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(54) **Title**: METHODS FOR IDENTIFYING AND COMPOUNDS USEFUL FOR THE DIAGNOSIS AND TREATMENT OF DISEASES INVOLVING INFLAMMATION

(57) **Abstract**: The present invention relates to agents, and methods for identifying compounds, which agents and compounds result in the stabilization of mast cells, in particular that inhibit mast cell degranulation. In addition, the invention relates to compositions and methods for the use thereof in treating conditions that are characterized by mast cell degranulation and/or inflammation, including allergic rhinitis.

METHODS FOR IDENTIFYING AND COMPOUNDS USEFUL FOR THE DIAGNOSIS AND TREATMENT OF DISEASES INVOLVING INFLAMMATION

Field of the Invention

[0001] The present invention relates to agents, and methods for identifying compounds, which agents and compounds result in the inhibition of degranulation of mast cells. The invention also relates to targets, the modulation of which results in the inhibition of degranulation of mast cells. In addition, the invention relates to compositions and methods for the use thereof in treating conditions that are characterized by degranulation of mast cells, including conditions characterized by inflammation.

Background of the Invention

[0002] Mast cells play an important role in immediate hypersensitivity and inflammatory reactions by releasing a large variety of mediators. The mast cell is a tissue-based inflammatory cell of bone marrow origin that responds to danger signals of innate and acquired immunity with immediate and delayed release of inflammatory mediators. Mast cells play an important role in the pathology of diseases such as asthma, allergic diseases such as allergy, allergic rhinitis, urticaria, angioedema, food allergy, allergic conjunctivitis, Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and other related disorders. When activated, a mast cell rapidly degranulates and releases its characteristic granules and various humoral and proinflammatory mediators. Mast cells and basophils, both have on their surfaces the high affinity IgE receptors; known as FcεRI. Binding of an antigen to the IgE on the mast cell or the basophil results in activation of the mast cell or basophil by cross-linking of the FcεRI receptors.

[0003] Mast cells express a complete and functional FcεRI receptor ($\alpha\beta\gamma 2$), aggregation of which leads to mast-cell activation, granule exocytosis, and mediator release. Mast cells may also be activated by complement-derived anaphylatoxins such as C3a and C5a through C3aR and C5aR (CD88), or by nerve growth factor through TRKA, or by IgG through FcγRI, or by reactions to cytokines derived from T-cells or mononuclear phagocytes.

[0004] The characteristic feature of the mast cell is the presence of dense cytoplasmic granules that occupy the cytoplasm. In human beings, these granules contain lattice or scroll-like structures. Mast cells are relatively abundant in skin, thymus, lymphoid tissue, lung, nasal mucosa, conjunctiva, uterus, urinary bladder, tongue, synovia, and mesentery; around large and small blood vessels; and in the subserosal and submucosal layers of the digestive tract. Mast cells occur chiefly in the loose connective tissue surrounding blood vessels, nerves, and glandular ducts and under epithelial, serous, and synovial membranes. In general, mast cells are scant in parenchymous tissues. In the lungs, mast cells are found both in bronchial airway connective tissues and in peripheral intra-alveolar spaces. In the skin, mast cells appear in greatest number near blood vessels, hair follicles, sebaceous glands, and sweat glands. Mast cells in human tissues are divided into two major subtypes according to the secretory protease content,

designated as MCT or MCTC according to the presence of tryptase with or without chymase. MCTC cells thus contain tryptase and chymase, as well as carboxypeptidase and cathepsin G. MCT cells contain only tryptase. Tryptase staining thus identifies all mast cells in tissues and has become the principal method of visualizing mast cells. MCTC cells predominate in skin and small bowel submucosa. MCT cells predominate in normal airway and small bowel mucosa. MCT cells appear selectively attenuated in the small bowel of patients with end-stage immunodeficiency diseases. Human mast cells are characterized as Kit+ (positive for receptor for stem cell factor (SCF)) and FcεRI+. They express a variety of membrane receptors, depending on their tissue source, state of differentiation, and conditions of culture. Human resting mast cells express the high-affinity IgE receptor (FcεRI) and FcγRIIb (CD32). After exposure to interferon (IFN)-γ in vitro, they express the high-affinity receptor for IgG (FcγRI, CD64). Mast cells may also express C3a and C5a receptors. They may be similarly positively staining for cytokine receptors (IL-3R, IL-4R, IL-5R, IL-9R, IL-10R, granulocyte-macrophage colony-stimulating factor [GM-CSF]R, IFN-γR), chemokine receptors (CCR3, CCR5, CXCR2, CXCR4), and nerve growth factor receptors, among others.

[0005] Mediators produced by mast cells have classically been divided into three categories: (1) preformed mediators, (2) newly synthesized lipid mediators, and (3) cytokines. These categories are not absolutely exclusive, with at least one cytokine, tumor necrosis factor (TNF)-alpha, occurring both preformed and as a newly synthesized molecule.

[0006] Preformed mediators are packaged within secretory granules. At activation, granule contents are released into the extracellular environment within minutes. Principal granule constituents include histamine, serine proteases, carboxypeptidase A, and proteoglycans (heparin and chondroitin sulfate E). Histamine is synthesized from histidine, and serotonin (present in mast cell granules in mice and rat) from tryptophan. Histamine and serotonin in granules is found in ionic association with acidic residues of the glycosaminoglycan side chains of heparin and chondroitin sulfate E, and it dissociates from these glycosaminoglycans in extracellular fluids by exchanging with sodium ions. After release into the extracellular space, they are metabolized within minutes. Histamine has effects on smooth muscle (contraction), endothelial cells, nerve endings, and mucous secretion. It is rapidly degraded to N-methyl histamine, methylimidazole acetic acid, and imidazole acetic acid. Heparin and chondroitin sulfate proteoglycans are believed to aid in storage of preformed molecules, which dissociate from these proteoglycans at variable rates in physiologic buffer solution. Most of the protein in mast cell granules is made up of neutral proteases, which catalyse the cleavage of peptide bonds at neutral pH: tryptase, chymase, carboxypeptidase, and cathepsin G. Tryptase is found in all mast cells populations, but chymase is present only in a subpopulation of mast cells, MCTC. The biological role of tryptase and chymase has not been clarified, but several potential biological functions have been demonstrated in vitro. Tryptase degrades fibrinogen, destroys high molecular weight kininogen and generates C3a. Chymase generates angiotensin II, degrades basement membrane and activates IL-1β precursor.

[0007] The major newly synthesized lipid mediators are metabolized from arachidonic acid and include prostaglandin D2 and leukotriene C4. Liberation of arachidonic acid from cellular lipid stores occurs with mast cell activation. Arachidonic acid is metabolized into prostaglandin D2 (PGD2) with cyclo-oxygenase, and into leukotriene (LT) C4 with lipoxygenase. Extracellular peptidolytic processing of LTC4 yields the active metabolites LTD4 and LTE4. These metabolites from arachidonic acid have a variety of biological functions, including vasodilation, inhibition of platelet aggregation and contraction of intestinal and bronchial smooth muscle. PGD2 and LTC4, LTD4, and LTE4 are all bronchoconstrictors. LTC4, LTD4, and LTE4 also enhance vascular permeability. PGD2 is also a neutrophil chemoattractant. PAF is a phospholipid mediator released from mast cells in allergic and inflammatory reactions. It is newly synthesized by the activated mast cells. The biological activity of PAF in vitro includes activation of platelets and neutrophils, and contraction of smooth muscle in ileum and pulmonary tissue.

[0008] Mast cells are capable of synthesizing and secreting an array of cytokines. TNF-alpha appears to be a major cytokine produced by human mast cells. It appears to be both stored and synthesized after mast-cell activation. Other cytokines reported to be produced by human mast cells include interleukin-13 (IL-13) associated with B cell isotype switch to IgE synthesis, eosinophil recruitment and activation, and smooth muscle cell contraction; IL-4 associated with TH2 cell differentiation and IgE synthesis; IL-3, GM-CSF, and IL-5, critical for eosinophil development and survival; and IL-6, IL-8, and IL-16. Human mast cells are also documented to produce chemokines, such as macrophage inflammatory protein (MIP)-1alpha. In mouse and rat upon activation of mast cells, the following cytokine mRNAs are newly transcribed: TNF-alpha, transforming growth factor beta(TGF-beta), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFN-gamma), IL-1, -2, -3, -4, -5, -6, - 10 and MIP-1 alpha, MIP-1 beta.

[0009] Mediators released upon activation of mast cells are central to the pathophysiology of diseases such as asthma, allergic diseases such as allergy, allergic rhinitis, urticaria, angioedema, food allergy, allergic conjunctivitis, Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, and eczema. Mast-cell activation through IgE-dependent mechanisms initiates a cascade of events, resulting in an immediate hypersensitivity reaction, as well as a late-phase reaction. The immediate reaction is reflected in the skin as erythema, edema, and itch; in the upper airways as sneezing, rhinorrhea, and mucous secretion; in the lungs as cough, bronchospasm, edema, and mucous secretion resulting in immediate breathlessness; and in the gastrointestinal tract as nausea, vomiting, diarrhea, and cramping. This reaction is coincident with histamine liberation and demonstration of production of PGD2 and LTC4. The reaction is then often followed 6 to 24 hours later by persistent edema and a leukocytic influx, the late phase reaction, which is due at least in part to the generation and release of the additional mast cell–derived substances listed above. In humans the production of cytokines like IL-5 and IL-13 are believed to be central to the

evolution of the chronic allergic/asthmatic states. In turn, recruited cells contribute additional inflammatory mediators at the cellular level. In the lungs, the late-phase reaction is believed to play a major role in the genesis of persistent asthma and the accompanying inflammation.

[0010] Asthma is clinically recognized by airway hyper-reactivity and reversible airway obstruction. Other pathological events include constriction of the airway smooth muscle cells, increased vascular permeability resulting in airway oedema, hypersecretion of mucus from goblet cells and mucus glands, removal from epithelial lining cells, influx of inflammatory cells. Exercise-induced asthma and aspirin-sensitive asthma have also been linked to undesired mast cell degranulation.

[0011] Despite an increased understanding of mast cells and their role in inflammatory diseases and pathological events such as asthma, allergic rhinitis, food allergies, rheumatoid arthritis, and other disorders and conditions, there is still a need for improved and specific therapies for these conditions and for the particular modulation of mast cell degranulation.

Summary of the Invention

[0012] The rat basophilic cell line (RBL), and the human mastocytoma cell lines HMC1 and LAD2 are often used as a model system for mast cells. Rat cells are not suitable for screening of shRNA libraries targeting human genes. The use of tumor cell lines for identification of TARGETS is not preferred as such cells have a gene expression profile significantly different from normal human mast cells. This may lead to false positive and false negative results. In the present invention cultivated human primary mast cells have been used directly. The advantage of using primary human mast cells is that their gene expression profile is similar or identical to that of mast cells in human tissue. TARGETS that are identified in human primary mast cells could be screened for drug discovery purposes in cell lines that do express the TARGET from the primary cell. The use of human primary mast cells for screening libraries (here adenoviral siRNA expression libraries or adenoviral overexpression libraries) is novel. The screening for novel targets for asthma has been hampered by poor transduction methods for cultivated human mast cells and the lack of appropriate knock-in or knock-down libraries. The use of adenoviruses with adapted capsids enables us to efficiently transduce primary human mast cells. This in combination with an adenoviral expression library of siRNAs directed against mRNA sequences of drugable targets allows us to hunt for drugable regulators for mast cell activation. In the knock-down approach (siRNA expression constructs), the siRNA expression constructs mimic antagonistic compounds. The invention also relates to the development of compounds that result in the modulation of mast cell activation. Preferably, the compound antagonizes the activation of mast cells, and/or inhibits the release of cytokines, and/or inhibits the release of leukotrienes and/or prostaglandins.

[0013] The present invention is based on the discovery that agents which inhibit the expression and / or activity of the TARGETS disclosed herein are able to result in inhibition of mast cell degranulation, as indicated by a suppression of the release of inflammatory mediators in mast cells, in particular a

suppression of the release of histamine. The present invention therefore provides TARGETS which are involved in the pathway involved in mast cell stabilisation, methods for screening for agents capable of inhibiting mast cell degranulation and uses of these agents in the prevention and / or treatment of diseases associated with mast cell degranulation and/or inflammation.

[0014] The present invention relates to a method for identifying compounds that inhibit mast cell degranulation, comprising contacting the compound with the identified TARGETS or their protein domain fragments (SEQ ID. NO: 20-38) under conditions that allow said TARGETS or their protein domain fragments to bind to the compound, and measuring a compound-polypeptide property related to mast cell degranulation. In one aspect the property is the release of inflammatory mediator(s) from mast cells.

[0015] In particular the present invention provides TARGETS which are involved in the stabilization of mast cells, particularly in mast cell degranulation, methods for screening for agents capable of modulating the expression and / or activity of TARGETS and uses of these agents in the prevention and / or treatment of diseases involving mast cell degranulation, in particular diseases involving inflammation. The present invention provides TARGETS which are involved in or otherwise associated with mast cell stabilisation. The invention provides TARGETS, the modulation of which results in inhibition of mast cell degranulation. The present invention provides TARGETS which are involved in inflammation and the inflammatory response, particularly associated with mast cells. The invention provides uses of agents directed against these targets in diseases involving an inflammation.

[0016] Aspects of the present method include the *in vitro* assay of compounds using identified TARGETS, and cellular assays wherein identified TARGET inhibition is followed by observing indicators of efficacy, including alteration of the release of inflammatory mediators, e.g. histamine, proteoglycans, and cytokines. Another aspect of the invention is a method of treatment or prevention of a condition involving mast cell degranulation, in a subject suffering or susceptible thereto, by administering a pharmaceutical composition comprising an agent which is able to inhibit mast cell degranulation.

[0017] The present invention relates to a method for identifying compounds that inhibit the TARGET(s), comprising contacting the compound with the identified TARGETS or their protein domain fragments (SEQ ID NO: 20-38) under conditions wherein the compounds may interact with or influence the TARGET(s), measuring the expression or release of inflammatory mediators, and selecting compounds which suppress the expression or release of inflammatory mediators from cells, in particular mast cells. In one such method the release of histamine from mast cells is measured.

[0018] The present invention relates to a method for identifying compounds that are able to stabilize mast cells, and particularly inhibit mast cell degranulation, comprising contacting a compound with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38 (hereinafter "TARGETS") and fragments thereof, under conditions that allow said polypeptide to

bind to said compound, and measuring a compound-polypeptide property related to mast cell stabilisation. In a specific embodiment, the present invention relates to a method for identifying compounds that are able to modulate the release of inflammatory mediators from mast cells, comprising contacting a compound with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38 (hereinafter "TARGETS") and fragments thereof, under conditions that allow said polypeptide to bind to said compound, and measuring a compound-polypeptide property related to mast cell stabilisation. In a specific embodiment the compound-polypeptide property measured is one related to the inhibition of mast cell degranulation. In a specific embodiment, the property measured is the release of inflammatory mediators from mast cells, in a particular aspect the inflammatory mediator is histamine. In addition, or in the alternative, the release of proteases, tryptase, carboxypeptidase, cathepsin G, or chymase may be measured.

[0019] Aspects of the present method include the *in vitro* assay of compounds using polypeptide of a TARGET, or fragments thereof, including the amino acid sequences described by SEQ ID NO: 20-38 and cellular assays wherein TARGET inhibition is followed by observing indicators of efficacy including, for example, TARGET expression levels, TARGET enzymatic activity, mast cell degranulation, inflammatory mediator release from mast cells, and/or other assessments of inflammation and inflammatory response.

[0020] The present invention also relates to

(1) expression inhibitory agents comprising a polynucleotide selected from the group of an antisense polynucleotide, a ribozyme, and a small interfering RNA (siRNA), wherein said polynucleotide comprises a nucleic acid sequence complementary to, or engineered from, a naturally occurring polynucleotide sequence encoding a TARGET polypeptide said polynucleotide sequence comprising a sequence selected from the group consisting of SEQ ID NO: 1-19 and

(2) pharmaceutical compositions comprising said agent(s), useful in the treatment, or prevention, of a disease characterized by mast cell activation and/or inflammation.

