said one or two biomarkers proteins may be identified based on the known "separation profile" e. g., retention time, for that compound and measured using standard techniques.

Alternatively, the separated compounds may be detected and measured by, for example, a mass spectrometer.

Typically, levels of soluble CD95L in a sample may be measured by an immunometric assay on the basis of a double-antibody "sandwich" technique, with a monoclonal antibody specific for soluble CD95L. According to said embodiment, said means for measuring the level of soluble CD95L are for example i) a buffer, ii) a monoclonal antibody that interacts specifically with soluble CD95L, iii) an enzyme-conjugated antibody specific for soluble CD95L and a predetermined reference value of said selected biomarker.

As used herein the term "RhoA kinase" or "ROCK" has its general meaning in the art. ROCK is a member of the serine-threonine protein kinase family. ROCK exists in two isoforms, ROCK1 and ROCK2 (T. Ishizaki et al, EMBO J., 1996, 15, 1885-1893). ROCK has been identified as an effector molecule of RhoA, a small GTP-binding protein (G protein) that plays a key role in multiple cellular signaling pathways.

As used herein, the term "ROCK inhibitor" refers to a natural or synthetic compound which inhibits ROCK1, and/or ROCK2 activity or expression. In some embodiments, the inhibitor is selective. The selective ROCK inhibiting compounds are not limited to a particular manner of selective ROCK inhibition. For example, in some embodiments, one or more of the selective ROCK inhibiting compounds selectively inhibit ROCK1 activity over ROCK2 activity. For example, in some embodiments, one or more of the selective ROCK inhibiting compounds selectively inhibit ROCK2 activity over ROCK1 activity. Moreover, in some embodiments, one or more of the selective ROCK inhibiting compounds selectively inhibit both ROCK1 activity and ROCK2 activity with similar capability.

ROCK inhibitors are well known in the art. For example, isoquinoline derivatives, especially fasudil, are typical ROCK inhibitors. Fasudil (hexahydro-1-(5-isoquinolylsulfonyl)-1H-1,4-diazepine), also named as HA-1077, is an isoquinoline sulfonamide derivative and the only clinically available ROCK inhibitor codeveloped by Asahi Kasei of Japan and Department of Pharmacology of Nagoya University. Hydroxyfasudil is an active metabolite of fasudil in vivo, which has higher affinity to ROCK than Fasudil. Another isoquinoline derivative, H-1152P, is optimized on the basis of fasudil. Through competitively binding to the ATP binding pocket, Y-27632, another type of ROCK inhibitor, inhibits both ROCK1 and ROCK2. Optimization of these compounds leads to a more potent ROCK inhibitor, Y-39983, which is benefit for the treatment of the glaucoma

(Kubo T, Yamaguchi A, Iwata N, The therapeutic effects of Rho-ROCK inhibitors on CNS disorders. *Ther Clin Risk Manag* 2008;4(3):605-15). SLx-2119, a ROCK2-specific inhibitor, has recently been developed (Boerma M, Fu Q, Wang J, Comparative gene expression profiling in three primary human cell lines after treatment with a novel inhibitor of Rho kinase or atorvastatin. *Blood Coagul Fibrinolysis* 2008;19(7):709-18). A series of fasudil analogs were synthesized and their selectivity and inhibitory activity against ROCK were evaluated (Sato N, Toyohira Y, Itoh H, Stimulation of norepinephrine transporter function by fasudil, a Rho kinase inhibitor, in cultured bovine adrenal medullary cells. *Naunyn Schmiedebergs Arch Pharmacol* 2012;385(9):921-31; Nakabayashi S, Nagaoka T, Tani T, Retinal arteriolar responses to acute severe elevation in systemic blood pressure in cats: role of endothelium-derived factors. *Exp Eye Res* 2012;103:63-70; Sun X, Minohara M, Kikuchi H, The selective Rho-kinase inhibitor Fasudil is protective and therapeutic in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2006;180(1-2):126-34; Yu JZ, Ding J, Ma CG, Therapeutic potential of experimental autoimmune encephalomyelitis by Fasudil, a Rho kinase inhibitor. *J Neurosci Res* 2010;88(8):1664-72; Hou SW, Liu CY, Li YH, Fasudil ameliorates disease progression in experimental autoimmune encephalomyelitis, acting possibly through antiinflammatory effect. *CNS Neurosci Ther* 2012;18(11):909-17; LoGrasso PV, Feng Y. Rho kinase (ROCK) inhibitors and their application to inflammatory disorders. *Curr Top Med Chem* 2009;9(8):704-23; Engel J Jr. A proposed diagnostic scheme for people with epileptic seizures and with epilepsy: report of the ILAE Task Force on Classification and Terminology. *Epilepsia* 2001;42(6):796-803; Fisher RS, van Emde Boas W, Blume W, Epileptic seizures and epilepsy: definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia* 2005;46(4):470-2. Inan S, Buyukafsar K. Antiepileptic effects of two Rho-kinase inhibitors, Y-27632 and fasudil, in mice. *Br J Pharmacol* 2008;155(1):44-51; Meihui Chen, Anmin Liu, Ying Ouyang, Yingjuan Huang, Xiaojuan Chao, Rongbiao Pi Fasudil and its analogs: a new powerful weapon in the long war against central nervous system disorders? *Expert Opinion on Investigational Drugs* Apr 2013, Vol. 22, No. 4, Pages 537-550.). Other examples of ROCK inhibitors include those described in the international patent publications WO98/06433, WO00/09162, WO00/78351, WO01/17562, WO02/076976, EP1256574, WO02/100833, WO03/082808, WO2004/009555, WO2004/024717, WO2004/108724, WO2005/003101, WO2005/035501, WO2005/035503, WO2005/035506, WO2005/058891, WO2005/074642, WO2005/074643, WO2005/Q80934, WO2005/082367, WO2005/082890, WO2005/097790, WO2005/100342, WO2005/103050, WO2005/105780, WO2005/108397, WO2006/044753,

WO2006/051311, WO2006/057270, WO2006/058120 , WO2006/072792WO2011107608A1, and WO2007026920A2.

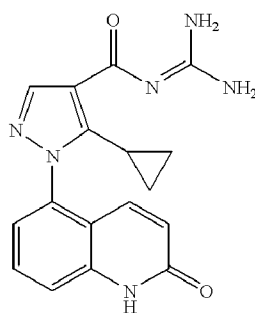
As used herein the term “NHE1” has its general meaning in the art and refers to the
5 Na⁺/H⁺ exchanger NHE1 is a ubiquitous plasma membrane transporter, which regulates intracellular pH, cell volume and is involved in cell fate programs such as proliferation or apoptosis (for review see(Boedtkjer et al, 2012).

As used herein, the term “NHE1 inhibitor” refers to a natural or synthetic compound
10 which inhibits NHE1 activity or expression. In some embodiments, the inhibitor is selective. The selective ROCK inhibiting compounds are not limited to a particular manner of selective NHE1 inhibition. For example, in some embodiments, one or more of the selective NHE1 inhibiting compounds selectively inhibit NHE1 activity over the other NHE (e.g., NHE3) activity. Moreover, in some embodiments, one or more of the NHE1 inhibitors inhibit NHE
15 activity with similar capability over the family.

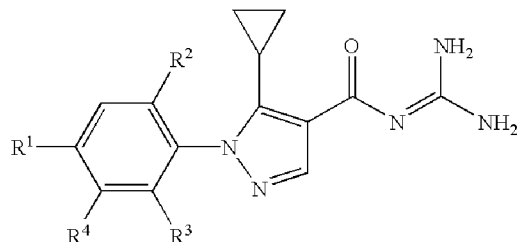
NHE1 inhibitors are well known in the art (see e.g. Pharmacological profile of SL
59.1227, a novel inhibitor of the sodium/hydrogen exchanger. Lorrain J, Briand V, Favennec
E, Duval N, Grosset A, Janiak P, Hoornaert C, Cremer G, Latham C, O'Connor SE. Br J
20 Pharmacol. 2000 Nov;131(6):1188-94; Clements-Jewery H, Sutherland FJ, Allen MC, Tracey
WR, Avkiran M. Cardioprotective efficacy of zoniporide, a potent and selective inhibitor of
Na⁺/H⁺ exchanger isoform 1, in an experimental model of cardiopulmonary bypass. Br J
Pharmacol. 2004 May;142(1):57-66. Epub 2004 Mar 22 and Masereel B, Pochet L,
Laeckmann D. An overview of inhibitors of Na(+)/H(+) exchanger. Eur J Med Chem. 2003
25 Jun;38(6):547-54. Review). Examples of NHE1 inhibitors include but are not limited to HOE-
694, cariporide, eniporide, BIIB-513, zoniporide, MS-31038, SM-20220, SM-20550, SMP-
300, KB-R9032, BMS-284640, T-162559, TY-12533, S-3226 or SL-591227. In some
embodiments, the NHE-1 inhibitor is cariporide, i.e. N-(aminoiminomethyl)-4-(1-
methylethyl)-3-(methylsulfonyl)-benzamide, that can be prepared as disclosed in U.S. Pat.
30 No. 5,591,754, the disclosure of which is incorporated herein by reference. The NHE-1
inhibitor eniporide, i.e. N-(aminoiminomethyl)-2-methyl-5-(methylsulfonyl)-4-(1H-pyrrol-1-
yl)-benzamide, may be prepared as disclosed in U.S. Pat. No. 5,753,680, the disclosure of
which is incorporated herein by reference. The NHE-1 inhibitor BIIB-513, i.e. N-
(aminoiminomethyl)-4-(4-(2-furanylcarbonyl)-1-piperazinyl)-3-(methylsulfonyl)-benzamide,

may be prepared as disclosed in U.S. Pat. No. 6,114,335, the disclosure of which is incorporated herein by reference. The NHE-1 inhibitor TY-12533, i.e. 6,7,8,9-tetrahydro-2-methyl-5H-cyclohepta[b]pyridine-3-carbonylguanidine, may be prepared as disclosed in PCT International Application Publication No. WO 98/39300, the disclosure of which is incorporated herein by reference. The NHE-1 inhibitor SM-15681, i.e. N-(aminoiminomethyl)-1-methyl-1H-indole-2-carboxamide, may be prepared as disclosed in EP0708091, the disclosure of which is incorporated herein by reference. Other examples typically include those described in WO 99/43663A1, WO 2001083470 and WO 2001030759.