[0021] Another aspect of the invention is a method of treatment or prevention of a disease or condition characterized by mast cell activation, in particular those diseases/conditions characterized by inflammation, in a subject suffering from or susceptible thereto, by administering a pharmaceutical composition comprising an effective TARGET-expression inhibiting amount of a expression-inhibitory agent or an effective TARGET activity inhibiting amount of an activity-inhibitory agent.

[0022] A further aspect of the present invention is a method for diagnosis of a disease characterized by mast cell activation, in particular those diseases characterized by inflammation, comprising measurement of indicators of levels of TARGET expression in a subject. In particular the present invention relates to a method for the diagnosis of allergic diseases, allergic airways disease (e.g. asthma,

rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[0023] Another aspect of this invention relates to the use of agents which inhibit a TARGET as disclosed herein in a therapeutic method, a pharmaceutical composition, and the manufacture of such composition, useful for the treatment of a disease or condition involving mast cell degranulation. In particular, the present method relates to the use of the agents which inhibit a TARGET in the treatment of a disease characterized by mast cell activation, suitable conditions include but are not limited to asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis.

[0024] Another aspect of this invention relates to the use of agents which inhibit a TARGET as disclosed herein in a therapeutic method, a pharmaceutical composition, and the manufacture of such composition, useful for the treatment of a disease involving inflammation. In particular said diseases are selected from the group consisting of allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[0025] Other objects and advantages will become apparent from a consideration of the ensuing description taken in conjunction with the following illustrative drawings.

Brief Description of the Figures

[0026] Figure 1: depicts mast cells positive for CD117 and FcεR1.

[0027] Figure 2 depicts the transduction efficiency of primary mast cells. Mast cells were transduced with either Ad5C20-empty or with AD5C20-GFP. The percentage of cells positive for GFP was determined by flow cytometry. Primary mast cells can be transduced with an efficiency of 70% or more.

[0028] Figure 3 depicts the results obtained with negative and positive control Ad5 shRNAs

[0029] Figure 4 depicts the results of screening the SilenceSelect® collection in biological duplicate. Histamine values of individual replicates are plotted against each other.

Detailed Description of the Invention

[0030] The following terms are intended to have the meanings presented therewith below and are useful in understanding the description and intended scope of the present invention.

[0031] The term 'agent' means any molecule, including polypeptides, antibodies, polynucleotides, chemical compounds and small molecules. In particular the term agent includes compounds such as test compounds or drug candidate compounds.

[0032] The term 'agonist' refers to a ligand that stimulates the receptor the ligand binds to in the broadest sense.

[0033] As used herein, the term 'antagonist' is used to describe a compound that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses, or prevents or reduces agonist binding and, thereby, agonist-mediated responses.

[0034] The term 'assay' means any process used to measure a specific property of an agent. A 'screening assay' means a process used to characterize or select agents based upon their activity from a collection of agents.

[0035] The term 'binding affinity' is a property that describes how strongly two or more compounds associate with each other in a non-covalent relationship. Binding affinities can be characterized qualitatively (such as 'strong', 'weak', 'high', or 'low') or quantitatively (such as measuring the K_D).

[0036] The term 'carrier' means a non-toxic material used in the formulation of pharmaceutical compositions to provide a medium, bulk and/or useable form to a pharmaceutical composition. A carrier may comprise one or more of such materials such as an excipient, stabilizer, or an aqueous pH buffered solution. Examples of physiologically acceptable carriers include aqueous or solid buffer ingredients including phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONICS®.

[0037] The term 'complex' means the entity created when two or more compounds bind to, contact, or associate with each other.

[0038] The term 'compound' is used herein in the context of a 'test compound' or a 'drug candidate compound' described in connection with the assays of the present invention. As such, these compounds comprise organic or inorganic compounds, derived synthetically, recombinantly, or from natural sources.

[0039] The compounds include inorganic or organic compounds such as polynucleotides, lipids or hormone analogs. Other biopolymeric organic test compounds include peptides comprising from about 2 to about 40 amino acids and larger polypeptides comprising from about 40 to about 500 amino acids, including polypeptide ligands, enzymes, receptors, channels, antibodies or antibody conjugates.

[0040] The term 'condition' or 'disease' means the overt presentation of symptoms (i.e., illness) or the manifestation of abnormal clinical indicators (for example, biochemical indicators or diagnostic indicators). Alternatively, the term 'disease' refers to a genetic or environmental risk of or propensity for developing such symptoms or abnormal clinical indicators.

[0041] The term 'contact' or 'contacting' means bringing at least two moieties together, whether in an *in vitro* system or an *in vivo* system.

[0042] The term 'derivatives of a polypeptide' relates to those peptides, oligopeptides, polypeptides, proteins and enzymes that comprise a stretch of contiguous amino acid residues of the polypeptide and that retain a biological activity of the protein, for example, polypeptides that have amino acid mutations compared to the amino acid sequence of a naturally-occurring form of the polypeptide. A derivative may further comprise additional naturally occurring, altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally occurring form of the polypeptide. It may also contain one or more non-amino acid substituents, or heterologous amino acid substituents, compared to the amino acid sequence of a naturally occurring form of the polypeptide, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence.

[0043] The term 'derivatives of a polynucleotide' relates to DNA-molecules, RNA-molecules, and oligonucleotides that comprise a stretch of nucleic acid residues of the polynucleotide, for example, polynucleotides that may have nucleic acid mutations as compared to the nucleic acid sequence of a naturally occurring form of the polynucleotide. A derivative may further comprise nucleic acids with modified backbones such as PNA, polysiloxane, and 2'-O-(2-methoxy) ethyl-phosphorothioate, non-naturally occurring nucleic acid residues, or one or more nucleic acid substituents, such as methyl-, thio-, sulphate, benzoyl-, phenyl-, amino-, propyl-, chloro-, and methanocarbanucleosides, or a reporter molecule to facilitate its detection.

[0044] The term 'endogenous' shall mean a material that a mammal naturally produces. Endogenous in reference to the term 'protease', 'kinase', 'factor', or 'receptor' shall mean that which is naturally produced by a mammal (for example, and not limitation, a human). In contrast, the term non-endogenous in this context shall mean that which is not naturally produced by a mammal (for example, and not limitation, a human). Both terms can be utilized to describe both *in vivo* and *in vitro* systems. For example, and without limitation, in a screening approach, the endogenous or non-endogenous TARGET may be in reference to an *in vitro* screening system. As a further example and not limitation, where the genome of a mammal has been manipulated to include a non-endogenous TARGET, screening of a candidate compound by means of an *in vivo* system is viable.

[0045] The term 'expressible nucleic acid' means a nucleic acid coding for a proteinaceous molecule, an RNA molecule, or a DNA molecule.

[0046] The term 'expression' comprises both endogenous expression and overexpression by transduction.

[0047] The term 'expression inhibitory agent' means a polynucleotide designed to interfere selectively with the transcription, translation and/or expression of a specific polypeptide or protein normally expressed within a cell. More particularly, 'expression inhibitory agent' comprises a DNA or RNA

molecule that contains a nucleotide sequence identical to or complementary to at least about 15-30, particularly at least 17, sequential nucleotides within the polyribonucleotide sequence coding for a specific polypeptide or protein. Exemplary expression inhibitory molecules include ribozymes, double stranded siRNA molecules, self-complementary single-stranded siRNA molecules (shRNA), genetic antisense constructs, and synthetic RNA antisense molecules with modified stabilized backbones.

[0048] The term 'fragment of a polynucleotide' relates to oligonucleotides that comprise a stretch of contiguous nucleic acid residues that exhibit substantially a similar, but not necessarily identical, activity as the complete sequence. In a particular aspect, 'fragment' may refer to a oligonucleotide comprising a nucleic acid sequence of at least 5 nucleic acid residues (preferably, at least 10 nucleic acid residues, at least 15 nucleic acid residues, at least 20 nucleic acid residues, at least 25 nucleic acid residues, at least 40 nucleic acid residues, at least 50 nucleic acid residues, at least 60 nucleic residues, at least 70 nucleic acid residues, at least 80 nucleic acid residues, at least 90 nucleic acid residues, at least 100 nucleic acid residues, at least 125 nucleic acid residues, at least 150 nucleic acid residues, at least 175 nucleic acid residues, at least 200 nucleic acid residues, or at least 250 nucleic acid residues) of the nucleic acid sequence of said complete sequence.

[0049] The term 'fragment of a polypeptide' relates to peptides, oligopeptides, polypeptides, proteins, monomers, subunits and enzymes that comprise a stretch of contiguous amino acid residues, and exhibit substantially a similar, but not necessarily identical, functional or expression activity as the complete sequence. In a particular aspect, 'fragment' may refer to a peptide or polypeptide comprising an amino acid sequence of at least 5 amino acid residues (preferably, at least 10 amino acid residues, at least 15 amino acid residues, at least 20 amino acid residues, at least 25 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, at least 150 amino acid residues, at least 175 amino acid residues, at least 200 amino acid residues, or at least 250 amino acid residues) of the amino acid sequence of said complete sequence.

[0050] The term 'hybridization' means any process by which a strand of nucleic acid binds with a complementary strand through base pairing. The term 'hybridization complex' refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary bases. A hybridization complex may be formed in solution (for example, C_{0t} or R_{0t} analysis) or formed between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (for example, paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate to which cells or their nucleic acids have been fixed). The term "stringent conditions" refers to conditions that permit hybridization between polynucleotides and the claimed polynucleotides. Stringent conditions can be defined by salt concentration, the concentration of organic solvent, for example, formamide, temperature, and other conditions well known in the art. In

particular, reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature can increase stringency. The term 'standard hybridization conditions' refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such 'standard hybridization conditions' are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20^{N}C below the predicted or determined T_m with washes of higher stringency, if desired.

[0051] The term 'inhibit' or 'inhibiting', in relationship to the term 'response' means that a response is decreased or prevented in the presence of a compound as opposed to in the absence of the compound.

[0052] The term 'inhibition' refers to the reduction, down regulation of a process or the elimination of a stimulus for a process, which results in the absence or minimization of the expression or activity of a protein or polypeptide.

[0053] The term 'induction' refers to the inducing, up-regulation, or stimulation of a process, which results in the expression or activity of a protein or polypeptide.

[0054] The term 'ligand' means a molecule, including an endogenous, naturally occurring or synthetic, non-natural molecules, specific for an endogenous, naturally occurring receptor.

[0055] The term 'pharmaceutically acceptable salts' refers to the non-toxic, inorganic and organic acid addition salts, and base addition salts, of compounds which inhibit the expression or activity of TARGETS as disclosed herein. These salts can be prepared *in situ* during the final isolation and purification of compounds useful in the present invention.

[0056] The term 'polypeptide' relates to proteins (such as TARGETS), proteinaceous molecules, fragments of proteins, monomers, subunits or portions of polymeric proteins, peptides, oligopeptides and enzymes (such as kinases, proteases, GPCR's etc.).

[0057] The term 'polynucleotide' means a polynucleic acid, in single or double stranded form, and in the sense or antisense orientation, complementary polynucleic acids that hybridize to a particular polynucleic acid under stringent conditions, and polynucleotides that are homologous in at least about 60 percent of its base pairs, and more particularly 70 percent of its base pairs are in common, most particularly 90 per cent, and in a particular embodiment, 100 percent of its base pairs. The polynucleotides include polyribonucleic acids, polydeoxyribonucleic acids, and synthetic analogues thereof. It also includes nucleic acids with modified backbones such as peptide nucleic acid (PNA), polysiloxane, and 2'-O-(2-methoxy)ethylphosphorothioate. The polynucleotides are described by sequences that vary in length, that range from about 10 to about 5000 bases, particularly about 100 to

about 4000 bases, more particularly about 250 to about 2500 bases. One polynucleotide embodiment comprises from about 10 to about 30 bases in length. A particular embodiment of polynucleotide is the polyribonucleotide of from about 17 to about 22 nucleotides, more commonly described as small interfering RNAs (siRNAs - double stranded siRNA molecules or self-complementary single-stranded siRNA molecules (shRNA)). Another particular embodiment are nucleic acids with modified backbones such as peptide nucleic acid (PNA), polysiloxane, and 2'-O-(2-methoxy)ethylphosphorothioate, or including non-naturally occurring nucleic acid residues, or one or more nucleic acid substituents, such as methyl-, thio-, sulphate, benzoyl-, phenyl-, amino-, propyl-, chloro-, and methanocarbanucleosides, or a reporter molecule to facilitate its detection. Polynucleotides herein are selected to be 'substantially' complementary to different strands of a particular target DNA sequence. This means that the polynucleotides must be sufficiently complementary to hybridize with their respective strands. Therefore, the polynucleotide sequence need not reflect the exact sequence of the target sequence. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the polynucleotide, with the remainder of the polynucleotide sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the polynucleotide, provided that the polynucleotide sequence has sufficient complementarity with the sequence of the strand to hybridize therewith under stringent conditions or to form the template for the synthesis of an extension product.

[0058] The term 'preventing' or 'prevention' refers to a reduction in risk of acquiring or developing a disease or disorder (i.e., causing at least one of the clinical symptoms of the disease not to develop) in a subject that may be exposed to a disease-causing agent, or predisposed to the disease in advance of disease onset.

[0059] The term 'prophylaxis' is related to and encompassed in the term 'prevention', and refers to a measure or procedure the purpose of which is to prevent, rather than to treat or cure a disease. Non-limiting examples of prophylactic measures may include the administration of vaccines; the administration of low molecular weight heparin to hospital patients at risk for thrombosis due, for example, to immobilization; and the administration of an anti-malarial agent such as chloroquine, in advance of a visit to a geographical region where malaria is endemic or the risk of contracting malaria is high.

[0060] The term 'solvate' means a physical association of a compound useful in this invention with one or more solvent molecules. This physical association includes hydrogen bonding. In certain instances the solvate will be capable of isolation, for example when one or more solvent molecules are incorporated in the crystal lattice of the crystalline solid. "Solvate" encompasses both solution-phase and isolable solvates. Representative solvates include hydrates, ethanolates and methanolates.

[0061] The term 'subject' includes humans and other mammals.

[0062] 'Therapeutically effective amount' means that amount of a drug, compound, expression inhibitory agent, or pharmaceutical agent that will elicit the biological or medical response of a subject that is being sought by a medical doctor or other clinician.

[0063] The term 'treating' or 'treatment' of any disease or disorder refers, in one embodiment, to ameliorating the disease or disorder (i.e., arresting the disease or reducing the manifestation, extent or severity of at least one of the clinical symptoms thereof). In another embodiment 'treating' or 'treatment' refers to ameliorating at least one physical parameter, which may not be discernible by the subject. In yet another embodiment, 'treating' or 'treatment' refers to modulating the disease or disorder, either physically, (e.g., stabilization of a discernible symptom), physiologically, (e.g., stabilization of a physical parameter), or both. In a further embodiment, 'treating' or 'treatment' relates to slowing the progression of the disease.

[0064] The term "vectors" also relates to plasmids as well as to viral vectors, such as recombinant viruses, or the nucleic acid encoding the recombinant virus.

[0065] The term "vertebrate cells" means cells derived from animals having vertera structure, including fish, avian, reptilian, amphibian, marsupial, and mammalian species. Preferred cells are derived from mammalian species, and most preferred cells are human cells. Mammalian cells include feline, canine, bovine, equine, caprine, ovine, porcine murine, such as mice and rats, and rabbits.

[0066] The term 'TARGET' or 'TARGETS' means the protein(s) identified in accordance with the assays described herein and determined to be involved in the modulation of mast cell activation . The term TARGET or TARGETS includes and contemplates alternative species forms, isoforms, and variants, such as splice variants, allelic variants, alternate in frame exons, and alternative or premature termination or start sites, including known or recognized isoforms or variants thereof such as indicated in Table 1.

[0067] The term 'disease characterized by mast cell activation' refers to a disease which involves, results at least in part from, or includes mast cell degranulation in response to the activation of mast cells, in particular where the mast cell degranulation results in the release of inflammatory mediators from mast cells. The term includes, but is not limited to, exemplary diseases selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis.

[0068] The term 'disease characterized by inflammation' refers to a disease which involves, results at least in part from or includes inflammation. The term includes, but is not limited to, exemplary diseases selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[0069] The term 'inflammatory mediators' refers to mediators which enhance, initiate or facilitate an inflammatory reaction or an inflammatory response, and may be selected from the following: Cytokines (e.g. TNFalpha, IL3, IL4, IL5, IL13, GM-CSF), Prostaglandins (e.g. PGD2), Leukotrienes (e.g. LTB4, LTC4, LTD4), metalloproteases, chymase, tryptase, growth factors (e.g. VEGF).

TARGETS

[0070] The present invention is based on the present inventors' discovery that the TARGETS are factors in the release of inflammatory mediators in particular histamine from human primary mast cells, whereby inhibition of the TARGETS results in suppression of the histamine release following activation of mast cells. The TARGETS are factors or protein molecules involved in the response of mast cells to activation such that their inhibition results in a suppression of the release of histamine and other inflammatory mediators. The TARGETS may also serve a role in inflammation and/or the inflammatory response in other cells, particularly in basophils and plasmacytoid dendritic cells.

[0071] The TARGETS listed in Table 1 below were identified herein as involved in the pathway that inhibits the release of inflammatory mediators and/or cytokines from mast cells, therefore, inhibitors of these TARGETS are able to inhibit degranulation of mast cells and are of use in the prevention and/or treatment of diseases characterised by mast cell degranulation. These TARGETS are proposed to have a general role in inflammatory responses via mast cells. Therefore these TARGETS are involved in diseases or conditions characterized by inflammation.

[0072] Therefore, in one aspect, the present invention relates to a method for assaying for drug candidate compounds that promote stabilization of mast cells comprising contacting the compound with a polypeptide comprising an amino acid sequence of SEQ ID NO: 20-38, or fragment thereof, under conditions that allow said polypeptide to bind to the compound, and detecting the formation of a complex between the polypeptide and the compound. In particular said method is used to identify an agent that inhibits the degranulation of mast cells. In particular said method may be used to identify drug candidate compounds that inhibit the release of inflammatory mediators from mast cells. One particular means of measuring the complex formation is to determine the binding affinity of said compound to said polypeptide.

[0073] More particularly, the invention relates to a method for identifying an agent or compound that inhibits the degranulation of mast cells said method comprising:

(a) contacting a population of mammalian cells with one or more compound that exhibits binding affinity for a TARGET polypeptide, or fragment thereof, and

(b) measuring a compound-polypeptide property related to mast cell degranulation.

[0074] In a further aspect of the present invention said method is used to identify a compound that inhibits the release of inflammatory mediators from mast cells. In particular the inhibition of the release of histamine, proteoglycans, and/or cytokines may be assessed.

[0075] In a further aspect, the present invention relates to a method for assaying for drug candidate compounds that inhibit mast cell degranulation comprising contacting the compound with a polypeptide comprising an amino acid sequence selected from SEQ ID NO: 20-38, or a fragment thereof, under conditions that allow said compound to modulate the activity or expression of the polypeptide, and determining the activity or expression of the polypeptide. In particular said method may be used to identify drug candidate compounds capable of suppressing the release of inflammatory mediators from mast cells. One particular means of measuring the activity or expression of the polypeptide is to determine the amount of said polypeptide using a polypeptide binding agent, such as an antibody, or to determine the activity of said polypeptide in a biological or biochemical measure, for instance the amount of phosphorylation of a target of a kinase polypeptide.

[0076] The compound-polypeptide property referred to above is related to the expression and/or activity of the TARGET, and is a measurable phenomenon chosen by the person of ordinary skill in the art. The measurable property may be, for example, the binding affinity of said compound for a peptide domain of the polypeptide TARGET, a property related to the folding or activity of the disease-related protein or the level of any one of a number of biochemical marker levels of inflammation or of inflammatory mediators. In a preferred method, degranulation of mast cells is measured by measuring release of inflammatory mediators from mast cells, in particular the release of histamine, proteoglycans, and/or cytokines.

[0077] In an additional aspect, the present invention relates to a method for assaying for drug candidate compounds that inhibit mast cell degranulation, comprising contacting the compound with a nucleic acid encoding a TARGET polypeptide, including a nucleic acid sequence selected from SEQ ID NO: 1-19, or fragment thereof, under conditions that allow said nucleic acid to bind to or otherwise associate with the compound, and detecting the formation of a complex between the nucleic acid and the compound. In particular, said method may be used to identify drug candidate compounds able to suppress the release of inflammatory mediators from mast cells. One particular means of measuring the complex formation is to determine the binding affinity of said compound to said nucleic acid or the presence of a complex by virtue of resistance to nucleases or by gel mobility assays. Alternatively, complex formation may be determined by inhibition of nucleic acid transcription or translation.

[0078] In a particular embodiment of the invention, the TARGET polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID No: 20-38 as listed in Table 1. In an embodiment of the invention, the nucleic acid capable of encoding the TARGET polypeptide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1-19 as listed in Table 1. Table 1 provides TARGET exemplary human nucleic acid and protein sequence, including recognized variants or isoforms where more than one accession number and SEQ ID NO: is indicated. Isoforms or variants of the TARGET(S) include nucleic acid or proteins with or utilizing alternate in frame exons, alternative splicing or splice variants, and alternative or premature termination variants.

Table 1: TARGETS

TARGET Gene Symbol	GenBank nucleic acid Acc#:	SEQ ID NO: DNA	GenBank protein Acc#:	SEQ ID NO: Protein	Protein class
HTR1E	NM_000865	1	NP_000856	20	GPCR
SHKBP1	NM_138392	2	NP_612401	21	Ion Channel
FOLH1/PSMAL	NM_001014986 NM_004476 NM_153696	3 4 5	NP_001014986 NP_004467 NP_710163	22 23 24	Protease
FZD4	NM_012193	6	NP_036325	25	GPCR
MAP2K3	NM_002756 NM_145109	7 8	NP_002747 NP_659731	26 27	Kinase
GPR83	NM_016540	9	NP_057624	28	GPCR
STK39	NM_013233	10	NP_037365	29	Kinase
ULK2	NM_014683	11	NP_055498	30	Kinase
TRPC7	NM_020389	12	NP_065122	31	Ion Channel
TRPC3	NM_001130698 NM_003305	13 14	NP_001124170 NP_003296	32 33	Ion Channel
P2RY13	NM_176894 NM_023914	15 16	NP_795713 NP_076403	34 35	GPCR
TRPM8	NM_024080	17	NP_076985	36	Ion Channel
EPHA4	NM_004438	18	NP_004429	37	Kinase
CHRNA9	NM_017581	19	NP_060051	38	Ion Channel

[0079] Depending on the choice of the skilled artisan, the present assay method may be designed to function as a series of measurements, each of which is designed to determine whether the drug candidate compound is indeed acting on the TARGET to thereby inhibit degranulation of mast cells. For example, an assay designed to determine the binding affinity of a compound to the TARGET, or fragment thereof, may be necessary, but not sufficient, to ascertain whether the test compound would be useful for inhibiting mast cell degranulation when administered to a subject. Nonetheless, such binding information would be useful in identifying a set of test compounds for use in an assay that would measure a different property, further down the biochemical pathway, for example suppression of the release of inflammatory mediators. Such additional assay(s) may be designed to confirm that the test compound, having binding affinity for the TARGET, actually inhibits mast cell degranulation.

[0080] Suitable controls should always be in place to insure against false positive readings. In a particular embodiment of the present invention the screening method comprises the additional step of comparing the compound to a suitable control. In one embodiment, the control may be a cell or a sample

that has not been in contact with the test compound. In an alternative embodiment, the control may be a cell that does not express the TARGET; for example in one aspect of such an embodiment the test cell may naturally express the TARGET and the control cell may have been contacted with an agent, e.g. an siRNA, which inhibits or prevents expression of the TARGET. Alternatively, in another aspect of such an embodiment, the cell in its native state does not express the TARGET and the test cell has been engineered so as to express the TARGET, so that in this embodiment, the control could be the untransformed native cell. The control may also or alternatively utilize a known mediator of degranulation and/or inflammation, or a known mast cell marker, such as FcεR1, SYK, and/or BTK. Whilst exemplary controls are described herein, this should not be taken as limiting; it is within the scope of a person of skill in the art to select appropriate controls for the experimental conditions being used.

[0081] The order of taking these measurements is not believed to be critical to the practice of the present invention, which may be practiced in any order. For example, one may first perform a screening assay of a set of compounds for which no information is known respecting the compounds' binding affinity for the TARGET. Alternatively, one may screen a set of compounds identified as having binding affinity for a TARGET protein domain, or a class of compounds identified as being an inhibitor of the TARGET. However, for the present assay to be meaningful to the ultimate use of the drug candidate compounds in diseases characterized by mast cell degranulation and/or inflammation, a measurement of the stabilization of mast cells and/or inhibition of mast cell degranulation is necessary. Validation studies, including controls, and measurements of binding affinity to the polypeptides of the invention are nonetheless useful in identifying a compound useful in any therapeutic or diagnostic application.

[0082] Analogous approaches based on art-recognized methods and assays may be applicable with respect to the TARGETS and compounds in any of various disease(s) characterized by mast cell degranulation or inflammatory diseases. An assay or assays may be designed to confirm that the test compound, having binding affinity for the TARGET, inhibits the degranulation of mast cells after activation. In one such method the release of inflammatory mediators from mast cells is measured.

[0083] The present assay method may be practiced *in vitro*, using one or more of the TARGET proteins, or fragments thereof, including monomers, portions or subunits of polymeric proteins, peptides, oligopeptides and enzymatically active portions thereof.

[0084] The binding affinity of the compound with the TARGET or a fragment thereof can be measured by methods known in the art, such as using surface plasmon resonance biosensors (Biacore), by saturation binding analysis with a labeled compound (e.g. Scatchard and Lindmo analysis), by differential UV spectrophotometer, fluorescence polarization assay, Fluorometric Imaging Plate Reader (FLIPR®) system, Fluorescence resonance energy transfer, and Bioluminescence resonance energy transfer. The binding affinity of compounds can also be expressed in dissociation constant (Kd)

or as IC_{50} or EC_{50}. The IC_{50} represents the concentration of a compound that is required for 50% inhibition of binding of another ligand to the polypeptide. The EC_{50} represents the concentration required for obtaining 50% of the maximum effect in any assay that measures the TARGET function. The dissociation constant, Kd, is a measure of how well a ligand binds to the polypeptide, it is equivalent to the ligand concentration required to saturate exactly half of the binding-sites on the polypeptide. Compounds with a high affinity binding have low Kd, IC_{50} and EC_{50} values, i.e. in the range of 100 nM to 1 pM; a moderate to low affinity binding relates to a high Kd, IC_{50} and EC_{50} values, i.e. in the micromolar range.

[0085] The present assay method may also be practiced in a cellular assay. A host cell expressing the TARGET can be a cell with endogenous expression or a cell over-expressing the TARGET e.g. by transduction. When the endogenous expression of the polypeptide is not sufficient to determine a baseline that can easily be measured, one may use host cells that over-express the TARGET. Over-expression has the advantage that the level of the TARGET substrate end products is higher than the activity level by endogenous expression. Accordingly, measuring such levels using presently available techniques is easier. In one such cellular assay, the biological activity of the TARGET may be measured by measuring the release of inflammatory mediators from mast cells.

[0086] One embodiment of the present method for identifying a compound that inhibits mast cell degranulation comprises culturing a population of mammalian cells expressing a TARGET polypeptide, or a functional fragment or derivative thereof; determining a first level of inflammatory mediator release in said population of cells on activation of the population of cells (e.g. by the binding of an antigen to the cell surface IgE); exposing said population of cells to a compound, or a mixture of compounds; determining a second level inflammatory mediator release in said population of cells after the same activation, during or after exposure of said population of cells to said compound, or the mixture of said compounds; and identifying the compound(s) that suppress the release of inflammatory mediators. In a specific embodiment, the cells are mast cells, basophils, or plasmacytoid dendritic cells. In a further embodiment the cells are mast cells. In a specific embodiment the cells are human cells.

[0087] The release of inflammatory mediators from mast cells can be determined by methods known in the art such as the methods as described herein.

[0088] The present inventors identified TARGET genes involved in the inhibition of mast cell degranulation by using a 'knock-down' library. This type of library is a screen in which siRNA molecules are transduced into cells by recombinant adenoviruses, which siRNA molecules inhibit or repress the expression of a specific gene as well as expression and activity of the corresponding gene product in a cell. Each siRNA in a viral vector corresponds to a specific natural gene. By identifying a siRNA that inhibits degranulation of mast cells, as measured by suppression of the release of histamine, a direct correlation can be drawn between the specific gene expression and the pathway for inhibiting mast cell degranulation. The TARGET genes identified using the knock-down library (the protein

expression products thereof herein referred to as "TARGET" polypeptides) are then used in the present inventive method for identifying compounds that can be used to stabilize mast cells. Indeed, shRNA compounds comprising the sequences listed in Table 2 (SEQ ID NOs: 39-83) inhibit the expression and/or activity of these TARGET genes and suppress histamine release, confirming the role of the TARGETS in the pathway leading to degranulation of mast cells in response to activation of IgE.

Table 2: KD TARGET sequences useful in the practice of the present expression-inhibitory agent invention

TARGET Gene Symbol	SEQ ID NO: DNA	Sequences	SEQ ID NO: Knock-Down
HTR1E	1	GGCTGAGTGTGGACATGAC	39
HTR1E	1	TCTGTTCTCTGGCCGTGAC	40
SHKBP1	2	ACCTCCCAAGATGAAGCTC	41
SHKBP1	2	CTATACCCAGTTTCTAGTC	42
FOLH1	3, 4	TGTTTGAGCTAGCCAATTC	43
FOLH1	3, 4	ATCCACAGGAAATGAAGAC	44
FOLH1	4	AATGATGAATGATCAACTC	45
FOLH1	4	ATAGGCATGTCATCTATGC	46
PSMAL	5	TGTTTGAGCTAGCCAATTC	47
PSMAL	5	AATGATGAATGATCAACTC	48
PSMAL	5	ATAGGCATGTCATCTATGC	49
PSMAL	5	ATCCACAGGAAATGAAGAC	50
FZD4	6	TGACCTTCCTGATCGATTC	51
FZD4	6	CTATGATGCTGGCTTATAC	52
FZD4	6	GGATGGGACAAAGACAGAC	53
MAP2K3	7, 8	GGACTTCACTGCTCAGTGC	54
MAP2K3	7, 8	CGTATGAGCTACCTGGAGC	55
MAP2K3	7, 8	TGTGAAGCCCTCCAATGTC	56
MAP2K3	7, 8	CCAGAAGGGCTACAATGTC	57
GPR83	9	TACAGTGAGGACATTGTGC	58
GPR83	9	TGATCACGCTGCTCAACAC	59
GPR83	9	TGACACTGACAGCCATTGC	60
STK39	10	ATACAGTCCCTCTCTGTGC	61
STK39	10	GCAATAATAGCAACAATTC	62
ULK2	11	CAATCATTGGCTCTCCTAC	63

TARGET Gene Symbol	SEQ ID NO: DNA	Sequences	SEQ ID NO: Knock-Down
ULK2	11	GAAGGCCTAAGTAGGATTC	64
ULK2	11	TTCAGGAATTACCCAACTC	65
ULK2	11	TCACCTTATTTGGCTAATC	66
TRPC7	12	GTTCGTTGCTCATCCTAAC	67
TRPC7	12	CAAGACCCTTAACTTCAAC	68
TRPC7	12	CGGCTTATCTGAAGTAATC	69
TRPC7	12	AAGTGGTGGCCTTCAGACC	70
TRPC3	13	GTCCAGGTTAAACCTCTTC	71
TRPC3	13	TAAGGGAGCAGACCATAGC	72
P2RY13	15	TGACAGATTCCTCAAGATC	73
P2RY13	15	TATGGATCCCTTAATATAC	74
P2RY13	16	ACGCCCAACTCTTGAAGTC	75
TRPM8	17	TGGAAGATTATCCTGTGTC	76
TRPM8	17	AGGCATCGATTTAGACAAC	77
TRPM8	17	TGAAGAACGACATCAATGC	78
EPHA4	18	CTTGGGTGGATAGCAAGCC	79
EPHA4	18	AAGTTACCTTATTGGATTC	80
EPHA4	18	GAACTTGATTTCAAATGTC	81
CHRNA9	19	TGGTGGCAGAAATCATGCC	82
CHRNA9	19	TTCGTCCAGTGGAAGATAC	83

[0089] The present invention further relates to a method for identifying a compound that inhibits the degranulation of mast cells, comprising:

(a) contacting a compound with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38;

(b) determining the binding affinity of the compound to the polypeptide;

(c) contacting a population of mammalian cells expressing said polypeptide with the compound that exhibits at least a moderate binding affinity; and

(d) identifying the compound that inhibits the degranulation of mast cells.