In some embodiments, the NHE1 inhibitor has the formula of:



In some embodiments, the NHE1 inhibitor has the general formula of:



wherein R^1 is methylsulfonyl or hydrogen, R^2 is hydrogen or a halogen, R^3 is hydrogen, R^4 is hydrogen or a halogen, or R^3 and R^4 form, together with the carbon atoms to which they are attached, a six member fully unsaturated ring having one hetero atom that is nitrogen.

In some embodiments, the NHE1 inhibitor is selected from the group consisting of:

- [1-(2-chlorophenyl)-5-methyl-1H-pyrazole-4-carbonyl]guanidine;
- [5-methyl-1-(2-trifluoromethylphenyl)-1H-pyrazole-4-carbonyl]guanidine;
- [5-ethyl-1-phenyl-1H-pyrazole-4-carbonyl]guanidine;
- [5-cyclopropyl-1-(2-trifluoromethylphenyl)-1H-pyrazole-4-carbonyl]guanidine;
- [5-cyclopropyl-1-phenyl-1H-pyrazole-4-carbonyl]guanidine;

- [5-cyclopropyl-1-(2,6-dichlorophenyl)-1H-pyrazole-4-carbonyl]guanidine;
[5-methyl-1-(quinolin-6-yl)-1H-pyrazole-4-carbonyl]guanidine;
[5-methyl-1-(naphthalen-1-yl)-1H-pyrazole-4-carbonyl]guanidine;
[5-cyclopropyl-1-(quinolin-5-yl)-1H-pyrazole-4-carbonyl]guanidine;
5 [5-cyclopropyl-1-(quinolin-8-yl)-1H-pyrazole-4-carbonyl]guanidine;
[3-methyl-1-phenyl-1H-pyrazole-4-carbonyl]guanidine;
[3-methyl-1-(naphthalen-1-yl)-1H-pyrazole-4-carbonyl]guanidine;
[3-methyl-1-(isoquinolin-5-yl)-1H-pyrazole-4-carbonyl]guanidine;
[2-methyl-5-phenyl-2H-pyrazole-3-carbonyl]guanidine;
10 [2-methyl-5-(naphthalen-1-yl)-2H-pyrazole-3-carbonyl]guanidine;
[5-methyl-2-phenyl-2H-1,2,3-triazole-4-carbonyl]guanidine;
[5-methyl-2-(3-methoxyphenyl)-2H-1,2,3-triazole-4-carbonyl]guanidine;
[2-(3-bromophenyl)-5-methyl-2H-1,2,3-triazole-4-carbonyl]guanidine;
[2-(naphthalen-1-yl)-5-methyl-2H-1,2,3-triazole-4-carbonyl]guanidine;
15 [2-(isoquinolin-5-yl)-5-methyl-2H-1,2,3-triazole-4-carbonyl]guanidine;
[5-methyl-2-(quinolin-5-yl)-2H-1,2,3-triazole-4-carbonyl]guanidine;
[1-(naphthalen-1-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
[1-(2-chloro-4-methylsulfonylphenyl)-5-cyclopropyl-1H-pyrazole-4-
carbonyl]guanidine;
20 [1-(2-chlorophenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
[1-(2-trifluoromethyl-4-fluorophenyl)-5-cyclopropyl-1H-pyrazole-4-
carbonyl]guanidine;
[1-(2-bromophenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
[1-(2-fluorophenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
25 [1-(2-chloro-5-methoxyphenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
[1-(2-chloro-4-methylaminosulfonylphenyl)-5-cyclopropyl-1H-pyrazole-4-
carbonyl]guanidine;
[1-(2,5-dichlorophenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
[1-(2,3-dichlorophenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
30 [1-(2-chloro-5-aminocarbonylphenyl)-5-cyclopropyl-1H-pyrazole-4-
carbonyl]guanidine;
[1-(2-chloro-5-aminosulfonylphenyl)-5-cyclopropyl-1H-pyrazole-4-
carbonyl]guanidine;

[1-(2-fluoro-6-trifluoromethylphenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(2-chloro-5-methylsulfonylphenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

5 [1-(2-chloro-5-dimethylaminosulfonylphenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(2-trifluoromethyl-4-chlorophenyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

10 [1-(8-bromoquinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;
[1-(6-chloroquinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(indazol-7-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(benzimidazol-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(1-isoquinolyl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine;

[5-cyclopropyl-1-(4-quinoliny)-1H-pyrazole-4-carbonyl]guanidine;

15 [1-(indazol-6-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(indazol-5-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(benzimidazol-5-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(1-methylbenzimidazol-6-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine

[1-(5-quinoliny)-5-n-propyl-1H-pyrazole-4-carbonyl]guanidine;

20 [1-(5-quinoliny)-5-isopropyl-1H-pyrazole-4-carbonyl]guanidine;

[5-ethyl-1-(6-quinoliny)-1H-pyrazole-4-carbonyl]guanidine;

[1-(2-methylbenzimidazol-5-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(1,4-benzodioxan-6-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(benzotriazol-5-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine;

25 [1-(3-chloroindazol-5-yl)-5-ethyl-1H-pyrazole-4-carbonyl]guanidine;

[1-(5-quinoliny)-5-butyl-1H-pyrazole-4-carbonyl]guanidine;

[5-propyl-1-(6-quinoliny)-1H-pyrazole-4-carbonyl]guanidine;

[5-isopropyl-1-(6-quinoliny)-1H-pyrazole-4-carbonyl]guanidine;

[1-(indazol-7-yl)-3-methyl-1H-pyrazole-4-carbonyl]guanidine;

30 [1-(2,1,3-benzothiadiazol-4-yl)-3-methyl-1H-pyrazole-4-carbonyl]guanidine; and

[3-methyl-1-(quinolin-5-yl)-1H-pyrazole-4-carbonyl]guanidine

In some embodiments, the NHE1 inhibitor Sabiporide (Touret et al. Characterization of sabiporide, a new specific NHE-1 inhibitor exhibiting slow dissociation kinetics and

cardioprotective effects. Eur. J. Pharmacol. 2003.;Wu et al. 2013 Plos One : Sabiporide Improves Cardiovascular Function, Decreases the Inflammatory Response and Reduces Mortality in Acute Metabolic Acidosis in Pigs.).

5 In one embodiment the ROCK or NHE1 inhibitor is an inhibitor of ROCK or NHE1 expression. An “inhibitor of expression” refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. Typically the inhibitor of gene expression is a siRNA, an antisense oligonucleotide or a ribozyme.

10 Inhibitors of gene expression for use in the present invention may be based on antisense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of the targeted mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of the targeted protein (e.g. ROCK), and thus
15 activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding the target protein can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in
20 the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

 Small inhibitory RNAs (siRNAs) can also function as inhibitors of gene expression for use in the present invention. Gene expression can be reduced by contacting the tumor, subject
25 or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that gene expression is specifically inhibited (i.e., RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT.
30 et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

 Ribozymes can also function as inhibitors of gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific

cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of the targeted mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Both antisense oligonucleotides and ribozymes useful as inhibitors of gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide siRNA or ribozyme nucleic acid to the cells. Typically, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the the antisense oligonucleotide siRNA or

ribozyme nucleic acid sequences. Viral vectors are a typical type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma
5 viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Typical viral vectors are based on non-cytopathic eukaryotic viruses in which non-
10 essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (*i.e.*, capable of directing synthesis of the desired
15 proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging
20 cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Clifton, N.J., 1991).

Typical viruses for certain applications are the adeno-viruses and adeno-associated
25 viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse
30 lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in

tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

5 Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly
10 advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be
15 custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface
20 using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

As used herein, the phrase "therapeutically-effective amount" as used herein means
25 that amount of ROCK inhibitor or NHE1 inhibitor that is effective for producing some desired therapeutic effect by CD95-mediated cell motility. It will be understood, however, that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of
30 factors including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors

well known in the medical arts. For example, it is well within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may varied over a wide range from 0.01 to 1,000 mg per adult per day.

5 Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, typically from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to
10 about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

In some embodiments, the compound of the invention (i.e. ROCK inhibitor or NHE1 inhibitor) may be administered sequentially or concomitantly with one or more therapeutic
15 active agent such as chemotherapeutic or radiotherapeutic agents. Examples of chemotherapeutics include but are not limited to fludarabine, gemcitabine, capecitabine, methotrexate, mercaptopurine, thioguanine, hydroxyurea, cytarabine, cyclophosphamide, ifosfamide, nitrosoureas, platinum complexes such as cisplatin, carboplatin and oxaliplatin, mitomycin, dacarbazine, procarbazine, epipodophyllotoxins such as etoposide and teniposide,
20 camptothecins such as irinotecan and topotecan, bleomycin, doxorubicin, idarubicin, daunorubicin, dactinomycin, plicamycin, mitoxantrone, L-asparaginase, doxorubicin, epirubicin, 5-fluorouracil and 5-fluorouracil combined with leucovorin, taxanes such as docetaxel and paclitaxel, levamisole, estramustine, nitrogen mustards, nitrosoureas such as carmustine and lomustine, vinca alkaloids such as vinblastine, vincristine, vindesine and
25 vinorelbine, imatinib mesylate, hexamethylmelamine, kinase inhibitors, phosphatase inhibitors, ATPase inhibitors, tyrphostins, protease inhibitors, inhibitors herbimycin A, genistein, erbstatin, and lavendustin A. In some embodiments, additional therapeutic active agents may be selected from, but are not limited to, one or a combination of the following class of agents: alkylating agents, plant alkaloids, DNA topoisomerase inhibitors, anti-folates,
30 pyrimidine analogs, purine analogs, DNA antimetabolites, taxanes, podophyllotoxins, hormonal therapies, retinoids, photosensitizers or photodynamic therapies, angiogenesis inhibitors, antimitotic agents, isoprenylation inhibitors, cell cycle inhibitors, actinomycin, bleomycin, anthracyclines, MDR inhibitors and Ca^{2+} ATPase inhibitors. The term “radiotherapeutic agent” as used herein, is intended to refer to any radiotherapeutic agent

known to one of skill in the art to be effective to treat or ameliorate cancer, without limitation. For instance, the radiotherapeutic agent can be an agent such as those administered in brachytherapy or radionuclide therapy. Such methods can optionally further comprise the administration of one or more additional cancer therapies, such as, but not limited to, chemotherapies, and/or another radiotherapy.