[0090] In one aspect, the assay method includes contacting cells expressing said polypeptide with the compound that exhibits a binding affinity in the micromolar range. In an aspect, the binding affinity exhibited is at least 10 micromolar. In an aspect, the binding affinity is at least 1 micromolar. In an aspect, the binding affinity is at least 500 nanomolar.

[0091] The assay method may be based on the particular expression or activity of the TARGET polypeptide, including but not limited to an enzyme activity. Thus, assays for the enzyme TARGETs identified as SEQ ID NOs: 22-24, 26, 27, 29, 30 or 37 may be based on enzymatic activity or enzyme expression. Assays for the protease TARGETs identified as SEQ ID NOs: 22-24 may be based on protease activity or expression. Assays for the kinase TARGETs identified as SEQ ID NOs: 26, 27, 29, 30 or 37 may be based on kinase activity or expression, including but not limited to phosphorylation of a kinase target. Assays for the GPCR TARGETs identified as SEQ ID NO: 20, 25, 28, 34 or 35 may be based on GPCR activity or expression, including downstream mediators or activators. Assays for the ion channel TARGETs identified as SEQ ID NOs: 21, 31, 32, 33, 36 or 38 may use techniques well known to those of skill in the art including classical patch clamping, high-throughput fluorescence based or tracer based assays which measure the ability of a compound to open or close an ion channel thereby changing the concentration of fluorescent dyes or tracers across a membrane or within a cell. The measurable phenomenon, activity or property may be selected or chosen by the skilled artisan. The person of ordinary skill in the art may select from any of a number of assay formats, systems or design one using his knowledge and expertise in the art.

[0092] Table 1 lists the TARGETS identified using applicants' knock-down library in the HTRF assay described below, including the class of polypeptides identified. TARGETS have been identified in polypeptide classes including kinase, protease, GPCR, and ion channel, for instance. Specific methods to determine the activity of a kinase by measuring the phosphorylation of a substrate by the kinase, which measurements are performed in the presence or absence of a compound, are well known in the art.

[0093] Specific methods to determine the inhibition by a compound by measuring the cleavage of the substrate by the polypeptide, which is a protease, are well known in the art. Classically, substrates are used in which a fluorescent group is linked to a quencher through a peptide sequence that is a substrate that can be cleaved by the target protease. Cleavage of the linker separates the fluorescent group and quencher, giving rise to an increase in fluorescence.

[0094] Ion channels are membrane protein complexes and their function is to facilitate the diffusion of ions across biological membranes. Membranes, or phospholipid bilayers, build a hydrophobic, low dielectric barrier to hydrophilic and charged molecules. Ion channels provide a high conducting, hydrophilic pathway across the hydrophobic interior of the membrane. The activity of an ion channel can be measured using classical patch clamping. High-throughput fluorescence-based or tracer-based assays are also widely available to measure ion channel activity. These fluorescent-based assays screen compounds on the basis of their ability to either open or close an ion channel thereby changing the concentration of specific fluorescent dyes across a membrane. In the case of the tracer based assay, the changes in concentration of the tracer within and outside the cell are measured by radioactivity measurement or gas absorption spectrometry.

[0095] G-protein coupled receptors (GPCR) are capable of activating an effector protein, resulting in changes in second messenger levels in the cell. The activity of a GPCR can be measured by measuring the activity level of such second messengers. Two important and useful second messengers in the cell are cyclic AMP (cAMP) and Ca^{2+}. The activity levels can be measured by methods known to persons skilled in the art, either directly by ELISA or radioactive technologies or by using substrates that generate a fluorescent or luminescent signal when contacted with Ca^{2+} or indirectly by reporter gene analysis. The activity level of the one or more secondary messengers may typically be determined with a reporter gene controlled by a promoter, wherein the promoter is responsive to the second messenger. Promoters known and used in the art for such purposes are the cyclic-AMP responsive promoter that is responsive for the cyclic-AMP levels in the cell, and the NF-AT responsive promoter that is sensitive to cytoplasmic Ca^{2+}-levels in the cell. The reporter gene typically has a gene product that is easily detectable. The reporter gene can either be stably infected or transiently transfected in the host cell. Useful reporter genes are alkaline phosphatase, enhanced green fluorescent protein, destabilized green fluorescent protein, luciferase and β-galactosidase.

[0096] It should be understood that the cells expressing the polypeptides, may be cells naturally expressing the polypeptides, or the cells may be transfected to express the polypeptides, as described above. Also, the cells may be transduced to overexpress the polypeptide, or may be transfected to express a non-endogenous form of the polypeptide, which can be differentially assayed or assessed.

[0097] In one particular embodiment the methods of the present invention further comprise the step of contacting the population of cells with an agonist of the polypeptide. This is useful in methods wherein the expression of the polypeptide in a certain chosen population of cells is too low for a proper detection of its activity. By using an agonist the polypeptide may be triggered, enabling a proper read-out if the compound inhibits the polypeptide. Similar considerations apply to the measurement of the release of inflammatory mediators. In a particular embodiment, the cells used in the present method are mammalian mast cells. The mast cells, in the assay contemplated, may be activated (e.g. by cross linking the IgE receptor on the mast cells with a combination of IgE and anti-IgE).

[0098] A method for identifying a compound that stabilises mast cells, comprising:

(a) contacting a compound with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38, and fragments thereof; and

(b) measuring a compound-polypeptide property related to mast cell degranulation.

[0099] In one embodiment of the present invention the method relates to identifying a compound that inhibits the degranulation of mast cells.

[00100] In one embodiment of the present invention the compound-polypeptide property related to mast cell stabilisation is binding affinity.

[00101] In one embodiment of the present invention the compound-polypeptide property related to mast cell stabilisation is the suppression of the release of inflammatory mediators.

[00102] In one embodiment of the present invention the compound-polypeptide property related to mast cell stabilisation is the activity of said polypeptide. In particular, in one embodiment the compound inhibits the activity of said polypeptide.

[00103] In one embodiment of the present invention the compound-polypeptide property related to mast cell stabilisation is the expression of said polypeptide. In particular, in one embodiment the compound inhibits the expression of said polypeptide.

[00104] The present invention further relates to a method for identifying a compound that stabilizes mast cells, wherein said compound exhibits at least a moderate binding affinity to an amino acid selected from the group of SEQ ID NOS: 20-38, said method comprising:

a) contacting a compound with a population of mammalian mast cells expressing a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 20-38, wherein the cells have been activated;

b) determining the release of inflammatory mediators from said cells; and

c) identifying the compound that stabilizes mast cells as the compound which suppresses the release of inflammatory mediators from the cells.

[00105] In one such method, the compound exhibits a binding affinity to an amino acid selected from the group of SEQ ID NOS: 20-38 of at least 10 micromolar. In an aspect, the binding affinity is at least 1 micromolar. In an aspect, the binding affinity is at least 500 nanomolar.

[00106] The present invention further relates to a method for identifying a compound that stabilizes mast cells, said method comprising:

a) contacting a compound with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38;

b) determining the binding affinity of the compound to the polypeptide;

c) contacting a population of mammalian cells expressing said polypeptide with the compound that exhibits a binding affinity of at least 10 micromolar; and

d) identifying the compound that stabilizes mast cells.

[00107] The present invention further relates to a method for identifying a compound that stabilizes mast cells said method comprising:

a) contacting a compound with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38;

b) determining the ability of the compound to inhibit the expression or activity of the polypeptide;

c) contacting a population of mammalian cells expressing said polypeptide with the compound that significantly inhibits the expression or activity of the polypeptide ; and

d) identifying the compound that stabilizes mast cells.

[00108] In a particular aspect of the present invention the methods described above include the additional step of comparing the compound to be tested to a control, where the control is a population of cells that have not been contacted with the test compound.

[00109] In a particular aspect of the present invention the methods described above include the additional step of comparing the compound to be tested to a control, where the control is a population of cells that do not express said polypeptide.

[00110] For high-throughput purposes, libraries of compounds may be used such as antibody fragment libraries, peptide phage display libraries, peptide libraries (e.g. LOPAP™, Sigma Aldrich), lipid libraries (BioMol), synthetic compound libraries (e.g. LOPAC™, Sigma Aldrich, BioFocus DPI) or natural compound libraries (Specs, TimTec).

[00111] Preferred drug candidate compounds are low molecular weight compounds. Low molecular weight compounds, i.e. with a molecular weight of 500 Dalton or less, are likely to have good absorption and permeation in biological systems and are consequently more likely to be successful drug candidates than compounds with a molecular weight above 500 Dalton (Lipinski et al. (1997) Adv Drug Del Rev 23: 3–25). Peptides comprise another preferred class of drug candidate compounds. Peptides may be excellent drug candidates and there are multiple examples of commercially valuable peptides such as fertility hormones and platelet aggregation inhibitors. Natural compounds are another preferred class of drug candidate compound. Such compounds are found in and extracted from natural sources, and which may thereafter be synthesized. The lipids are another preferred class of drug candidate compound.

[00112] Another preferred class of drug candidate compounds is an antibody. The present invention also provides antibodies directed against the TARGETS. These antibodies may be endogenously produced to bind to the the TARGETS within the cell, or added to the tissue to bind to the TARGET polypeptide present outside the cell. These antibodies may be monoclonal antibodies or polyclonal antibodies. The present invention includes chimeric, single chain, and humanized antibodies, as well as FAb fragments and the products of a FAb expression library, and Fv fragments and the products of an Fv expression library.

[00113] In certain embodiments, polyclonal antibodies may be used in the practice of the invention. The skilled artisan knows methods of preparing polyclonal antibodies. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. Antibodies may also be generated against the intact TARGET protein or polypeptide, or against a fragment, derivatives including conjugates, or other epitope of the the TARGET protein or polypeptide, such as the TARGET embedded in a cellular membrane, or a library of antibody variable regions, such as a phage display library.

[00114] It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants that may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). One skilled in the art without undue experimentation may select the immunization protocol.

[00115] In some embodiments, the antibodies may be monoclonal antibodies. Monoclonal antibodies may be prepared using methods known in the art. The monoclonal antibodies of the present invention may be "humanized" to prevent the host from mounting an immune response to the antibodies. A "humanized antibody" is one in which the complementarity determining regions (CDRs) and/or other portions of the light and/or heavy variable domain framework are derived from a non-human immunoglobulin, but the remaining portions of the molecule are derived from one or more human immunoglobulins. Humanized antibodies also include antibodies characterized by a humanized heavy chain associated with a donor or acceptor unmodified light chain or a chimeric light chain, or vice versa. The humanization of antibodies may be accomplished by methods known in the art (see, e.g. Mark and Padlan, (1994) "Chapter 4. Humanization of Monoclonal Antibodies", The Handbook of Experimental Pharmacology Vol. 113, Springer-Verlag, New York). Transgenic animals may be used to express humanized antibodies.

[00116] Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom and Winter, (1991) J. Mol. Biol. 227:381-8; Marks *et al.* (1991). J. Mol. Biol. 222:581-97). The techniques of Cole, *et al.* and Boerner, *et al.* are also available for the preparation of human monoclonal antibodies (Cole, *et al.* (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77; Boerner, *et al* (1991). J. Immunol., 147(1):86-95).

[00117] Techniques known in the art for the production of single chain antibodies can be adapted to produce single chain antibodies to the TARGETS. The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain cross-linking. Alternatively; the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent cross-linking.

[00118] Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens and preferably for a cell-surface protein or receptor or receptor subunit. In one such embodiment, one of the binding specificities is for one domain of the TARGET; the other one is for another domain of the the TARGET.

[00119] Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, (1983) Nature 305:537-9). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. Affinity chromatography steps usually accomplish the purification of the correct molecule. Similar procedures are disclosed in Trauneeker, et al. (1991) EMBO J. 10:3655-9.

[00120] According to another preferred embodiment, the assay method uses a drug candidate compound identified as having a binding affinity for the TARGET, and/or has already been identified as having down-regulating activity such as antagonist activity for the TARGET.

[00121] *In vivo* animal models of inflammation or inflammatory diseases may be utilized by the skilled artisan to further or additionally screen, assess, and/or verify the agents or compounds identified in the present invention, including further assessing TARGET modulation *in vivo*. Such animal models include, but are not limited to, ulcerative colitis models, multiple sclerosis models (including EAE, lysolecithin-induced), arthritis models, allergic asthma models, airway inflammation models, and acute inflammation models.

[00122] The present invention further relates to a method for stabilizing mast cells comprising contacting said cells with an expression inhibitory agent comprising a polynucleotide sequence that complements at least about 15 to about 30, particularly at least 17 to about 30, most particularly at least 17 to about 25 contiguous nucleotides of a nucleotide sequence encoding a polypeptide TARGET or portion thereof including the nucleotide sequences selected from the group consisting of SEQ ID NO: 1-19.

[00123] Another aspect of the present invention relates to a method for stabilizing mast cells, comprising contacting said cell with an expression-inhibiting agent that inhibits the translation in the cell of a polyribonucleotide encoding the TARGET. A particular embodiment relates to a composition comprising a polynucleotide including at least one antisense strand that functions to pair the agent with the the TARGET mRNA, and thereby down-regulate or block the expression of the TARGET. The inhibitory agent preferably comprises antisense polynucleotide, a ribozyme, and a small interfering RNA (siRNA), wherein said agent comprises a nucleic acid sequence complementary to, or engineered from, a naturally-occurring polynucleotide sequence encoding a portion of a polypeptide comprising the amino acid sequence SEQ ID NO: 20-38. In a preferred embodiment the expression-inhibiting agent is complementary to a polynucleotide sequence consisting of SEQ ID NO: 1-19. In another preferred embodiment the expression-inhibiting agent is complementary to a polynucleotide sequence selected from the group consisting of SEQ ID NO: 39-83.

[00124] An embodiment of the present invention relates to a method wherein the expression-inhibiting agent is selected from the group consisting of antisense RNA, antisense oligodeoxynucleotide (ODN), a

ribozyme that cleaves the polyribonucleotide coding for SEQ ID NO: 1-19, a small interfering RNA (siRNA, preferably shRNA,) that is sufficiently complementary to a portion of the polyribonucleotide coding for SEQ ID NO: 20-38 such that the siRNA, preferably shRNA, interferes with the translation of the TARGET polyribonucleotide to the TARGET polypeptide. Preferably the expression-inhibiting agent is an antisense RNA, ribozyme, antisense oligodeoxynucleotide, or siRNA, preferably shRNA, complementary to a nucleotide sequence selected from the group consisting of SEQ ID NO: 1-19. In another preferred embodiment, the nucleotide sequence is complementary to a polynucleotide selected from the group consisting of SEQ ID NO: 39-83.

[00125] The down regulation of gene expression using antisense nucleic acids can be achieved at the translational or transcriptional level. Antisense nucleic acids of the invention are preferably nucleic acid fragments capable of specifically hybridizing with all or part of a nucleic acid encoding the TARGET or the corresponding messenger RNA. In addition, antisense nucleic acids may be designed which decrease expression of the nucleic acid sequence capable of encoding the TARGET by inhibiting splicing of its primary transcript. Any length of antisense sequence is suitable for practice of the invention so long as it is capable of down-regulating or blocking expression of a nucleic acid coding for the TARGETS. Preferably, the antisense sequence is at least about 17 nucleotides in length. The preparation and use of antisense nucleic acids, DNA encoding antisense RNAs and the use of oligo and genetic antisense is known in the art.

[00126] One embodiment of expression-inhibitory agent is a nucleic acid that is antisense to a nucleic acid selected from the group consisting of SEQ ID NO: 1-19. For example, an antisense nucleic acid (e.g. DNA) may be introduced into cells *in vitro*, or administered to a subject *in vivo*, as gene therapy to inhibit cellular expression of a nucleic acid selected from the group constisting of SEQ ID NO: 1-19. Antisense oligonucleotides preferably comprise a sequence containing from about 15 to about 100 nucleotides and more preferably the antisense oligonucleotides comprise from about 17 to about 30, most particularly at least 17 to about 25. Antisense nucleic acids may be prepared from about 10 to about 30 contiguous nucleotides complementary to a nucleic acid sequence selected from the sequences of SEQ ID NO: 1-19.

[00127] The skilled artisan can readily utilize any of several strategies to facilitate and simplify the selection process for antisense nucleic acids and oligonucleotides effective in inhibition of TARGET expression and/or inhibition of degranulation of mast cells. Predictions of the binding energy or calculation of thermodynamic indices between an olionucleotide and a complementary sequence in an mRNA molecule may be utilized (Chiang et al. (1991) J. Biol. Chem. 266:18162-18171; Stull et al. (1992) Nucl. Acids Res. 20:3501-3508). Antisense oligonucleotides may be selected on the basis of secondary structure (Wickstrom et al (1991) in Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, Wickstrom, ed., Wiley-Liss, Inc., New York, pp. 7-24; Lima et al. (1992) Biochem. 31:12055-12061). Schmidt and Thompson (U.S. Patent 6,416,951) describe a method for identifying a

functional antisense agent comprising hybridizing an RNA with an oligonucleotide and measuring in real time the kinetics of hybridization by hybridizing in the presence of an intercalation dye or incorporating a label and measuring the spectroscopic properties of the dye or the label's signal in the presence of unlabelled oligonucleotide. In addition, any of a variety of computer programs may be utilized which predict suitable antisense oligonucleotide sequences or antisense targets utilizing various criteria recognized by the skilled artisan, including for example the absence of self-complementarity, the absence hairpin loops, the absence of stable homodimer and duplex formation (stability being assessed by predicted energy in kcal/mol). Examples of such computer programs are readily available and known to the skilled artisan and include the OLIGO 4 or OLIGO 6 program (Molecular Biology Insights, Inc., Cascade, CO) and the Oligo Tech program (Oligo Therapeutics Inc., Wilsonville, OR). In addition, antisense oligonucleotides suitable in the present invention may be identified by screening an oligonucleotide library, or a library of nucleic acid molecules, under hybridization conditions and selecting for those which hybridize to the target RNA or nucleic acid (see for example U.S. Patent 6,500,615). Mishra and Toulme have also developed a selection procedure based on selective amplification of oligonucleotides that bind target (Mishra et al (1994) Life Sciences 317:977-982). Oligonucleotides may also be selected by their ability to mediate cleavage of target RNA by RNAse H, by selection and characterization of the cleavage fragments (Ho et al (1996) Nucl Acids Res 24:1901-1907; Ho et al (1998) Nature Biotechnology 16:59-630). Generation and targeting of oligonucleotides to GGGA motifs of RNA molecules has also been described (U.S. Patent 6,277,981).

[00128] The antisense nucleic acids are preferably oligonucleotides and may consist entirely of deoxyribo-nucleotides, modified deoxyribonucleotides, or some combination of both. The antisense nucleic acids can be synthetic oligonucleotides. The oligonucleotides may be chemically modified, if desired, to improve stability and/or selectivity. Since oligonucleotides are susceptible to degradation by intracellular nucleases, the modifications can include, for example, the use of a sulfur group to replace the free oxygen of the phosphodiester bond. This modification is called a phosphorothioate linkage. Phosphorothioate antisense oligonucleotides are water soluble, polyanionic, and resistant to endogenous nucleases. In addition, when a phosphorothioate antisense oligonucleotide hybridizes to its target site, the RNA-DNA duplex activates the endogenous enzyme ribonuclease (RNase) H, which cleaves the mRNA component of the hybrid molecule. Oligonucleotides may also contain one or more substituted sugar moieties. Particular oligonucleotides comprise one of the following at the 2' position: OH, SH, SCH_3, F, OCN, heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide.

[00129] In addition, antisense oligonucleotides with phosphoramidite and polyamide (peptide) linkages can be synthesized. These molecules should be very resistant to nuclease degradation. Furthermore, chemical groups can be added to the 2' carbon of the sugar moiety and the 5 carbon (C-5) of pyrimidines to enhance stability and facilitate the binding of the antisense oligonucleotide to its target site. Modifications may include 2'-deoxy, O-pentoxy, O-propoxy, O-methoxy, fluoro, methoxyethoxy phosphorothioates, modified bases, as well as other modifications known to those of skill in the art.

[00130] Another type of expression-inhibitory agent that can reduce the level of the TARGETS is the ribozyme. Ribozymes are catalytic RNA molecules (RNA enzymes) that have separate catalytic and substrate binding domains. The substrate binding sequence combines by nucleotide complementarity and, possibly, non-hydrogen bond interactions with its target sequence. The catalytic portion cleaves the target RNA at a specific site. The substrate domain of a ribozyme can be engineered to direct it to a specified mRNA sequence. The ribozyme recognizes and then binds a target mRNA through complementary base pairing. Once it is bound to the correct target site, the ribozyme acts enzymatically to cut the target mRNA. Cleavage of the mRNA by a ribozyme destroys its ability to direct synthesis of the corresponding polypeptide. Once the ribozyme has cleaved its target sequence, it is released and can repeatedly bind and cleave at other mRNAs.

[00131] Ribozyme forms include a hammerhead motif, a hairpin motif, a hepatitis delta virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) motif or Neurospora VS RNA motif. Ribozymes possessing a hammerhead or hairpin structure are readily prepared since these catalytic RNA molecules can be expressed within cells from eukaryotic promoters (Chen, et al. (1992) Nucleic Acids Res. 20:4581-9). A ribozyme of the present invention can be expressed in eukaryotic cells from the appropriate DNA vector. If desired, the activity of the ribozyme may be augmented by its release from the primary transcript by a second ribozyme (Ventura, et al. (1993) Nucleic Acids Res. 21:3249-55).

[00132] Ribozymes may be chemically synthesized by combining an oligodeoxyribonucleotide with a ribozyme catalytic domain (20 nucleotides) flanked by sequences that hybridize to the target mRNA after transcription. The oligodeoxyribonucleotide is amplified by using the substrate binding sequences as primers. The amplification product is cloned into a eukaryotic expression vector.

[00133] Ribozymes are expressed from transcription units inserted into DNA, RNA, or viral vectors. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol (I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on nearby gene regulatory sequences. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Gao and Huang, (1993) Nucleic Acids Res. 21:2867-72). It has been demonstrated that ribozymes expressed

from these promoters can function in mammalian cells (Kashani-Sabet, et al. (1992) Antisense Res. Dev. 2:3-15).

[00134] A particularly preferred inhibitory agent is a small interfering RNA (either double stranded siRNA molecules, or in a particular embodiment self-complementary single-stranded siRNA molecules (shRNA)). siRNA, particularly shRNA, mediate the post-transcriptional process of gene silencing by double stranded RNA (dsRNA) that is homologous in sequence to the silenced RNA. siRNA according to the present invention comprises a sense strand of 15-30, particularly 17-30, most particularly 17-25 nucleotides complementary or homologous to a contiguous 17-25 nucleotide sequence of a sequence selected from the group consisting of SEQ ID NO: 1-19, and an antisense strand of 17-25 nucleotides complementary to the sense strand. Exemplary sequences are described as sequences complementary to SEQ ID NO: 39-83. The most preferred siRNA comprises sense and anti-sense strands that are 100 per cent complementary to each other and the target polynucleotide sequence. Preferably the siRNA further comprises a loop region linking the sense and the antisense strand.

[00135] A self-complementing single stranded siRNA molecule polynucleotide according to the present invention comprises a sense portion and an antisense portion connected by a loop region linker. Preferably, the loop region sequence is 4-30 nucleotides long, more preferably 5-15 nucleotides long and most preferably 12 nucleotides long. In a most particular embodiment the linker sequence is UUGCUAUA or GUUUGCUAUAAC (SEQ ID NO: 84). Self-complementary single stranded siRNAs form hairpin loops and are more stable than ordinary dsRNA. In addition, they are more easily produced from vectors.

[00136] Analogous to antisense RNA, the siRNA can be modified to confer resistance to nucleolytic degradation, or to enhance activity, or to enhance cellular distribution, or to enhance cellular uptake, such modifications may consist of modified internucleoside linkages, modified nucleic acid bases, modified sugars and/or chemical linkage of the siRNA to one or more moieties or conjugates. The nucleotide sequences are selected according to siRNA designing rules that give an improved reduction of the TARGET sequences compared to nucleotide sequences that do not comply with these siRNA designing rules (For a discussion of these rules and examples of the preparation of siRNA, WO 2004/094636, and US 2003/0198627, are hereby incorporated by reference).

[00137] The present invention also relates to compositions, and methods using said compositions, comprising a DNA expression vector capable of expressing a polynucleotide capable of stabilizing mast cell, in particular capable of inhibiting mast cell degranulation, and described hereinabove as an expression inhibition agent.

[00138] A particular aspect of these compositions and methods relates to the down-regulation or blocking of the expression of the TARGET by the induced expression of a polynucleotide encoding an intracellular binding protein that is capable of selectively interacting with the TARGET. An intracellular binding protein includes any protein capable of selectively interacting, or binding, with the

polypeptide in the cell in which it is expressed and neutralizing the function of the polypeptide. Preferably, the intracellular binding protein is a neutralizing antibody or a fragment of a neutralizing antibody having binding affinity to an epitope of a TARGET selected from the group consisting of SEQ ID NO: 20-38. More preferably, the intracellular binding protein is a single chain antibody.

[00139] A particular embodiment of this composition comprises the expression-inhibiting agent selected from the group consisting of antisense RNA, antisense oligodeoxynucleotide (ODN), a ribozyme that cleaves the polyribonucleotide coding for a TARGET selected from the group consisting of SEQ ID NO: 20-38, and a small interfering RNA (siRNA) that is sufficiently homologous to a portion of the polyribonucleotide coding for a TARGET selected from the group consisting of SEQ ID NO: 20-38, such that the siRNA interferes with the translation of the TARGET polyribonucleotide to the TARGET polypeptide.

[00140] The polynucleotide expressing the expression-inhibiting agent, or a polynucleotide expressing the TARGET polypeptide in cells, is particularly included within a vector. The polynucleic acid is operably linked to signals enabling expression of the nucleic acid sequence and is introduced into a cell utilizing, preferably, recombinant vector constructs, which will express the antisense nucleic acid once the vector is introduced into the cell. A variety of viral-based systems are available, including adenoviral, retroviral, adeno-associated viral, lentiviral, herpes simplex viral or a sendaiviral vector systems, and all may be used to introduce and express polynucleotide sequence for the expression-inhibiting agents or the polynucleotide expressing the TARGET polypeptide in the target cells.

[00141] Particularly, the viral vectors used in the methods of the present invention are replication defective. Such replication defective vectors will usually pack at least one region that is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), or be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution, partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques may be performed in vitro (on the isolated DNA) or in situ, using the techniques of genetic manipulation or by treatment with mutagenic agents. Preferably, the replication defective virus retains the sequences of its genome, which are necessary for encapsidating, the viral particles.

[00142] In a preferred embodiment, the viral element is derived from an adenovirus. Preferably, the vehicle includes an adenoviral vector packaged into an adenoviral capsid, or a functional part, derivative, and/or analogue thereof. Adenovirus biology is also comparatively well known on the molecular level. Many tools for adenoviral vectors have been and continue to be developed, thus making an adenoviral capsid a preferred vehicle for incorporating in a library of the invention. An adenovirus is capable of infecting a wide variety of cells. However, different adenoviral serotypes have different preferences for cells. To combine and widen the target cell population that an adenoviral capsid of the invention can enter in a preferred embodiment, the vehicle includes adenoviral fiber

proteins from at least two adenoviruses. Preferred adenoviral fiber protein sequences are serotype 17, 45 and 51. Techniques or construction and expression of these chimeric vectors are disclosed in US 2003/0180258 and US 2004/0071660, hereby incorporated by reference.

[00143] In a preferred embodiment, the nucleic acid derived from an adenovirus includes the nucleic acid encoding an adenoviral late protein or a functional part, derivative, and/or analogue thereof. An adenoviral late protein, for instance an adenoviral fiber protein, may be favorably used to target the vehicle to a certain cell or to induce enhanced delivery of the vehicle to the cell. Preferably, the nucleic acid derived from an adenovirus encodes for essentially all adenoviral late proteins, enabling the formation of entire adenoviral capsids or functional parts, analogues, and/or derivatives thereof. Preferably, the nucleic acid derived from an adenovirus includes the nucleic acid encoding adenovirus E2A or a functional part, derivative, and/or analogue thereof. Preferably, the nucleic acid derived from an adenovirus includes the nucleic acid encoding at least one E4-region protein or a functional part, derivative, and/or analogue thereof, which facilitates, at least in part, replication of an adenoviral derived nucleic acid in a cell. The adenoviral vectors used in the examples of this application are exemplary of the vectors useful in the present method of treatment invention.

[00144] Certain embodiments of the present invention use retroviral vector systems. Retroviruses are integrating viruses that infect dividing cells, and their construction is known in the art. Retroviral vectors can be constructed from different types of retrovirus, such as, MoMuLV ("murine Moloney leukemia virus") MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Lentiviral vector systems may also be used in the practice of the present invention.

[00145] In other embodiments of the present invention, adeno-associated viruses ("AAV") are utilized. The AAV viruses are DNA viruses of relatively small size that integrate, in a stable and site-specific manner, into the genome of the infected cells. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies.

[00146] In the vector construction, the polynucleotide agents of the present invention may be linked to one or more regulatory regions. Selection of the appropriate regulatory region or regions is a routine matter, within the level of ordinary skill in the art. Regulatory regions include promoters, and may include enhancers, suppressors, etc.

[00147] Promoters that may be used in the expression vectors of the present invention include both constitutive promoters and regulated (inducible) promoters. The promoters may be prokaryotic or eukaryotic depending on the host. Among the prokaryotic (including bacteriophage) promoters useful for practice of this invention are lac, lacZ, T3, T7, lambda P_R, P_L, and trp promoters. Among the eukaryotic (including viral) promoters useful for practice of this invention are ubiquitous promoters (e.g. HPRT, vimentin, actin, tubulin), therapeutic gene promoters (e.g. MDR type, CFTR, factor VIII), tissue-

specific promoters, including animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals, e.g. chymase gene control region which is active in mast cells (Liao *et al.*, (1997), Journal of Biological Chemistry, 272,: 2969-2976), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl, et al. (1984) Cell 38:647-58; Adames, et al. (1985) Nature 318:533-8; Alexander, et al. (1987) Mol. Cell. Biol. 7:1436-44), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder, et al. (1986) Cell 45:485-95), and beta-globin gene control region which is active in myeloid cells (Mogram, et al. (1985) Nature 315:338-40; Kollias, et al. (1986) Cell 46:89-94). .

[00148] Other promoters which may be used in the practice of the invention include promoters which are preferentially activated in dividing cells, promoters which respond to a stimulus (e.g. steroid hormone receptor, retinoic acid receptor), tetracycline-regulated transcriptional modulators, cytomegalovirus immediate-early, retroviral LTR, metallothionein, SV-40, E1a, and MLP promoters. Further promoters which may be of use in the practice of the invention include promoters which are active and/or expressed in mast cells, or other degranulating cells.

[00149] Additional vector systems include the non-viral systems that facilitate introduction of polynucleotide agents into a patient. For example, a DNA vector encoding a desired sequence can be introduced in vivo by lipofection. Synthetic cationic lipids designed to limit the difficulties encountered with liposome-mediated transfection can be used to prepare liposomes for in vivo transfection of a gene encoding a marker (Felgner, et. al. (1987) Proc. Natl. Acad Sci. USA 84:7413-7); see Mackey, et al. (1988) Proc. Natl. Acad. Sci. USA 85:8027-31; Ulmer, et al. (1993) Science 259:1745-8). The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner and Ringold, (1989) Nature 337:387-8). Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in International Patent Publications WO 95/18863 and WO 96/17823, and in U.S. Pat. No. 5,459,127. The use of lipofection to introduce exogenous genes into the specific organs in vivo has certain practical advantages and directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, for example, pancreas, liver, kidney, and the brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides, e.g., hormones or neurotransmitters, and proteins for example, antibodies, or non-peptide molecules could be coupled to liposomes chemically. Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, for example, a cationic oligopeptide (e.g., International Patent Publication WO 95/21931), peptides derived from DNA binding proteins (e.g., International Patent Publication WO 96/25508), or a cationic polymer (e.g., International Patent Publication WO 95/21931).