According to the invention, the compound of the present invention (i.e. ROCK inhibitor or NHE1 inhibitor) is administered to the subject in the form of a pharmaceutical composition. Typically, the compound of the invention (i.e. ROCK inhibitor or NHE1 inhibitor) may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions. "Pharmaceutically" or "pharmaceutically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

Typically, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or

dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The compound of the invention (i.e. ROCK inhibitor or NHE1 inhibitor) can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin. Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the typical methods of preparation

are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: cl-CD95L increases cell migration by activation of NHE1. A- Indicated cells were incubated in Boyden chambers for 16 h in the presence or absence of 100 ng/ml of cl-CD95L. Migrating cells present on the lower part of the membrane were fixed with methanol and stained by Giemsa. For each experiment, five pictures of random fields were taken and a representative picture is shown (Bars = 70 nm). **B-** Giemsa-stained migrating cells were lysed, and absorbance was measured at 560 nm. Values represent means and SD of three independently performed experiments. **C-** Cell migration of PS120-CD95 cells (pcDNA3, ▲) or stably expressing wild type NHE1 () was monitored using wound healing assay in the presence (filled symbols) or absence (empty symbols) of cl-CD95L (100 ng/ml). Quantification was performed using ImageJ (see Materials and Methods). For each condition,

images were acquired at five different positions along each scratch. The graphs are representative of 3 independent experiments. **D-** Cells were treated for 10 minutes with 100 ng/ml of cl-CD95L or control medium and acidified at different intracellular pH values as described in Materials and Methods. Initial rates of NHE1 activity were measured using Li⁺ uptake. -Grey Lines : Ig-CD95L, -dotted lines: untreated; -lines: cl-CD95 ligand. Data are representative of at least five independent experiments. Error bars are SEM. **E-** Migration of PS120-CD95 cells stably expressing the transporter dead NHE1 mutant (D267V) was measured by wound healing and quantified as in C. Inset: Histogram bars correspond to the surface covered after 24 hours. Error bars are SEM.

Figure 2: CD95-driven Akt stimulation activates NHE1. **A-** After treatment with 100 ng/ml of cl-CD95L, Cells were lysed and the levels of phosphorylated Akt (Ser 473) were analyzed by immunoblotting. Akt serves as loading control. **B-** PS120-CD95-NHE1 cells were pre-treated with non-toxic dose of Triciribine (20 nM) or DMSO for 1 hour and then incubated in presence or absence of cl-CD95L (100 ng/ml). Cell migration was then assayed using wound-healing assay. **C-** The dose response of the AKTA mutant (full lines, ▲) to intracellular protons was measured in the presence (filled symbols) or absence of cl-CD95L (100 ng/ml) (empty symbols) as described in Fig. 1D and compared to that the wild type of NHE1 response. (dotted lines,). **D-** Cell migration of PS120-CD95 cells stably expressing AKTA or AKTD NHE1 mutants was assessed by wound healing assays. Histogram bars correspond to the surface covered after 24 hours. Error bars are SEMs. **E-** The dose response of the AKTD mutant (full lines,) to intracellular protons was measured in the presence (filled symbols) or absence of 100 ng/ml cl-CD95 (empty symbols) as described in Fig. 1D and compared to that of NHE1 (dotted lines,).

Figure 3: CD95-driven RhoA stimulation and activates NHE1. **A-** Jurkat T-cells and PS120-CD95-NHE1 cells were loaded with 1 μM Fura-2AM for 30 minutes at room temperature. Cells were transferred at 37°C with 2 mM extracellular calcium and then treated with 100 ng/ml of cl-CD95L (black arrow). Values were recorded every 6 s. **B-** After treatment with 100 ng/ml of cl-CD95L for the indicated times, cells were lysed and active RhoA was pulled down using GST-agarose Rhotekin Rho-binding domain. Active and total RhoA (used as loading control) were then loaded on gel and revealed by Western Blotting. Data are representative of three independently performed experiments. **C-** Cells were pre-treated in presence or absence of a non cytotoxic dose of ROCK1 inhibitor Y27632 (20μM)

for 1 hour. Then cell migration of PS120-CD95 cells expressing NHE1 or the AKTD mutant was assessed by wound healing in presence or absence of cl-CD95L (100ng/ml). Histogram bars correspond to the surface covered after 24 hours. Error bars are SEMs. **D-** The dose response of the ROCKA mutant (full lines) to intracellular protons was measured in the presence (filled symbols) or absence of 100 ng/ml cl-CD95 (empty symbols) and compared to wild type NHE1 response (dotted lines).

Figure 4: AKT and RhoA pathways cooperate to activate NHE1 and stimulate migration in presence of cl-CD95L. **A-** Cell migration of PS120-CD95 cells stably expressing the ROCKA NHE1 mutants was assessed by wound healing assay. Histogram bars correspond to the surface covered after 24 hours. Error bars are SEMs. **B-** Cell migration was assessed by wound healing assay. PS120-CD95 stably expressing the AKTD mutant were pre-treated with or without a non-cytotoxic dose of the ROCK1 inhibitor Y27632 (20 μ M) for 1 hour and then cells were incubated for 24 hours in presence or absence of cl-CD95L (100 ng/mL). Histogram bars correspond to the surface covered after 24 hours. Error bars are SEMs. **C.** Cell migration of PS120-CD95 cells stably expressing the AKTD-ROCKA NHE1 double mutants was measured by wound healing assay. Histogram bars correspond to the surface covered after 24 hours. Error bars are SEMs. **D-** Summary of NHE1 activation by CD95 engagement.

EXAMPLE:

Material & Methods

Antibodies and reagents

Anti-human CD95 mAb (DX2) was from BD Biosciences, Anti-Akt, anti-phosphoS473 Akt (Akt-^{P473}) were from Cell Signaling Technology. Anti-human NHE1 was from Millipore. The metalloprotease-cleaved CD95L (CD95L) and its multi-aggregated counterpart (Ig-CD95L) were generated in our laboratory, as described previously (Tauzin et al, 2011).

Cell Culture and Transfection

PS120 fibroblasts (Pouyssegur et al, 1984) were grown in DMEM with 50 µg/mL streptomycin, 50 unit/mL penicillin, and 8% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO₂. Transfections were performed using Lipofectamin 2000. Cell populations stably expressing wild-type or mutant NHE1 constructs were selected using 10µg/ml puromycin for 3 weeks.

Plasmids construction

CD95 mutant and WT cDNAs have been inserted in the pcDNA3 vector (Edmond et al, 2012). All NHE1 WT and mutant cDNAs have been inserted in a pECE-NHE1-Ires-Puro polycistronic vector derived from the pECE-NHE1-IresNeo described in (Lacroix et al, 2004).

Site-directed mutagenesis

Site-directed mutagenesis was performed using Quickchange Site-Directed Mutagenesis kit (Stratagene). Each mutant cDNA was sequenced before and after stable transfection. Mutations in AKT and ROCK phosphorylation sites, were generated using the primers (S648A), (S648D), (T653A), and (T653E).

Immunoblot analysis and Pull down assay.

Unless stated, proteins prepared from PS120 cells transfected with WT and mutant NHE-1 or CD95 were run in 7.5% acrylamide mini gels (Biorad). Immunoblots were revealed using commercial monoclonal antibodies against NHE1, AKT, phospho-AKT and RhoA.

In vitro motility assays

10⁵ cells were added to the top chamber of Boyden chambers (Millipore, Molsheim, France) containing 8 µm pore membranes. The bottom chamber was filled with medium containing 1% serum in the presence or absence of cl-CD95L (100 ng/ml). After 16 hours, cells were fixed with methanol and stained with Giemsa, for visualization. To quantify invading cells were then lysed and absorbance was measured at 560 nm as described previously (Malleter et al, 2013).

Wound healing assays

Confluent cells were incubated in 0.5% FCS for 3 hours and straight scratches were executed in the monolayer. Then cells were incubated in presence or absence of cl-CD95L (100 ng/ml) and images were acquired for 24 hours using phase contrast microscopy (10x

objective Axio Observer D1, Zeiss). Distance between the borders of the wound was quantified using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2012). For each condition, images were acquired at five different positions along the scratch. The graphs are representative of 3 independent experiments.

Calcium Video Imaging

Cells were loaded with 1 μ M Fura2-AM for 30 min in HBSS. After washing ratiometric imaging of calcium was performed at 37°C on a motorized microscope (lens x40, AxioObserver Z1, Zeiss) equipped with an EMCCD camera (Evolve, Photometrics) at 340 and 380 nm. The fluorescence ratios ($F_{\text{ratio}} 340/380$) were quantified using MetaFluor and Metamorph. Each experiment was repeated 3 times with 60 single-cell traces for each experimental condition.

RhoA activation

Cells were incubated for 16 hours in 0.5% FCS followed by 3 hours in 0% FCS. Then, cells were stimulated by 100 ng/ml cl-CD95 for the indicated durations. Cells were then lysed and active RhoA was precipitated using the RhoA pull down Rhotekin assay (Thermo Scientific) and revealed by Western Blotting according to the manufacturers instructions. Quantification was performed using Image J.

Data Treatment

Data were compiled using Microsoft Excel software. Curves are compiled from at least five independent experiments, in which experimental points are at least duplicates. Data are presented as mean values and Standard Error of the mean (error bars).

Results

CI-CD95L implements a NHE1-driven cell migration.

The fibroblast cell line PS120 lacking CD95 and NHE1 was reconstituted with both wild type or a DD-truncated CD95 (CD95⁽¹⁻²¹⁰⁾) and WT-NHE1. To mimic the cytotoxic activity of transmembrane CD95L, we have generated a multi-aggregated CD95L by fusing its extracellular region to a dimerization Ig domain derived from the LIF receptor gp190

(Daburon et al, 2013). Unlike cl-CD95L, which is homotrimeric, Ig-CD95L showed a dodecameric stoichiometry and triggered cell death (Tauzin et al, 2011). While CD95⁽¹⁻²¹⁰⁾ expression in PS120-NHE1 cells failed to transmit cell death, expression of wild type CD95 restored the apoptotic signal in response to Ig-CD95L, indicating that in these cells, the human CD95 could couple to the apoptotic machinery. Next, CD95-mediated cell migration was evaluated using Boyden chamber and wound healing assays. While cl-CD95L failed to induce migration in PS120-NHE1 cells, reconstitution of these cells with wild type CD95 was sufficient to trigger this process (Fig.1A-C). Deletion of the DD totally abrogated the CD95-mediated cell motility indicating that this domain was required to implement this non-conventional signaling pathway. Of note, PS120-NHE1-CD95 cells exposed to cl-CD95L showed an increased NHE1 cooperative response to intracellular proton, whereas this response remained unaffected in cells exposed to its multi-aggregated counterpart (Fig.1D). To confirm that NHE1 participated in the CD95-dependent cell motility, we next investigated if PS120 cell line expressing a “transport dead” NHE1 mutant (D267V) underwent cell migration in presence of cl-CD95L. This substitution resulted in a NHE1 molecule that was matured in a similar way to WT-NHE1 but was unable to mediate Na⁺/H⁺ exchange. The expression of this mutant blocked the CD95-mediated cell motility in Boyden chamber (Fig.1A-B) and wound healing (Fig.1C) assays demonstrating that cl-CD95L relies on NHE1 activity to induce cell migration pathway.