[00150] It is also possible to introduce a DNA vector in vivo as a naked DNA plasmid (see U.S. Pat. Nos. 5,693,622, 5,589,466 and 5,580,859). Naked DNA vectors for therapeutic purposes can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation,

microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (see, e.g., Wilson, et al. (1992) J. Biol. Chem. 267:963-7; Wu and Wu, (1988) J. Biol. Chem. 263:14621-4; Hartmut, et al. Canadian Patent Application No. 2,012,311, filed Mar. 15, 1990; Williams, et al (1991). Proc. Natl. Acad. Sci. USA 88:2726-30). Receptor-mediated DNA delivery approaches can also be used (Curiel, et al. (1992) Hum. Gene Ther. 3:147-54; Wu and Wu, (1987) J. Biol. Chem. 262:4429-32).

[00151] The present invention also provides biologically compatible, mast-cell degranulation inhibiting compositions comprising an effective amount of one or more compounds identified as TARGET inhibitors, and/or the expression-inhibiting agents as described hereinabove.

[00152] A biologically compatible composition is a composition, that may be solid, liquid, gel, or other form, in which the compound, polynucleotide, vector, and antibody of the invention is maintained in an active form, e.g., in a form able to effect a biological activity. For example, a compound of the invention would have inverse agonist or antagonist activity on the TARGET; a nucleic acid would be able to replicate, translate a message, or hybridize to a complementary mRNA of the TARGET; a vector would be able to transfect a target cell and express the antisense, antibody, ribozyme or siRNA as described hereinabove; an antibody would bind a the TARGET polypeptide domain.

[00153] A particular biologically compatible composition is an aqueous solution that is buffered using, e.g., Tris, phosphate, or HEPES buffer, containing salt ions. Usually the concentration of salt ions will be similar to physiological levels. Biologically compatible solutions may include stabilizing agents and preservatives. In a more preferred embodiment, the biocompatible composition is a pharmaceutically acceptable composition. Such compositions can be formulated for administration by topical, oral, parenteral, intranasal, subcutaneous, and intraocular, routes. Parenteral administration is meant to include intravenous injection, intramuscular injection, intraarterial injection or infusion techniques. The composition may be administered parenterally in dosage unit formulations containing standard, well-known non-toxic physiologically acceptable carriers, adjuvants and vehicles as desired.

[00154] A particular embodiment of the present composition invention is a pharmaceutical composition comprising a therapeutically effective amount of an expression-inhibiting agent as described hereinabove, in admixture with a pharmaceutically acceptable carrier. Another particular embodiment is a pharmaceutical composition for the treatment or prevention of a disease or condition involving mast cell degranulation, or a susceptibility to the condition, comprising an effective amount of the TARGET antagonist or inverse agonist, its pharmaceutically acceptable salts, hydrates, solvates, or prodrugs thereof in admixture with a pharmaceutically acceptable carrier. A further particular embodiment is a pharmaceutical composition for the treatment or prevention of a disease or condition involving inflammation, or a susceptibility to the condition, comprising an effective amount of the TARGET antagonist or inverse agonist, its pharmaceutically acceptable salts, hydrates, solvates, or prodrugs thereof in admixture with a pharmaceutically acceptable carrier.

[00155] Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient. Pharmaceutical compositions for oral use can be prepared by combining active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethyl-cellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate. Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinyl-pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

[00156] Pharmaceutical preparations that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

[00157] Preferred sterile injectable preparations can be a solution or suspension in a non-toxic parenterally acceptable solvent or diluent. Examples of pharmaceutically acceptable carriers are saline, buffered saline, isotonic saline (e.g. monosodium or disodium phosphate, sodium, potassium; calcium or magnesium chloride, or mixtures of such salts), Ringer's solution, dextrose, water, sterile water, glycerol, ethanol, and combinations thereof 1,3-butanediol and sterile fixed oils are conveniently employed as solvents or suspending media. Any bland fixed oil can be employed including synthetic mono- or di-glycerides. Fatty acids such as oleic acid also find use in the preparation of injectables.

[00158] The agents or compositions of the invention may be combined for administration with or embedded in polymeric carrier(s), biodegradable or biomimetic matrices or in a scaffold. The carrier, matrix or scaffold may be of any material that will allow composition to be incorporated and expressed and will be compatible with the addition of cells or in the presence of cells. Particularly, the carrier matrix or scaffold is predominantly non-immunogenic and is biodegradable. Examples of biodegradable materials include, but are not limited to, polyglycolic acid (PGA), polylactic acid (PLA), hyaluronic

acid, catgut suture material, gelatin, cellulose, nitrocellulose, collagen, albumin, fibrin, alginate, cotton, or other naturally-occurring biodegradable materials. It may be preferable to sterilize the matrix or scaffold material prior to administration or implantation, e.g., by treatment with ethylene oxide or by gamma irradiation or irradiation with an electron beam. In addition, a number of other materials may be used to form the scaffold or framework structure, including but not limited to: nylon (polyamides), dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (e.g., polyvinylchloride), polycarbonate (PVC), polytetrafluorethylene (PTFE, teflon), thermanox (TPX), polymers of hydroxy acids such as polylactic acid (PLA), polyglycolic acid (PGA), and polylactic acid-glycolic acid (PLGA), polyorthoesters, polyanhydrides, polyphosphazenes, and a variety of polyhydroxyalkanoates, and combinations thereof. Matrices suitable include a polymeric mesh or sponge and a polymeric hydrogel. In the particular embodiment, the matrix is biodegradable over a time period of less than a year, more particularly less than six months, most particularly over two to ten weeks. The polymer composition, as well as method of manufacture, can be used to determine the rate of degradation. For example, mixing increasing amounts of polylactic acid with polyglycolic acid decreases the degradation time. Meshes of polyglycolic acid that can be used can be obtained commercially, for instance, from surgical supply companies (e.g., Ethicon, N.J). In general, these polymers are at least partially soluble in aqueous solutions, such as water, buffered salt solutions, or aqueous alcohol solutions that have charged side groups, or a monovalent ionic salt thereof.

[00159] The composition medium can also be a hydrogel, which is prepared from any biocompatible or non-cytotoxic homo- or hetero-polymer, such as a hydrophilic polyacrylic acid polymer that can act as a drug absorbing sponge. Certain of them, such as, in particular, those obtained from ethylene and/or propylene oxide are commercially available. A hydrogel can be deposited directly onto the surface of the tissue to be treated, for example during surgical intervention.

[00160] Embodiments of pharmaceutical compositions of the present invention comprise a replication defective recombinant viral vector encoding the polynucleotide inhibitory agent of the present invention and a transfection enhancer, such as poloxamer. An example of a poloxamer is Poloxamer 407, which is commercially available (BASF, Parsippany, N.J.) and is a non-toxic, biocompatible polyol. A poloxamer impregnated with recombinant viruses may be deposited directly on the surface of the tissue to be treated, for example during a surgical intervention. Poloxamer possesses essentially the same advantages as hydrogel while having a lower viscosity.

[00161] The active expression-inhibiting agents may also be entrapped in microcapsules prepared, for example, by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980) 16th edition, Osol, A. Ed.

[00162] Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semi-permeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™. (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

[00163] As defined above, therapeutically effective dose means that amount of protein, polynucleotide, peptide, or its antibodies, agonists or antagonists, which ameliorate the symptoms or condition. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED_{50} (the dose therapeutically effective in 50% of the population) and LD_{50} (the dose lethal to 50% of the population). The dose ratio of toxic to therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD_{50}/ED_{50}. Pharmaceutical compositions that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies are used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

[00164] For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs. The animal model is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans. The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state, age, weight and gender of the patient; diet, desired duration of treatment, method of administration, time and frequency of administration, drug

combination(s), reaction sensitivities, and tolerance/response to therapy. Long acting pharmaceutical compositions might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

[00165] The pharmaceutical compositions according to this invention may be administered to a subject by a variety of methods. They may be added directly to target tissues, complexed with cationic lipids, packaged within liposomes, or delivered to target cells by other methods known in the art. Localized administration to the desired tissues may be done by direct injection, transdermal absorption, catheter, infusion pump or stent. The DNA, DNA/vehicle complexes, or the recombinant virus particles are locally administered to the site of treatment. Alternative routes of delivery include, but are not limited to, intravenous injection, intramuscular injection, subcutaneous injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. Examples of ribozyme delivery and administration are provided in Sullivan et al. WO 94/02595.

[00166] Antibodies according to the invention may be delivered as a bolus only, infused over time or both administered as a bolus and infused over time. Those skilled in the art may employ different formulations for polynucleotides than for proteins. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

[00167] As discussed hereinabove, recombinant viruses may be used to introduce DNA encoding polynucleotide agents useful in the present invention. Recombinant viruses according to the invention are generally formulated and administered in the form of doses of between about 10^4 and about 10^{14} pfu. In the case of AAVs and adenoviruses, doses of from about 10^6 to about 10^{11} pfu are preferably used. The term pfu ("plaque-forming unit") corresponds to the infective power of a suspension of virions and is determined by infecting an appropriate cell culture and measuring the number of plaques formed. The techniques for determining the pfu titre of a viral solution are well documented in the prior art.

[00168] In one aspect the present invention provides methods of preventing and/or treating disorders involving mast cell degranulation, said methods comprising administering to a subject a therapeutically effective amount of an agent as disclosed herein. In a particular embodiment, the agent is selected from an expression-inhibiting agent and an antibody. In a particular embodiment the disorder is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis.

[00169] In a further aspect the present invention provides a method of preventing and/or treating a disease characterized by inflammation, said method comprising administering to a subject a therapeutically effective amount of an agent as disclosed herein. In a particular embodiment, the agent is selected from an expression-inhibiting agent and an antibody. In a particular embodiment, the disease is selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant

rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[00170] A further aspect of the invention relates to a method of treating or preventing a disease involving activation of mast cells, comprising administering to said subject a therapeutically effective amount of an agent as disclosed herein. In a particular embodiment, the agent is selected from an expression-inhibiting agent and an antibody. In a particular embodiment the disorder is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis.

[00171] The invention also relates to the use of an agent as described above for the preparation of a medicament for treating or preventing a disease involving mast cell degranulation. In a particular embodiment, the disease is characterised by inflammation. In a particular embodiment of the present invention the disease is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis. In a particular embodiment the disease is selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[00172] The present invention also provides a method of treating and/or preventing a disease involving mast cell degranulation said method comprising administering, to a subject suffering from, or susceptible to, a disease involving mast cell degranulation., a pharmaceutical composition or compound as described herein, particularly a therapeutically effective amount of an agent which inhibits the expression or activity of a TARGET as identified herein. In one embodiment, the disease is characterized by inflammation. In a particular embodiment the disorder is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis. In a particular embodiment the disease is selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[00173] The invention also relates to an agent or a pharmaceutical composition as described above for use in the treatment and/or prevention of a disease involving mast cell degranulation. In a particular embodiment, the disease is characterised by inflammation. In a particular embodiment the disorder is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis,

urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis. In a particular embodiment the disease is selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[00174] Administration of the agent or pharmaceutical composition of the present invention to the subject patient includes both self-administration and administration by another person. The patient may be in need of treatment for an existing disease or medical condition, or may desire prophylactic treatment to prevent or reduce the risk for diseases and medical conditions characterized by mast cell degranulation. The agent of the present invention may be delivered to the subject patient orally, transdermally, via inhalation, injection, nasally, rectally or via a sustained release formulation.

[00175] Still another aspect of the invention relates to a method for diagnosing a pathological condition involving mast cell degranulation, comprising determining the amount of a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38 in a biological sample, and comparing the amount with the amount of the polypeptide in a healthy subject, wherein an increase of the amount of polypeptide compared to the healthy subject is indicative of the presence of the pathological condition. In a particular embodiment the disorder is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis. In one embodiment, the disease is characterized by inflammation. In a particular embodiment the disorder is selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[00176] Still another aspect of the invention relates to a method for diagnosing a pathological condition involving mast cell degranulation, comprising determining the activity of a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38 in a biological sample, and comparing the activity with the activity of the polypeptide in a healthy subject, wherein an increase of the activity of polypeptide compared to the healthy subject is indicative of the presence of the pathological condition. In a particular embodiment the disorder is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis. In one embodiment, the disease is characterized by inflammation. In a further embodiment the disease is selected from allergic airways disease (e.g.

asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[00177] Still another aspect of the invention relates to a method for diagnosing a pathological condition involving involving mast cell degranulation, comprising determining the nucleic acid sequence of at least one of the genes of SEQ ID NO: 1-19 within the genomic DNA of a subject; comparing the sequence with the nucleic acid sequence obtained from a database and/or a healthy subject; and identifying any difference(s) related to the onset or prevalence of the pathological conditions disclosed herein. In a particular embodiment the disorder is selected from asthma, allergic diseases (for example, allergy, allergic rhinitis, atopic dermatitis, urticaria, angioedema, food allergy, allergic conjunctivitis, anaphylaxis resulting from an allergic reaction), Chronic Obstructive Pulmonary Disease (COPD), Type I hypersensitivity reactions, multiple sclerosis, rheumatoid arthritis, parasitic infections, eczema, and atherosclerosis. In one embodiment, the disease is characterized by inflammation. In a further embodiment the disease is selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease, rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

[00178] The polypeptides or the polynucleotides of the present invention employed in the methods described herein may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. To perform the methods it is feasible to immobilize either the polypeptide of the present invention or the compound to facilitate separation of complexes from uncomplexed forms of the polypeptide, as well as to accommodate automation of the assay. Interaction (e.g., binding of) of the polypeptide of the present invention with a compound can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and microcentrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the polypeptide to be bound to a matrix. For example, the polypeptide of the present invention can be "His" tagged, and subsequently adsorbed onto Ni-NTA microtitre plates, or ProtA fusions with the polypeptides of the present invention can be adsorbed to IgG, which are then combined with the cell lysates (*e.g.*, $^{(35)}$S-labelled) and the candidate compound, and the mixture incubated under conditions favorable for complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the plates are washed to remove any unbound label, and the matrix is immobilized. The amount of radioactivity can be determined directly, or in the supernatant after dissociation of the complexes. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of the protein binding to the protein of the present invention quantitated from the gel using standard electrophoretic techniques.

[00179] Other techniques for immobilizing protein on matrices can also be used in the method of identifying compounds. For example, either the polypeptide of the present invention or the compound can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated protein molecules of

the present invention can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the polypeptides of the present invention but which do not interfere with binding of the polypeptide to the compound can be derivatized to the wells of the plate, and the polypeptide of the present invention can be trapped in the wells by antibody conjugation. As described above, preparations of a labeled candidate compound are incubated in the wells of the plate presenting the polypeptide of the present invention, and the amount of complex trapped in the well can be quantitated.

[00180] The polynucleotides encoding the TARGET polypeptides are identified as SEQ ID NO: 1-19. The present inventors show herein that transfection of mammalian cells with Ad-siRNAs targeting these genes decrease the functional activity of human primary mast cells.

[00181] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

[00182] The invention is further illustrated in the following figures and examples.

EXPERIMENTAL SECTION

Example 1: Culturing human mast cells from cord blood

[00183] A vial (10^8 cells/vial) of frozen Cord Blood Mononuclear Cells (CBMC) is thawed according the protocol supplied with the cells (purchased from Cambrex or AllCells). The cells are grown at 37°C, 5% CO_2 in Complete RPMI (add per 500 mL bottle RPMI 1640 (InVitrogen) Fetal Bovine Serum Heat Inactivated (ICN Biomedicals) (50mL); MEM non essential a.a. (InVitrogen) (5mL); L-Glutamin (200mM, 100x) (5mL) (InVitrogen); Pen/Strep (10000U:µg/mL) (5mL) (InVitrogen); Gentamicin (10mg/mL) (0.5 mL) (InVitrogen); 2-Mercaptoethanol (0.5mL) (InVitrogen); with the following cytokines added: human Stem Cell Factor (100 ng/mL final concentration) (Peprotech); human IL-6 (50 ng/mL final concentration) (Peprotech); human IL-10 (10 ng/mL final concentration) (Peprotech). The media with cytokines may be stored at 4°C for up to 2 weeks.