CD95-dependent activation of NHE1 occurs through an Akt-mediated mechanism

These results prompted us to ascertain the molecular mechanism linking CD95 engagement to Na⁺/H⁺ exchange activation. In presence of cl-CD95L, PS120-NHE1-CD95 cells triggered Akt activation (Fig.2A), while fibroblasts devoid of CD95 or expressing a DD-deficient CD95 did not induce phosphorylation of Akt at serine 473, a hallmark of its complete activation (Fig.2A). In addition, triciribine, a selective and reversible Akt inhibitor (Yang et al, 2004) prevented cell motility in PS120-NHE1-CD95 cells exposed to cl-CD95L (Fig.2B). As Akt is reported to activate NHE1 by phosphorylating its serine 648 (Meima et al, 2009; Snabaitis et al, 2008), we generated a S648A NHE1 mutant that we designated AKTA mutant. AKTA was functional and could still be activated by 20% FCS, but was unable to respond to cl-CD95 (Fig.2C). Consistently, AKTA did not enhance CD95-mediated cell migration when expressed in PS120-CD95 cells (Fig.2D). We next constructed a S648D mutant mimicking the phosphorylation of this crucial site (AKTD). This mutant was

functional, showed a cooperative response to intracellular protons that was increased in presence of serum. In contrast to AKTA, AKTD was still capable of being stimulated by the naturally-processed CD95L (Fig.2E), resulting in an enhanced cell migration when cells were challenged with cl-CD95L (Fig. 2D). Because mimicking the phosphorylation of the Akt site by replacement of serine 648 with an aspartate did not generate a constitutive active NHE1, this result suggested that a second event was required for full activation of NHE1 in presence of cl-CD95L.

NHE1 activity is stimulated by the serine/threonine kinase ROCK upon CD95 engagement

We recently showed that CD95 induced a Ca^{2+} response in activated T lymphocytes or breast cancer cells. However, no Ca^{2+} signal was detected in PS120-NHE1-CD95 cells exposed to cl-CD95L (Fig.3A) indicating that at least in this cell line, Ca^{2+} did not contribute to the CD95-mediated NHE1 activation.

The Rho GTPase family plays pivotal roles in the regulation of actin cytoskeleton reorganization, by activating effector proteins such as p160ROCK, which in turn promotes actomyosin contractile force generation (Rath & Olson, 2012). P160ROCK can be activated upon CD95 stimulation to trigger CD95 capping and operate the apoptotic signaling pathway (Hebert et al, 2008; Rebillard et al, 2010; Soderstrom et al, 2005). This kinase has also been shown to activate NHE1 at threonine 653 (Tominaga et al, 1998), in close vicinity of the Akt phosphorylation site at serine 648. In presence of cl-CD95L, PS120-NHE1-CD95 cells implemented Rho activation (Fig.3B), while fibroblasts devoid of CD95 or expressing a DD-deficient CD95 did not induce this signal, as assessed using RhoA pull down assay (Fig. 3B). Moreover, pre-incubation of PS120-NHE1-CD95 cells with the selective p160ROCK inhibitor Y-27632 abolished the CD95-mediated cell motility (Fig.3C). To confirm the involvement of p160ROCK in the CD95-mediated NHE1 activation, we next engineered the T653A NHE1 mutant (ROCKA mutant) that cannot be phosphorylated by these kinase. ROCKA was fully active and was stimulated by serum. However, this mutant did not enhance its response to intracellular pH in the presence of cl-CD95L (Fig.3D), and failed to promote cell motility in PS120 cells stimulated with cl-CD95L (Fig.4A). Pre-treatment with Y-27632 (Fig. 4B) or the addition of a ROCKA mutation (T653A) in AKTD abolished its ability to respond to cl-CD95L (Fig.4C) indicating that cl-CD95L activates this transporter by a two-step mechanism that requires the cooperation of Akt and p160ROCK (Fig.4D).

NHE1 C-terminal region contains multiple phosphorylation sites (for review see Hendus-Altenburger et al. 2014) and integrates multiple cellular signals to modulate NHE1 response to intracellular protons. The fact that the disruption of the Akt and ROCK phosphorylation sites suppresses the cl-CD95 signaling while leaving the global serum response unaffected shows that a combination of key phosphorylation can convey a very specific signal on NHE1.

Crystal structure of CD95 revealed that CD95L triggers a main conformational modification of its DD consisting in the shift of helix 6 and its fusion with helix 5 (Scott et al, 2009). This promotes both the self-association of the receptor and the recruitment of the adaptor protein FADD. However, this shift was only observed in an acidic context (pH 4) and not in a more neutral pH (pH 6.2) (Esposito et al, 2010). It is tempting to propose that low pH favors a DD structure promoting CD95 self-association and FADD binding (Scott et al, 2009), leading to the induction of cell death (Holler et al, 2003). By contrast, activation of NHE1 may prevent the fusion of helix 5 and 6 and thereby may further counteract FADD recruitment and the execution of the apoptotic signal.

To our knowledge, this is the first study revealing that the allosteric activation of the Na^+/H^+ exchanger NHE1 by CD95 contributes to a pro-migratory signaling pathway downstream Akt and ROCK activations. According to our recent studies showing that serum cl-CD95L is a strong prognostic marker associated with metastatic dissemination in women affected by triple negative breast cancer (Malleter et al, 2013), we predict that activation of NHE1 by cl-CD95L in these patients may contribute to the clinical outcomes of these women. In this context, the numerous NHE1 inhibitors that have been developed for the management of hypertension and heart failure may represent new and very attractive medicament candidates to reduce metastasis in triple negative breast cancers showing high concentrations of serum CD95L.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method for reducing CD95-mediated cell motility in a subject in need thereof comprising the steps consisting of i) determining the level of soluble CD95L in a sample obtained from the subject, ii) comparing the expression level determined at
5 step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of at least one compound selected from the group consisting of NHE1 inhibitors and ROCK inhibitors when the level determined at step i) is higher than the predetermined reference value.
2. The method of claim 1 for reducing CD95-mediated cancer cell motility.
- 10 3. The method of claim 1 for the treatment of cancer in a subject in need thereof.
4. The method of claim 3 wherein the cancer is selected from the group consisting of cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may
15 specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma,
20 malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil
25 carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous; adenocarcinoma;
30 mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous

adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; and roblastoma, malignant; Sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paraganuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic

leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

5. The method of claim 3 wherein the subject suffers from a cancer selected from the group consisting of breast cancer, colon cancer, lung cancer, prostate cancer, testicular cancer, brain cancer, skin cancer, rectal cancer, gastric cancer, esophageal cancer, sarcomas, tracheal cancer, head and neck cancer, pancreatic cancer, liver cancer, ovarian cancer, lymphoid cancer, cervical cancer, vulvar cancer, melanoma, mesothelioma, renal cancer, bladder cancer, thyroid cancer, bone cancers, carcinomas, sarcomas, and soft tissue cancers.
6. The method of claim 3 wherein the subject suffers from a triple negative breast cancer.
7. The method of claim 1 for reducing CD95-mediated lymphocyte motility.
8. The method of claim 1 for the treatment of an auto-immune disease.
9. The method of claim 8 wherein the auto-immune disease is selected from the group consisting of Addison's Disease, Allergy, Alopecia Areata, Alzheimer's disease, Antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, Ankylosing Spondylitis, Antiphospholipid Syndrome (Hughes Syndrome), arthritis, Asthma, Atherosclerosis, Atherosclerotic plaque, autoimmune disease (e.g., lupus, RA, MS, Graves' disease, etc.), Autoimmune Hemolytic Anemia, Autoimmune Hepatitis, Autoimmune inner ear disease, Autoimmune Lymphoproliferative syndrome, Autoimmune Myocarditis, Autoimmune Oophoritis, Autoimmune Orchitis, Azoospermia, Behcet's Disease, Berger's Disease, Bullous Pemphigoid, Cardiomyopathy, Cardiovascular disease, Celiac Sprue/Coeliac disease, Chronic Fatigue Immune Dysfunction Syndrome (CFIDS), Chronic idiopathic polyneuritis, Chronic Inflammatory Demyelinating, Polyradicalneuropathy (CIPD), Chronic relapsing polyneuropathy (Guillain-Barré syndrome), Churg-Strauss Syndrome (CSS), Cicatricial Pemphigoid, Cold Agglutinin Disease (CAD), chronic obstructive pulmonary disease (COPD), CREST syndrome, Crohn's disease, Dermatitis, Herpetiformis, Dermatomyositis, diabetes, Discoid Lupus, Eczema, Epidermolysis bullosa acquisita, Essential Mixed Cryoglobulinemia, Evan's Syndrome, Exophthalmos, Fibromyalgia, Goodpasture's Syndrome, Hashimoto's Thyroiditis, Idiopathic Pulmonary Fibrosis, Idiopathic Thrombocytopenia Purpura (ITP), IgA Nephropathy,

immunoproliferative disease or disorder (e.g., psoriasis), Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, Insulin Dependent Diabetes Mellitus (IDDM), Interstitial lung disease, juvenile diabetes, Juvenile Arthritis, juvenile idiopathic arthritis (JIA), Kawasaki's Disease, Lambert-Eaton Myasthenic Syndrome, Lichen Planus, lupus, Lupus Nephritis, Lymphocytic Lymphoproliferative Disease, Ménière's Disease, Miller Fish Syndrome/acute disseminated encephalomyeloradiculopathy, Mixed Connective Tissue Disease, Multiple Sclerosis (MS), muscular rheumatism, Myalgic encephalomyelitis (ME), Myasthenia Gravis, Ocular Inflammation, Pemphigus Foliaceus, Pemphigus Vulgaris, Pernicious Anaemia, Polyarteritis Nodosa, Polychondritis, Polyglandular Syndromes (Whitaker's syndrome), Polymyalgia Rheumatica, Polymyositis, Primary Agammaglobulinemia, Primary Biliary Cirrhosis/Autoimmune cholangiopathy, Psoriasis, Psoriatic arthritis, Raynaud's Phenomenon, Reiter's Syndrome/Reactive arthritis, Restenosis, Rheumatic Fever, rheumatic disease, Rheumatoid Arthritis, Sarcoidosis, Schmidt's syndrome, Scleroderma, Sjögren's Syndrome, Stiff-Man Syndrome, Systemic Lupus Erythematosus (SLE), systemic scleroderma, Takayasu Arteritis, Temporal Arteritis/Giant Cell Arteritis, Thyroiditis, Type 1 diabetes, Type 2 diabetes, Ulcerative colitis, Uveitis, Vasculitis, Vitiligo, and Wegener's Granulomatosis.

10. The method of claim 8 for the treatment of systemic lupus erythematosus.

11. The method of claim 1 for the treatment of an inflammatory condition.

12. The method of claim 11 wherein the inflammatory condition is selected from the group consisting of: sepsis, septicemia, pneumonia, septic shock, systemic inflammatory response syndrome (SIRS), Acute Respiratory Distress Syndrome (ARDS), acute lung injury, aspiration pneumonia, infection, pancreatitis, bacteremia, peritonitis, abdominal abscess, inflammation due to trauma, inflammation due to surgery, chronic inflammatory disease, ischemia, ischemia-reperfusion injury of an organ or tissue, tissue damage due to disease, tissue damage due to chemotherapy or radiotherapy, and reactions to ingested, inhaled, infused, injected, or delivered substances, glomerulonephritis, bowel infection, opportunistic infections, and for subjects undergoing major surgery or dialysis, subjects who are immunocompromised, subjects on immunosuppressive agents, subjects with HIV/AIDS, subjects with suspected endocarditis, subjects with fever, subjects with fever of unknown origin,

subjects with cystic fibrosis, subjects with diabetes mellitus, subjects with chronic renal failure, subjects with bronchiectasis, subjects with chronic obstructive lung disease, chronic bronchitis, emphysema, or asthma, subjects with febrile neutropenia, subjects with meningitis, subjects with septic arthritis, subjects with urinary tract infection, subjects with necrotizing fasciitis, subjects with other suspected Group A streptococcus infection, subjects who have had a splenectomy, subjects with recurrent or suspected enterococcus infection, other medical and surgical conditions associated with increased risk of infection, Gram positive sepsis, Gram negative sepsis, culture negative sepsis, fungal sepsis, meningococemia, post-pump syndrome, cardiac stun syndrome, stroke, congestive heart failure, hepatitis, epiglottitis, E. coli 0157:H7, malaria, gas gangrene, toxic shock syndrome, pre-eclampsia, eclampsia, HELP syndrome, mycobacterial tuberculosis, Pneumocystic carinii, pneumonia, Leishmaniasis, hemolytic uremic syndrome/thrombotic thrombocytopenic purpura, Dengue hemorrhagic fever, pelvic inflammatory disease, Legionella, Lyme disease, Influenza A, Epstein-Barr virus, encephalitis, inflammatory diseases and autoimmunity including Rheumatoid arthritis, osteoarthritis, progressive systemic sclerosis, systemic lupus erythematosus, inflammatory bowel disease, idiopathic pulmonary fibrosis, sarcoidosis, hypersensitivity pneumonitis, systemic vasculitis, Wegener's granulomatosis, transplants including heart, liver, lung kidney bone marrow, graftversus-host disease, transplant rejection, sickle cell anemia, nephrotic syndrome, toxicity of agents such as OKT3, cytokine therapy, and cirrhosis.

13. A compound selected from the group consisting of NHE1 inhibitors and ROCK inhibitors for use in a method for treating cancer in a subject in need thereof comprising the steps consisting of i) determining the level of soluble CD95L in a sample obtained from the subject, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) administering the subject with a therapeutically effective amount of said compound when the level determined at step i) is higher than the predetermined reference value.