[00184] Typically, the cells are passed once a week in pre-warmed Complete RPMI (including cytokines SCF, IL-6, IL-10). For counting the cells, an aliquot of the cells is fixated by mixing 50 µL of cell suspension with 50 µL of 4% paraformaldehyde solution (PFA, 4%) (Sigma) in Coulter counter vials (Beckman Coulter). Cells are counted in 10 mL Isoton (Beckman Coulter) with the Coulter Counter using the protocol recommended by the supplier and the following settings: above 6 µm, dilution factor 200x. The cells are maintained in culture at $1*10^6$ cells/mL based on this counting each week. For passing the cells, cells are put in 50 mL Greiner tubes and then spun for 10 min. at 1000 rpm in an Eppendorf centrifuge 5810R. The supernatant is discarded and the cells are resuspended in the new

media, containing cytokines. Cells are incubated at 37°C, 5% CO_2. Typically, cultures with volumes of 4-10 mL are grown in T25 flasks, 10-25mL in T75 flasks, and 25-100mL in T175 flasks.

[00185] Cells are used for mast cell activation experiments after 6 to 12 weeks of culturing, preferably after 8 weeks. Typically, 3 to 8 days (preferably 6 days) prior to the activation of the mast cells, IgE is added to the culture in a final concentration of 0.5-5µg/mL (preferably 2µg/mL; Biodesign) and hIL-4 is added in a final concentration of 2-25 ng/mL, (preferably 10 ng/mL; Peprotech). When adenoviral infections are done in the experiment, the additions of IgE and hIL-4 may or may not be performed at the same day as the additions of the viruses.

Example 2: Characterization of mast cells

[00186] To monitor the maturation of the mononuclear cell mixture to mast cells, typically after 6-12 weeks of culturing a Toluidine Blue staining is performed. A sample of the culture (200µL) is loaded in the Cytofunnel (ThermoShandon) and spun for 7 min. at 700 rpm in the Cytospin4 (Thermoshandon) onto an object glass according to the manufacturer's instructions. Subsequently the slides are stained with 100-500 µL staining solution for approximately 10 min. at room temperature (RT) with a Toluidine Blue solution (0.2 grams Toluidine Blue O (Sigma) and 1.92 g citric acid monohydrate (Calbiochem) dissolved in 100 mL of 50% EthOH (Riedel-de Haën) in water). Slides are washed once by dipping the slide carefully into a container with excessive demineralized water. Slides then follow a sequence of baths to dehydrate the cells; Ethanol 70% bath, 5 min.; Ethanol 100% bath, 5 min.; Xylene bath, 5 min. A drop of Eukitt (O. Kindler GmbH & CO) was added onto the slide and covered with a coverslip. Pictures are taken with a High Resolution Microscope (Zeiss, Axioplan 2 imaging, 630x). Only if the cells were clearly granular, are they used for further characterization.

[00187] The cells may then be further characterized for expression of both receptors FcεRI and c-kit (i.e. the SCF receptor) by FACS analysis. Therefore, an aliquot of cells (1 mL ~ 10^6) is incubated o/n with 2 µg/mL IgE (purified human myeloma IgE Biodesign), cells are harvested to FACS tubes (5mL polystyrene Falcon tubes, Becton Dickinson), spun for 5 minutes at 1500 rpm (Eppendorf, 5810R) washed once with 0.5mL washing buffer (PBS (InVitrogen) with 5% BSA (Sigma)), cells are then spun for 5 minutes at 1500 rpm. Aliquots (0.1mL) are incubated with 1:20 diluted antibodies (FITC anti-human IgE (epsilon chains, DAKO, APC anti-Human CD117, BD and isotype controls) for 30 min on ice. Cells are washed twice with 0.5mL washing buffer. Cells are taken in 250µL wash buffer with 1% paraformaldehyde. The samples are analysed by standard FACS analysis using the FACSCalibur from Becton Dickinson. Results given in Figure 1 show mast cells positive for CD117 (c-kit) and Fc epsilon receptor I.

Example 3: Transduction of HMC by adenoviruses

[00188] Cord blood derived human cultivated mast cells may be used after 6-12 weeks of cultivation as described in Example 1 and Example 2. Typically over 90% of the cells in culture have a mast cell phenotype after cultivation as described herein. The mast cells (seeded at 75 000 cells per well in a 96-well plate) are infected with the adenoviruses Ad5C20Att01/A010800-AcGFP and Ad5C20Att01/A010800-empty at MOI 1000 to 2000. After 3 days post infection the cell are harvested in FACS tubes and washed once using washing buffer as in Example 2 and analysed by standard FACS analysis for fluorescent protein expression using the FACSCalibur from Becton Dickinson. The samples infected with Ad5C20Att01/A010800-AcGFP are compared to the negative control samples Ad5C20Att01/A010800-empty. The results shown in Figure 2 clearly show that human mast cells can be efficiently transduced by adenoviral vectors with capsid C20, 58 +/-11% (n=19) AcGFP positive cells using 2000 virus particles per cell.

Example 4: Degranulation assay (histamine)

[00189] Mediators produced by human mast cells have classically been divided into three categories: (1) preformed mediators, (2) newly synthesized lipid mediators, and (3) cytokines. To study the effect on the release of the preformed mediators, typically a histamine assay is performed upon stimulation of the mast cells, such as anti-IgE stimulation. For example, to determine the released histamine levels, matured human mast cell cultures (e.g. see Example 1) are primed with IgE (2 µg/mL) and IL-4 (10 ng/mL) for 6 days. Then the cells are then stimulated using complete RPMI (including SCF, IL-6 and IL-10) containing anti-IgE antibody at a concentration of 1500 ng/mL and prewarmed at 37°C or mock stimulation (no addition). After incubating the cells at 37°C, 5% CO_2 for 1 hrs, the supernatants of the samples are collected (50 µL). The remaining cells were lysed using a 0.5% triton X-100 solution and histamine present in the supernatants and the remaining cells was measured. The histamine levels were determined and the release of histamine was calculated using the following equation: % of histamine released = histamine in supernatant / total histamine present in the cells (cells + supernatant) *100%.

[00190] For detection of histamine, the HTRF histamine assay from Cisbio was performed according to the manufacturer's recommendations. The assay has a two-step protocol: acylation and detection. Acylation and detection can both be carried out in a single 384 low volume plate. HTRF (homogeneous time resolved fluorescence) is a technology based on TR-FRET (time-resolved fluorescence resonance energy transfer) chemistry. The HTRF signal is detected at two different wavelengths (620 nm and 665 nm) which are used to calculate the fluorescence ratio and compensate for compound interference and sample quenching.

[00191] The HTRF ratio is calculated using the equation: (665nm/620nm)*10000. Delta F is used for the comparison of day-to-day data and greatly improves inter-assay reproducibility. The negative control (no anti IgE) was used as an internal assay control. The calculation is done according to the following

formula: DFP (ΔF%): (Standard or sample HTRF Ratio – HTRF Ratio neg ctrl)/(HTRF Ratio neg ctrl)*100.

[00192] The HTRF Assay is used to screen an arrayed collection of 4224 different recombinant adenoviruses mediating the expression of shRNAs in mast cells. These shRNAs cause a reduction in expression levels of genes that contain homologous sequences by a mechanism known as RNA interference (RNAi). The 4224 Ad-shRNAs contained in the arrayed collection target approximately 1900 different transcripts. On average, every transcript is targeted by 2 to 3 independent Ad-shRNAs.

[0100] In the screen Ad-shRNA control viruses were utilized. The control viruses include two sets of negative control viruses: Ad5-empty, which does not contain an shRNA construct and Ad5-Luc_v13,which encodes an shRNA construct targeting luciferase, a gene that is not expressed in human cells, together with a positive control virus, Ad5-FCER1G v5, which encodes an shRNA targeting the human FcεR1. A representative example of the performance of the control viruses is shown in Figure 3. In Figure 4, datapoints obtained in the screening of the SilenceSelect collection in the histamine release assay are shown.

[00193] From these experiments it is demonstrated that inhibition of the TARGETS disclosed herein results in inhibition of histamine release from the human mast cell.

Example 5: Rescreen of the primary hits using independent repropagation material

[00194] To confirm the results of the identified Ad-shRNA in the HTRF Assay, the following approach may be taken. New aliquots of the viruses that express the shRNA hits were made. Titered aliquots of previously propagated control viruses luc_v13 are used on each plate to enable hitcalling per plate. The rescreen was performed on 96 well format.

[00195] 5E+04 cells in 100 µL priming media are plated per well of a 96well, V-bottom plate. Virus stocks are diluted according to titer & MOI in priming media as per the following calculation

$$\text{Volume} = \frac{\text{cell number } (5\text{x}10^4) \text{ x MOI } (2000)}{\text{Viral titer}}$$

[00196] 80uL of diluted virus is added per well i.e. cells in a total of 180uL per well

[00197] The cells are then allowed to be infected and primed for 5-6days. On day 6 the plates are spun at 1100 rpm for 5 minutes and viral supernatant is removed. Cells are washed with 100µL cbmc assay buffer (which is cbmc culture media without cytokines and containing 4% FCS) and plates are spun at 1100rpm. After removal of the wash, cells are stimulated by addition of 150µL of 1500ng/mL anti-IgE (DAKO) in cbmc assay buffer medium for 1hour. Plates are spun at 1100rpm for 5 minutes and 140µL supernatant is transferred to a second V-bottom plate. After a 2nd spin, 125µL supernatant is removed and stored at -20°C for histamine determination using ELISA (obtained from IBL international, catalog number RE59221).

Histamine Elisa -96 well format

[00198] This assay is based on the competition principle and the microtiter plate separation. An unknown amount of antigen present in the sample and a fixed amount of enzyme labeled antigen compete for the binding sites of the antibodies coated onto the wells. After incubation the wells are washed to stop the competition reaction. Having added the TMB substrate solution the concentration of antigen is inversely proportional to the optical density measured. The measured OD values of the standards are used to construct a calibration curve against which the unknown samples are calculated.

[00199] ELISA is performed according to Manufacturer's protocol. In brief, 25µL supernatant or standard are acylated. Samples are diluted appropriately based on previous test results for each batch of cells (usually1:3) with assay buffer and incubated with histamine-antiserum and histamine-peroxidase conjugate in a anti-rabbit Ig coated microtiter plate. Unbound reagent is washed off and bound histamine-peroxidase developed using TMB substrate. Reaction is stopped by addition of sulphuric acid and signal detected at 450nm using ThermoMax (Molecular Devices) microplate reader. High OD values represent low amounts of histamine in samples.

[00200] A quality control of target Ad-siRNAs was performed as follows: Target Ad-siRNAs are propagated using derivatives of PERC6.E2A cells (Crucell, Leiden, The Netherlands) in 96-well plates, followed by sequencing the siRNAs encoded by the target Ad-siRNA viruses. PERC6.E2A cells are seeded in 96 well plates at a density of 40,000 cells/well in 180 µL PER.E2A medium. Cells are then incubated overnight at 39°C in a 10% CO_2 humidified incubator. One day later, cells are infected with 1 µL of crude cell lysate from SilenceSelect® stocks containing target Ad-siRNAs. Cells are incubated further at 34°C, 10% CO_2 until appearance of cytopathic effect (as revealed by the swelling and rounding up of the cells, typically 7 days post infection). The supernatant is collected, and the virus crude lysate is treated with proteinase K by adding 4 µL Lysis buffer (1x Expand High Fidelity buffer with MgCl2 (Roche Molecular Biochemicals, Cat. No 1332465) supplemented with 1 mg/mL proteinase K (Roche Molecular Biochemicals, Cat No 745 723) and 0.45% Tween-20 (Roche Molecular Biochemicals, Cat No 1335465) to 12 µL crude lysate in sterile PCR tubes. These tubes are incubated at 55°C for 2 hours followed by a 15 minutes inactivation step at 95°C. For the PCR reaction, 1 µL lysate is added to a PCR master mix composed of 5 µL 10x Expand High Fidelity buffer with MgCl2, 0.5 µL of dNTP mix (10 mM for each dNTP), 1 µL of "Forward primer" (10 mM stock, sequence: 5' CCG TTT ACG TGG AGA CTC GCC 3' (SEQ. ID NO: 85), 1 µL of "Reverse Primer" (10 mM stock, sequence: 5' CCC CCA CCT TAT ATA TAT TCT TTC C 3') (SEQ. ID NO: 86), 0.2 µL of Expand High Fidelity DNA polymerase (3.5 U/µL, Roche Molecular Biochemicals) and 41.3 µL of H2O. PCR is performed in a PE Biosystems GeneAmp PCR system 9700 as follows: the PCR mixture (50 µL in total) is incubated at 95°C for 5 minutes; each cycle runs at 95°C for 15 sec., 55°C for 30 sec., 68°C for 4 minutes, and is repeated for 35 cycles. A final incubation at 68°C is performed for 7 minutes. For sequencing analysis, the siRNA constructs expressed by the target adenoviruses are amplified by PCR using primers complementary to vector sequences flanking the SapI site of the pIPspAdapt6-U6

plasmid. The sequence of the PCR fragments is determined and compared with the expected sequence. All sequences are found to be identical to the expected sequence.

Example 6. Toxicity assay

[00201] For the validation assays the virus stocks were repropagated to have enough material to perform the additional experiments. The average titer and sequences of the repropagated viruses were determined prior to use.

[00202] The CellTiter-Blue® Cell Viability assay from Promega was used as a toxicity assay. The CellTiter-Blue® Cell Viability Assay provides a homogeneous, fluorometric method for estimating the number of viable cells present in multiwell plates. It uses the indicator dye resazurin to measure the metabolic capacity of cells, an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent and was measured at 530/590nm using CytofluorII (PerSeptive Biosystems). A standard curve was generated by titrating cells to a known density on d0.

Example 7. On-target assay

[00203] For the on-target assay, the methods similar to the screen and rescreen were used as described above. For each target, 5 extra virus constructs were taken along to determine target specificity. The histamine release assay was performed as per the rescreen (see Example 5) except that the control viruses (luc_v13) were repropagated alongside the on-target viruses.

Example 8: Screening using IL-13 release assay / MTS data

[00204] As an alternative, a screen may be performed using IL-13 as an indicator molecule for IgE dependent cytokine release from human mast cells. Human mast cells (cultured as described in example 1) may be seeded in 96-well V-bottom plates at a density of $7.5x10^4$ cells / 150 µL. Mast cells may be seeded in complete RPMI supplemented with IgE and hIL-4 (as described in culture method).

[00205] Knockdown virus is added in 30µL to the cells. A typical titer of the library that may be used is 5.0E+09 Vp/mL (Vp=Virus particles). The virus is left on for 6 days. Six days post infection the cells are spun down at 1000 rpm for 10 minutes. Supernatants are discarded and the cell pellets are resuspended in 100 µL culture medium containing anti-IgE (DAKO, 069(501) at a concentration of 1500ng/mL. Cells are then incubated at 37°C, 5% CO_2 for 6 hours. After 6 hours of incubation, the cells are spun down at 1000 rpm for 10 minutes and 80 µL of the supernatant is collected in a 96-well V-bottom plate and stored at –20°C till further use.

[00206] When needed, the supernatants may be thawed and measured in an IL-13 ELISA assay. For this assay an IL-13 ELISA kit (CLB, the Netherlands), may be used basically according to the manufacturer's recommendation. Briefly, 50 µL dilution buffer (supplied with kit) was added to the 80

µL of supernatant taken from the cells after 6 hour stimulation with anti-IgE and mixed. Of this mix 100 µL was used in the IL-13 ELISA that was performed according to the manual supplied with the kit. A basal level of IL-13 is produced upon 6 hr stimulation with anti-IgE. Hits may be identified that regulate the production of hIL-13 in human mast cells.

[00207] When needed the cell pellets may be used for determining the number of viable mast cells in IL-13 release assay, the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS assay, cat. no. G5421, Promega) may be used. In this assay enzymatic activity of dehydrogenase in metabolically active cells is measured. This assay is performed according to the protocol provided in the kit. After taking supernatant (80 µL) for the IL-13 measurement (see above), additional amount of fresh medium is added to the cells (30 µL) to get a final volume (50 µL) that can be used in this assay. Combined MTS/PMS (phenazine methosulfate) solution (10 µL) is added to the cells which are then incubated at 37°C, 5% CO_2 for 1 hour. The conversion of MTS into aqueous, soluble formazan in metabolically active cells is measured by the amount of 490nm absorbance which is directly proportional to the number of living cells in measured samples.