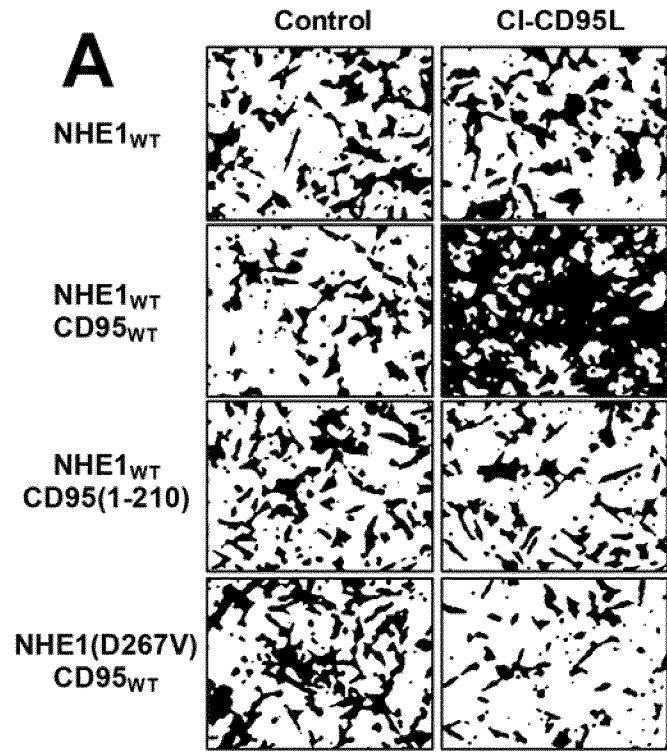


Figure 1A

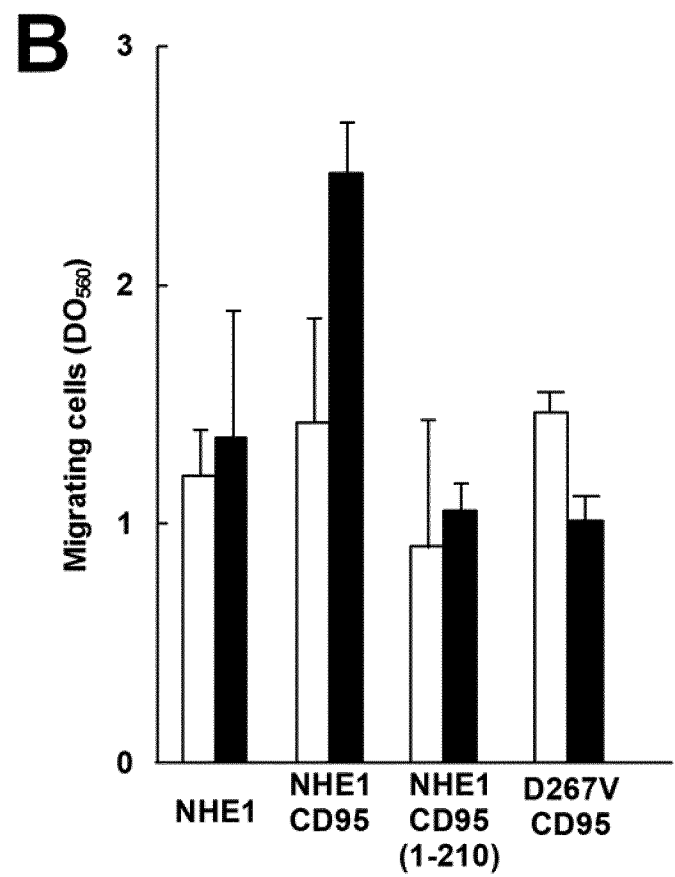


Figure 1 B

C

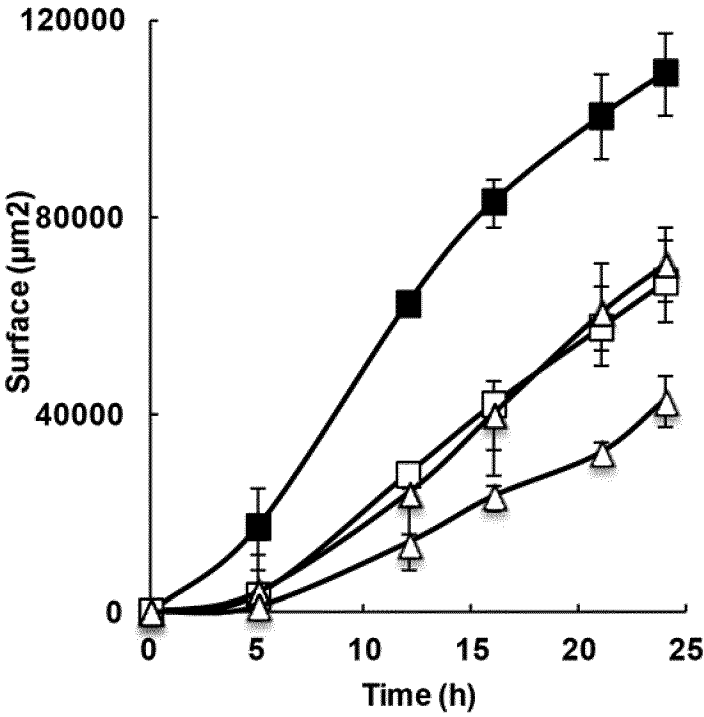


Figure 1C

D

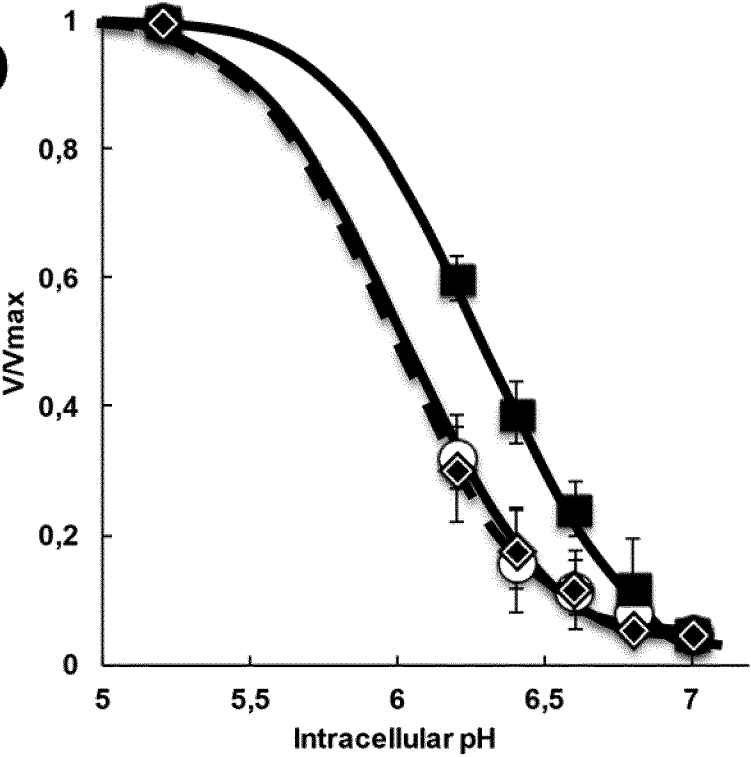


Figure 1D

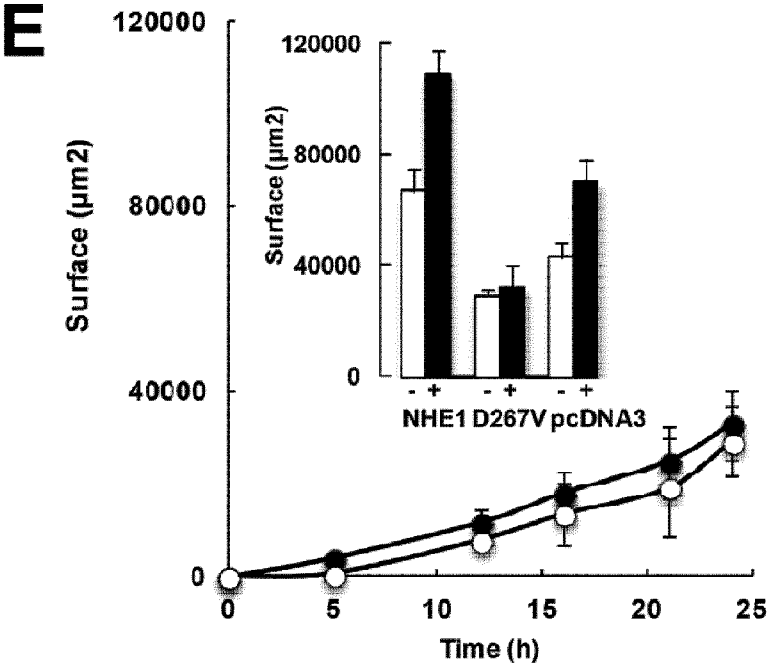


Figure 1E

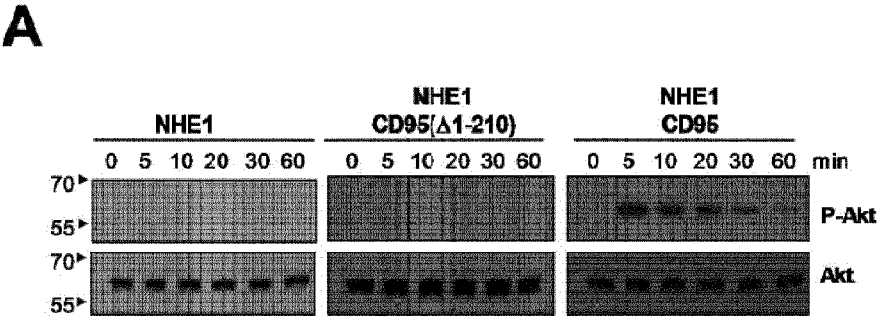


Figure 2A

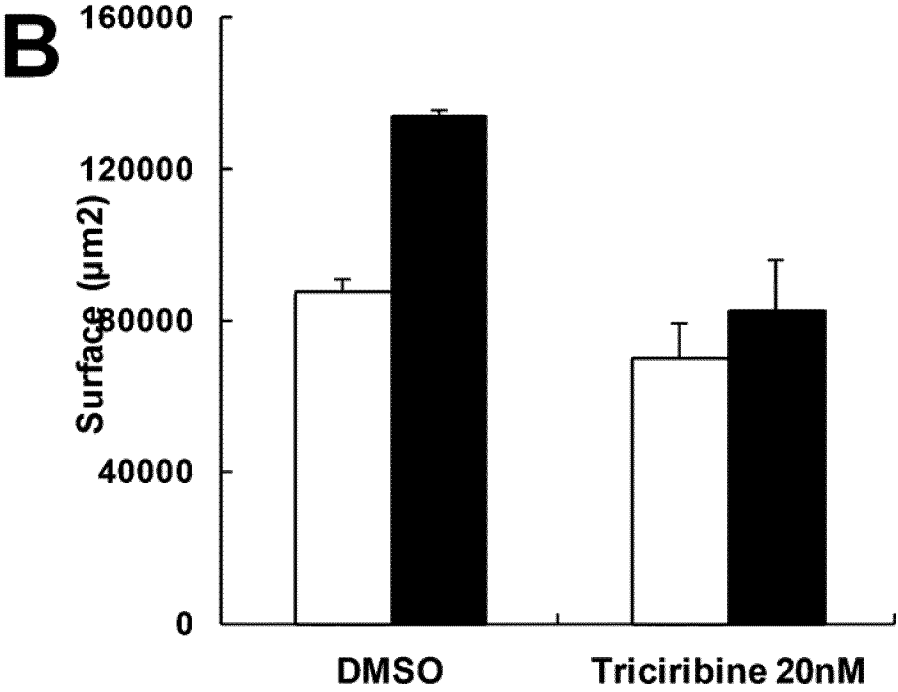


Figure 2B

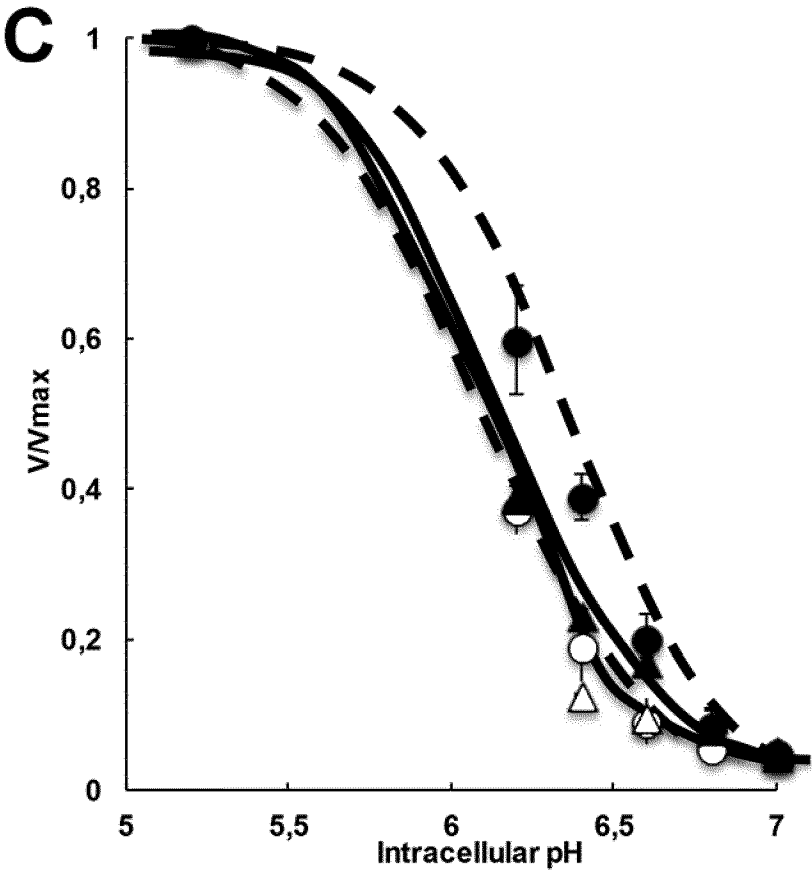


Figure 2C

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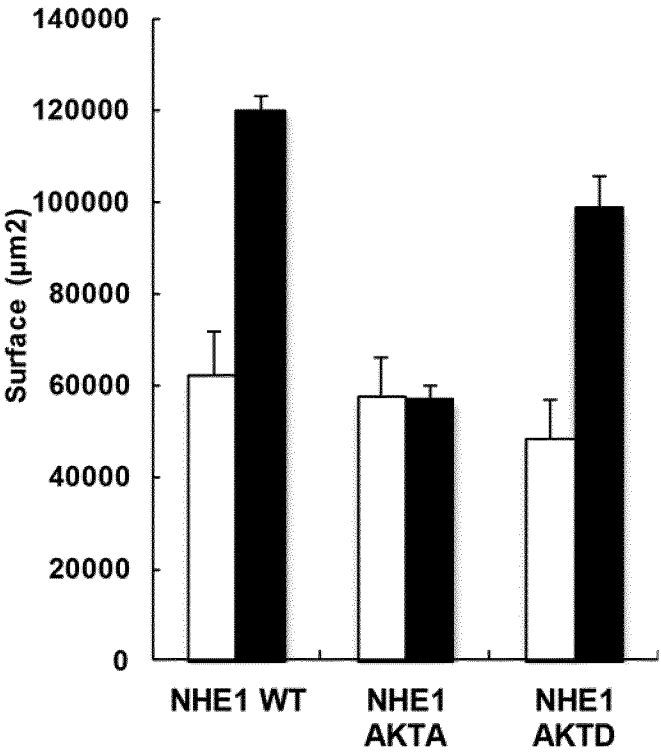