[00208] These absorption values were used to calculate percentages of viable cells in all samples with untreated mast cells as a reference (= 100% viable cells) according to the following equation:

$$\% \text{ viable mast cells} = (A_{490} \text{ sample} / A_{490} \text{ untreated mast cells}_{\text{average of duplo}}) * 100.$$

Example 9: Release of additional cytokines

[00209] To measure other cytokines besides IL-13 measured in Example 9 above the supernatant that is pipetted from the cells after 6 hours of stimulation with anti-IgE as in example 9 may be used in an assay to determine TNFalpha, TSLP, and/or IL-25 release upon IgE-dependent mast cell stimulation. The levels of TNFalpha, TSLP, and/or IL-25 in the supernatant may be determined with the TNFalpha ELISA (e-Bioscience or other supplier) according to the manufacturer's recommendations.

Example 10: Assay determining the arachidonic acid products (LTC)

[00210] Mediators produced by human mast cells have classically been divided into three categories: (1) preformed mediators, (2) newly synthesized lipid mediators, and (3) cytokines. The major newly synthesized lipid mediators are metabolized from arachidonic acid and include prostaglandin D2 and leukotriene C4. Liberation of arachidonic acid from cellular lipid stores occurs with mast cell activation. To study the effect on the release of the newly synthesized mediators, the release of prostaglandin D2 and/or leukotriene C4 can be determined upon mast cell stimulation (e.g. anti-IgE stimulation). For example, to determine the released leukotriene C4 levels, matured human mast cell cultures (e.g. see Example 1) may be primed with IgE (2 µg/mL) and IL-4 (10 ng/mL) for 6 days. Then the cells are stimulated with an anti-IgE antibody or mock stimulation (no addition). After incubating the cells at 37°C 5% CO_2 for 1 to 6 hrs, the supernatants of the samples are collected. The assay used for

determining LTC4 levels was the ACE™ Competitive Enzyme Immunoassay (Cayman Chemical). The assay is based on the competition between LTC4 and an LTC4-acetylcholinesterase (AChE) conjugate (LTC4 tracer) for a limited amount of LTC4 antiserum. Because the concentration of the LTC4 tracer is held constant while the concentration of LTC4 varies, the amount of LTC4 tracer that is able to bind to the LTC4 antiserum will be inversely proportional to the concentration of LTC4 in the well. This antibody-LTC4 complex binds to a mouse monoclonal anti-rabbit antibody that has been previously attached to the well. The plate is then washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of LTC4 tracer bound to the well, which is inversely proportional to the amount of free LTC4 present in the well during the incubation.

We claim:

1. A method for identifying a compound that stabilizes mast cells, comprising:
   (a) contacting a compound with a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38, and fragments thereof; and
   (b) measuring a compound-polypeptide property related to mast cell activity.
2. The method according to claim 1, wherein said polypeptide is in an *in vitro* cell-free preparation.
3. The method according to claim 1, wherein said polypeptide is present in a mammalian cell.
4. The method of claim 2, wherein said property is a binding affinity of said compound to said polypeptide.
5. The method of any one of claims 1 to 4, wherein the method is used to identify compounds that inhibit mast cell degranulation.
6. The method of claim 4, which additionally comprises the steps of:
   c) contacting a population of mammalian cells expressing said polypeptide with the compound that exhibits a binding affinity of at least 10 micromolar; and
   d) identifying a compound that stabilizes mast cells.
7. The method of claim 1 or 3, wherein said property is the release of inflammatory mediators from mast cells
8. The method according to any one of claims 1 to 3, wherein said property is the activity of said polypeptide.
9. The method according to claims 1 or 3, wherein said property is the expression of said polypeptide.
10. The method according to claims 8 or 9, which additionally comprises the steps of:
    c) contacting a population of mammalian cells expressing said polypeptide with the compound that significantly inhibits the expression or activity of the polypeptide ; and
    d) identifying the compound that stabilizes mast cells.
11. The method according to any one of claims 1 to 10, which additionally comprises the step of comparing the compound to be tested to a control.
12. The method according to claim 11, wherein said control is where the polypeptide has not been contacted with said compound.
13. The method according to claim 6 or 10, which additionally comprises the step of comparing the compound to a control, wherein said control is a population of mammalian cells that does not express said polypeptide.
14. The method according to any one of claims 1 to 13, wherein said compound is selected from the group consisting of compounds of a commercially available screening library and compounds

having binding affinity for a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 20-38.

15. The method according to claim 1, wherein said compound is a peptide in a phage display library or an antibody fragment library.

16. An agent effective in stabilizing mast cells, selected from the group consisting of an antisense polynucleotide, a ribozyme, and a small interfering RNA (siRNA), wherein said agent comprises a nucleic acid sequence complementary to, or engineered from, a naturally-occurring polynucleotide sequence of about 17 to about 30 contiguous nucleotides of a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1-19.

17. The agent according to claim 16, wherein a vector in a mammalian cell expresses said agent.

18. The agent according to claim 16, which inhibits mast cell degranulation.

19. The agent according to claim 17, wherein said vector is an adenoviral, retroviral, adeno-associated viral, lentiviral, a herpes simplex viral or a sendaiviral vector.

20. The agent according to claim 16, wherein said antisense polynucleotide and said siRNA comprise an antisense strand of 17-25 nucleotides complementary to a sense strand, wherein said sense strand is selected from 17-25 continuous nucleotides of a nucleic acid sequence selected from the group consisting of SEQ ID NO: 1-19.

21. The agent according to claims 20, wherein said siRNA further comprises said sense strand.

22. The agent according to claim 21, wherein said sense strand is selected from the group consisting of SEQ ID NO: 39-83.

23. The agent according to claim 20, wherein said siRNA further comprises a loop region connecting said sense and said antisense strand.

24. The agent according to claim 23, wherein said loop region comprises a nucleic acid sequence selected from the group consisting of UUGCUAUA or GUUUGCUAUAAC (SEQ ID NO: 84).

25. The agent according to any one of claims 16 to 24, wherein said agent is an antisense polynucleotide, ribozyme, or siRNA comprising a nucleic acid sequence complementary to a nucleic acid sequence selected from the group consisting of SEQ ID NO: 39-83.

26. A pharmaceutical composition comprising a therapeutically effective amount of an agent according to any one of claims 16 to 25 in admixture with a pharmaceutically acceptable carrier.

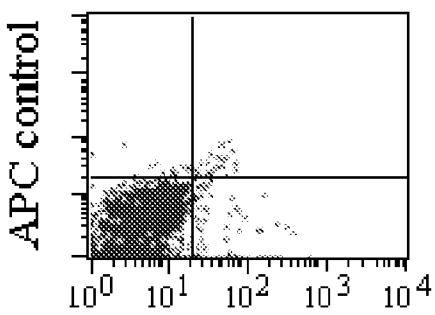
27. An agent according to any one of claims 16 to 25 for use in the treatment and/or prevention of a disease involving mast cell degranulation.

28. An agent according to any one of claims 16 to 25 for use in the treatment and/or prevention of a disease involving inflammation

29. The agent according to claim 27 or 28, wherein the disease is selected from allergic airways disease (e.g. asthma, rhinitis), autoimmune diseases, transplant rejection, Crohn's disease,

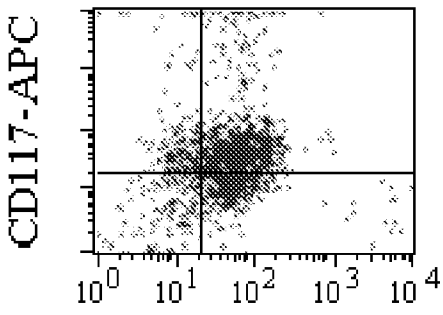
rheumatoid arthritis, psoriasis, juvenile idiopathic arthritis, colitis, and inflammatory bowel diseases.

Figure 1



FITC control

Control Strain



IgE-FITC

Double stained for FcεR and CD117

Figure 2

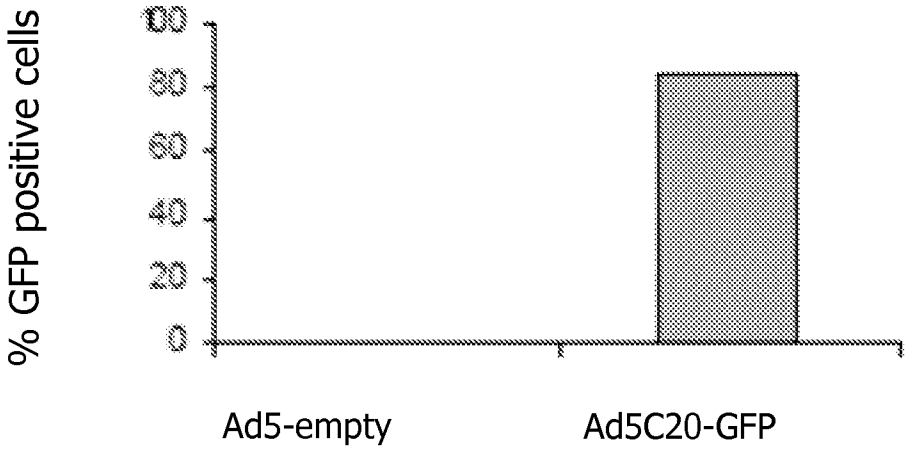


Figure 3

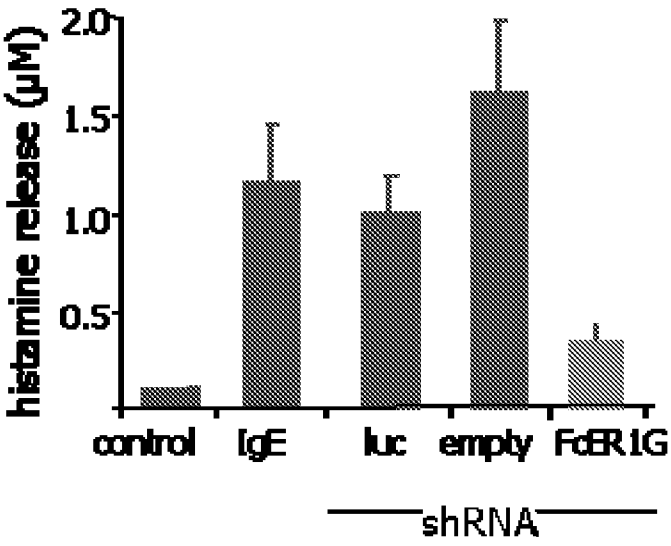
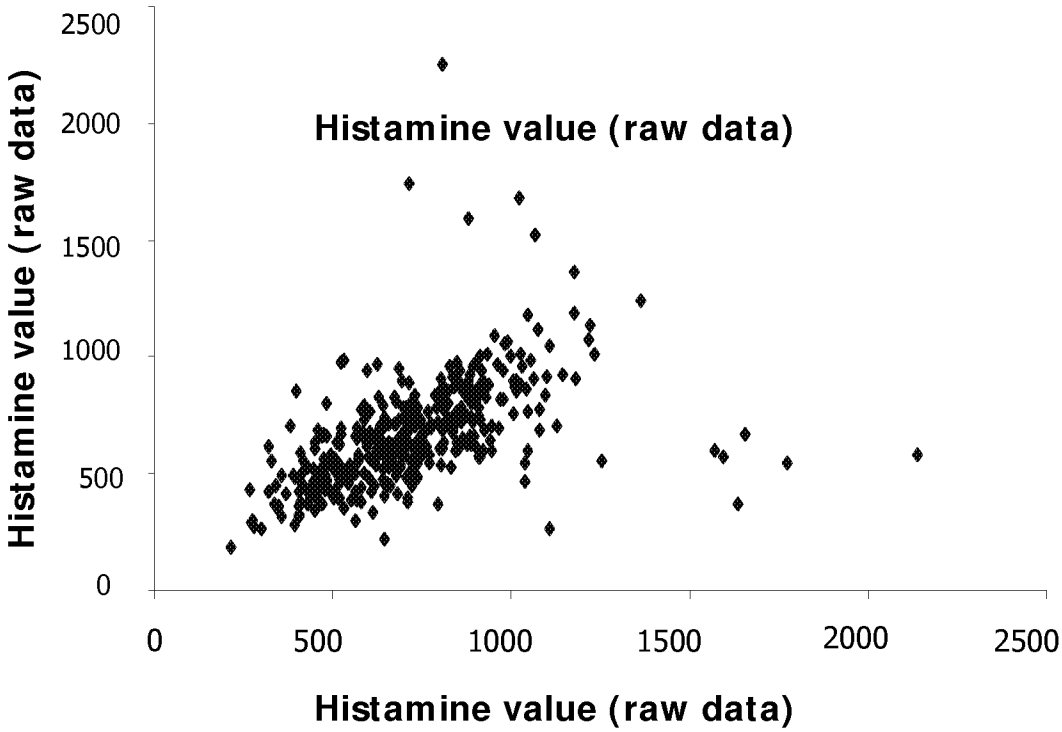

histamine release (µM)
2.0
1.5
1.0
0.5
control
IgE
luc
empty
FcεR1G
shRNA

Figure 4


Histamine value (raw data)
2500
2000
1500
1000
500
0
0
500
1000
1500
2000
2500
Histamine value (raw data)
Histamine value (raw data)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/052028

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/7105 A61K31/713 G01N33/50 C12N15/113

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 G01N C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, 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.
A	RUSSO ANNAPINA ET AL: "Inhibition of granuloma-associated angiogenesis by controlling mast cell mediator release: role of mast cell protease-5" BRITISH JOURNAL OF PHARMACOLOGY, vol. 145, no. 1, May 2005 (2005-05), pages 24-33, XP002577880 ISSN: 0007-1188 abstract figures 1,2,8 ----- -/--	1-29

[X] Further documents are listed in the continuation of Box C.

[X] 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 document 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	Date of mailing of the international search report
16 April 2010	15/06/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Hohwy, Morten

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/052028

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIVELY ET AL: "Effect of chemically modified IL-13 short interfering RNA on development of airway hyperresponsiveness in mice" JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, MOSBY, INC, US LNKD-DOI:10.1016/J.JACI.2007.08.029, vol. 121, no. 1, 22 October 2007 (2007-10-22), pages 88-94, XP022421015 ISSN: 0091-6749 page 91, column 1 figure 2	1-29
A	WO 01/29263 A1 (GENAISSANCE PHARMACEUTICALS [US]; CHOI JULIE Y [US]; DENTON R REX [US]) 26 April 2001 (2001-04-26) page 13	1-29
Y	KUSHNIR-SUKHOV NATALIYA M ET AL: "5-hydroxytryptamine induces mast cell adhesion and migration." JOURNAL OF IMMUNOLOGY (BALTIMORE, MD. : 1950) 1 NOV 2006 LNKD- PUBMED:17056574, vol. 177, no. 9, 1 November 2006 (2006-11-01), pages 6422-6432, XP002577881 ISSN: 0022-1767 Fig. 4c; Fig. 8b; p. 6431, col. 1, par. 1; p. 6431, col. 2 abstract	1-29
Y	DIETSCH G N ET AL: "THE ROLE OF MAST CELLS IN THE ELICITATION OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS" JOURNAL OF IMMUNOLOGY, vol. 142, no. 5, 1989, pages 1476-1481, XP002577882 ISSN: 0022-1767 abstract table 2	1-29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2010/052028

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-29(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-29(partially)

A method of identifying a compound that stabilizes mast cells by interacting with a polypeptide, an agent which stabilizes mast cells, wherein the agent is an oligonucleotide and targets said polypeptide, a pharmaceutical composition comprising said agent, and said agent for treatment of a disease involving inflammation, wherein said polypeptide is SEQ. ID. NO.:20.

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2. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:21.

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3. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:22.

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4. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:23.

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5. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:24.

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6. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:25.

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7. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:26.

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8. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:27.

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9. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:28.

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10. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:29.

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11. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:30.

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12. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:31.

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13. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:32.

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14. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:33.

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15. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:34.

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16. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:35.

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17. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:36.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

18. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:37

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19. claims: 1-29(partially)

Defined as Invention 1 but, wherein said polypeptide is SEQ. ID. NO.:38.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/052028

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0129263 A1	26-04-2001	AU 1570701 A	30-04-2001