Figure 2D

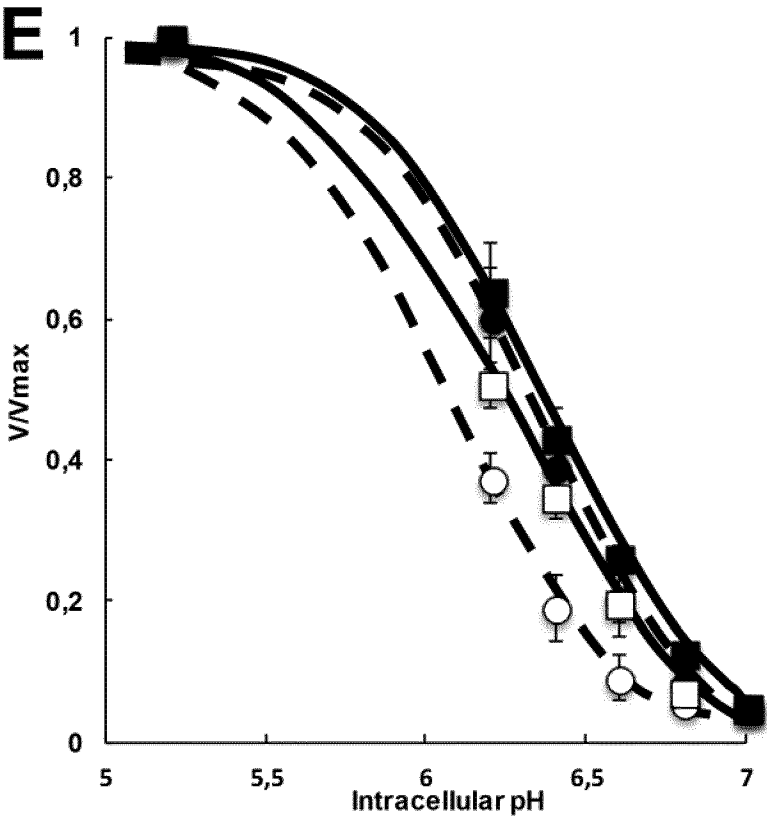


Figure 2E

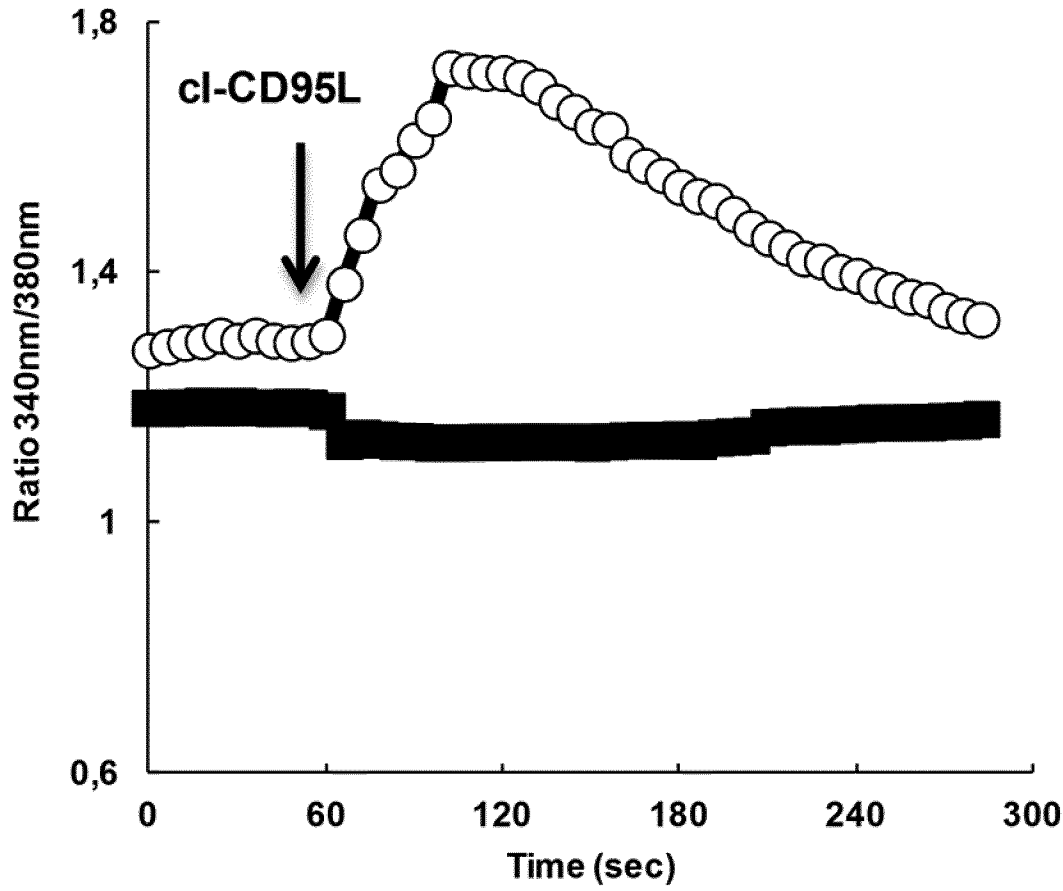


Figure 3A

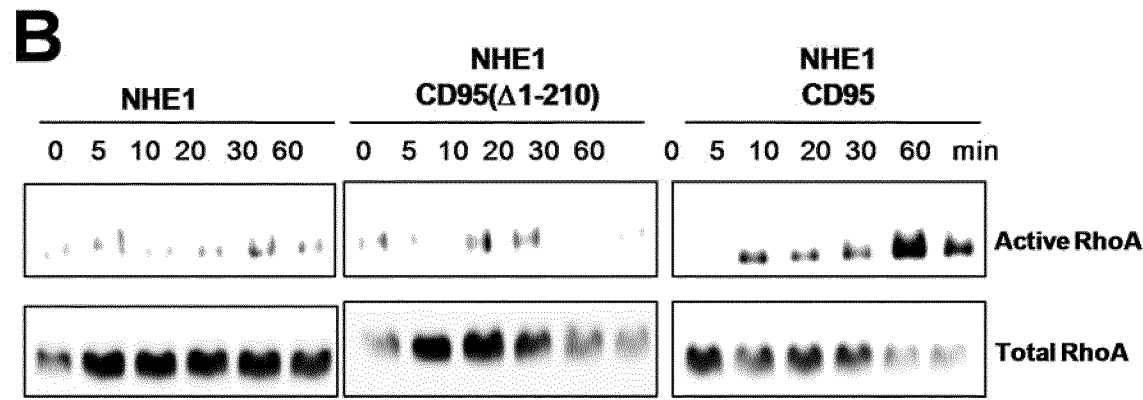


Figure 3B

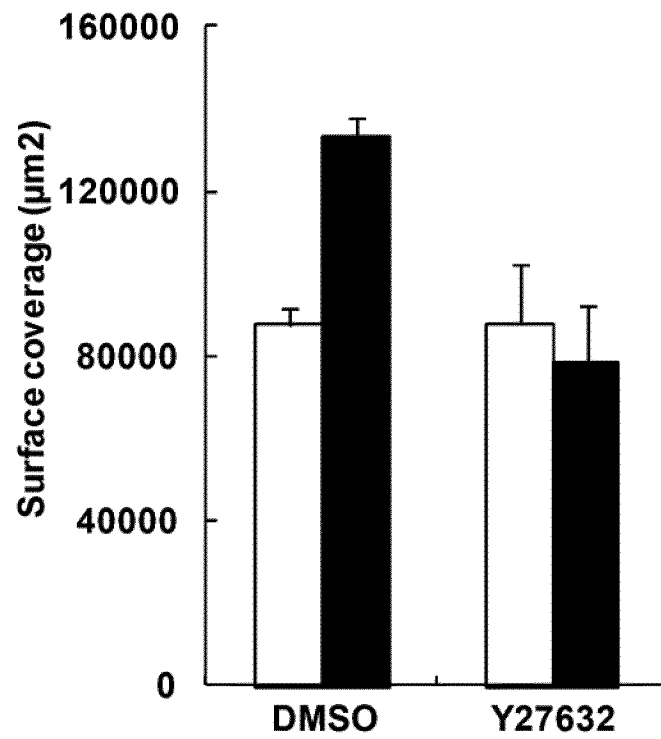
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Figure 3C

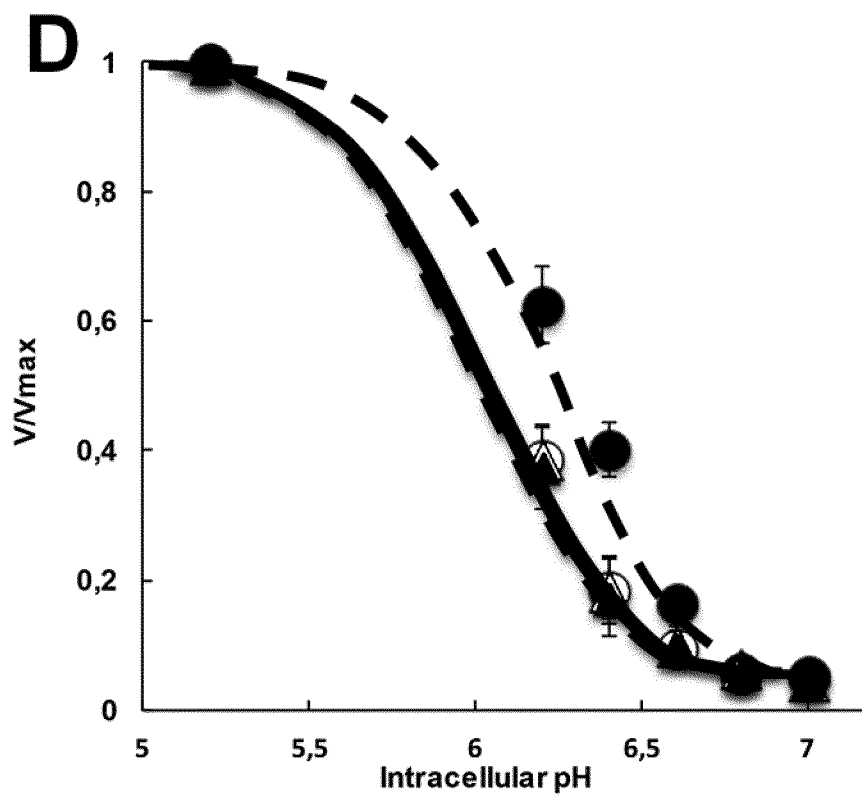


Figure 3D

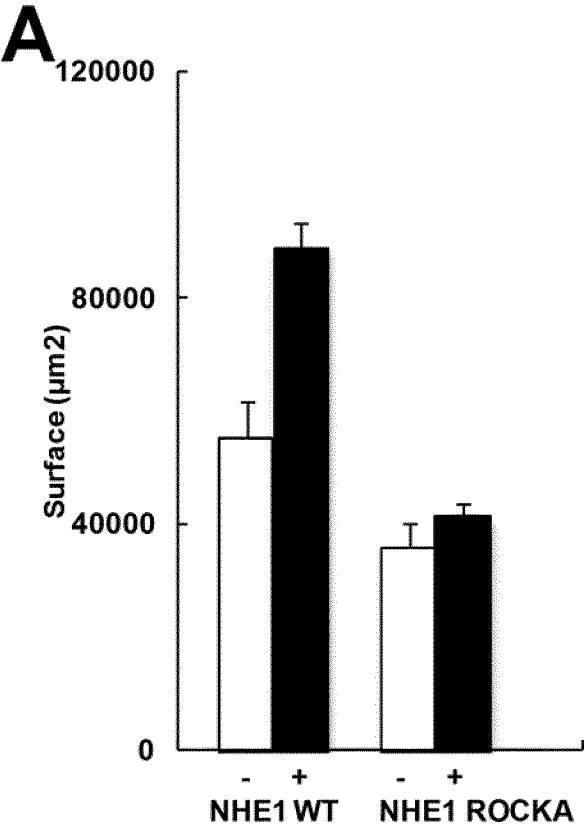


Figure 4A

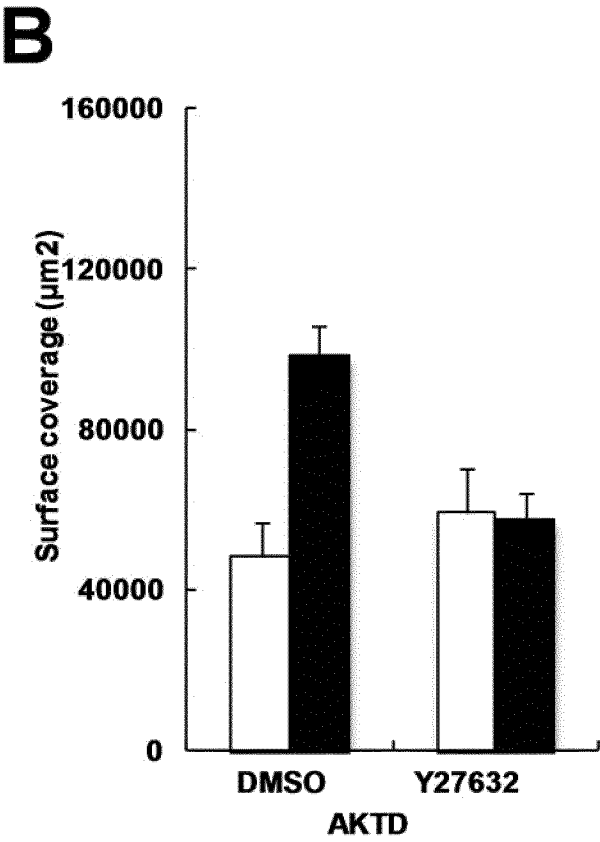


Figure 4B

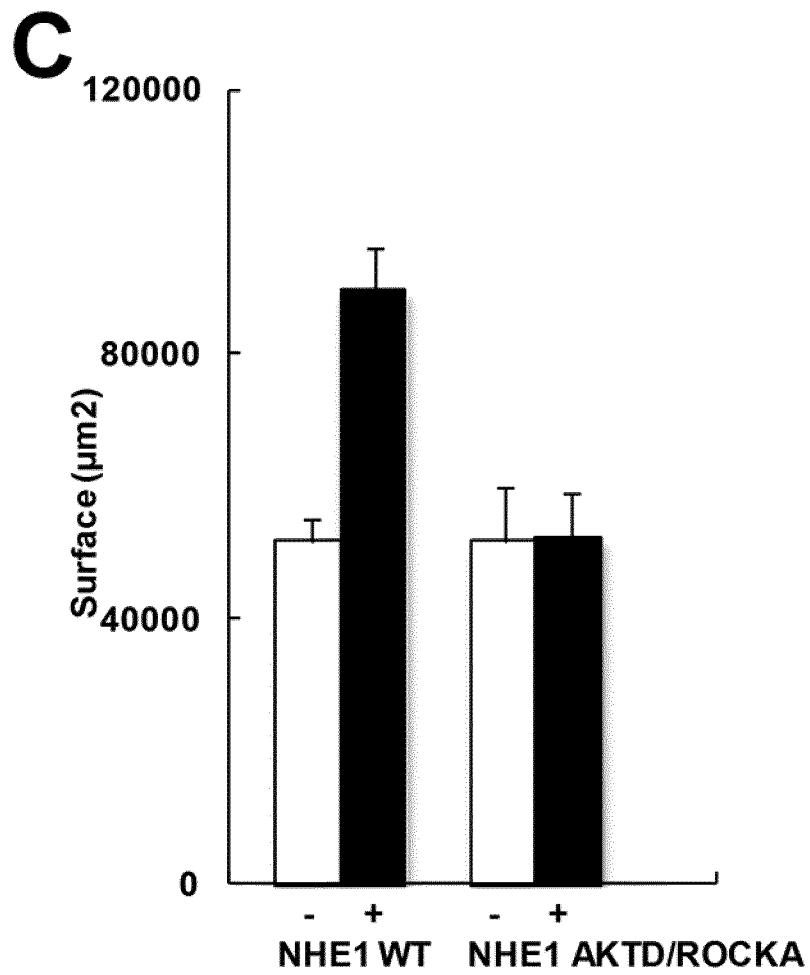


Figure 4C

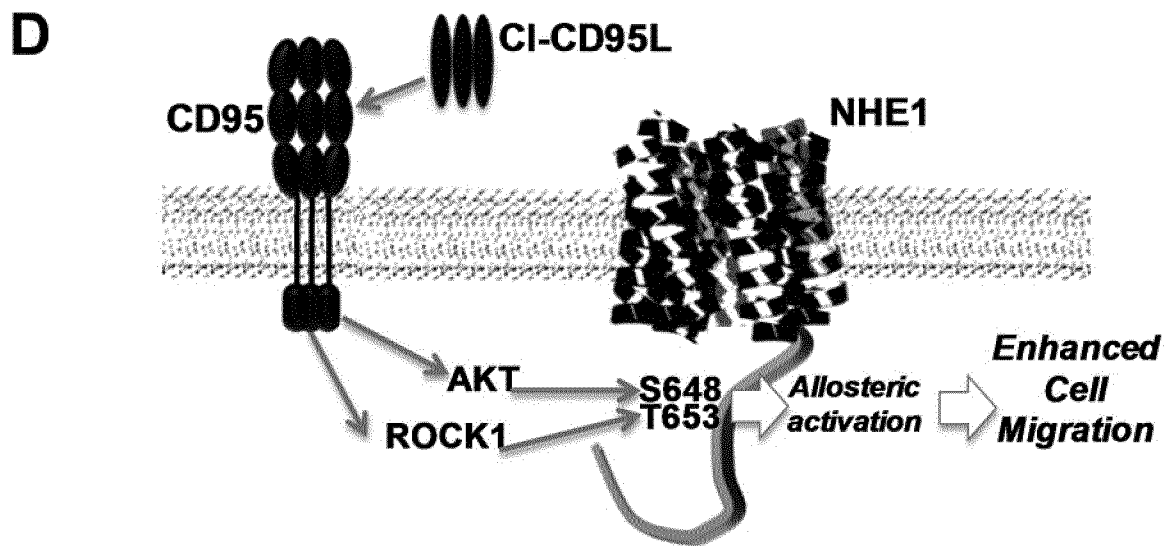


Figure 4D

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/062880

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00 A61P35/00 A61P37/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. MALLETER ET AL: "CD95L Cell Surface Cleavage Triggers a Prometastatic Signaling Pathway in Triple-Negative Breast Cancer", CANCER RESEARCH, vol. 73, no. 22, 26 September 2013 (2013-09-26), pages 6711-6721, XP055150022, ISSN: 0008-5472, DOI: 10.1158/0008-5472.CAN-13-1794 cited in the application the whole document page 6720 ----- -/--	1-13



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 August 2015

Date of mailing of the international search report

21/08/2015

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Loher, Florian

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2015/062880

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SÉBASTIEN TAUZIN ET AL: "The Naturally Processed CD95L Elicits a c-Yes/Calcium/PI3K-Driven Cell Migration Pathway", PLOS BIOLOGY, vol. 9, no. 6, 21 June 2011 (2011-06-21), XP055150223, US ISSN: 1544-9173, DOI: 10.1371/journal.pbio.1001090 the whole document	1-13
X	SOMLYO A V ET AL: "Rho-kinase inhibitor retards migration and in vivo dissemination of human prostate cancer cells", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 269, no. 3, 24 March 2000 (2000-03-24), pages 652-659, XP002277882, ISSN: 0006-291X, DOI: 10.1006/BBRC.2000.2343 table 1	13
Y	----- WO 2006/134305 A2 (CENTRE NAT RECH SCIENT [FR]; ROUX PIERRE [FR]; DE TOLEDO MARION [FR]) 21 December 2006 (2006-12-21) page 23, paragraph 1	1-13
X	----- PAPADOPOULOU NATALIA ET AL: "Membrane androgen receptor activation triggers down-regulation of PI-3K/Akt/NF-kappaB activity and induces apoptotic responses via Bad, FasL and caspase-3 in DU145 prostate cancer cells", MOLECULAR CANCER, BIOMED CENTRAL, LONDON, GB, vol. 7, no. 1, 3 December 2008 (2008-12-03), page 88, XP021050158, ISSN: 1476-4598, DOI: 10.1186/1476-4598-7-88 figure 6a	13
Y	----- -/--	1-13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/062880

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. LV ET AL: "Blocking the Na ⁺ /H ⁺ exchanger 1 with cariporide (HOE642) reduces the hypoxia-induced invasion of human tongue squamous cell carcinoma", INTERNATIONAL JOURNAL OF ORAL AND MAXILLOFACIAL SURGERY, vol. 41, no. 10, 1 October 2012 (2012-10-01), pages 1206-1210, XP055149777, DK ISSN: 0901-5027, DOI: 10.1016/j.ijom.2012.03.001	13
Y	page 1206 page 1207, column 1, paragraph 1 - paragraph 2 page 1209, right-hand column, paragraph 2 -----	1-13
Y	L BRISSON ET AL: "NaV1.5 enhances breast cancer cell invasiveness by increasing NHE1-dependent H ⁺ efflux in caveolae", ONCOGENE, vol. 30, no. 17, 20 December 2010 (2010-12-20), pages 2070-2076, XP055150070, GB ISSN: 0950-9232, DOI: 10.1038/onc.2010.574 page 2017, right-hand column, paragraph 2 page 2072 -----	1-13
X	US 6 355 410 B1 (RICH IVAN N [US]) 12 March 2002 (2002-03-12) column 4, line 6 - line 50 -----	13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/062880

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