Abstract:
The present invention relates to methods and pharmaceutical compositions for the treatment of cardiovascular fibrosis. In particular, the present invention relates to an inhibitor of Neutrophil Gelatinase-Associated Lipocalin (NGAL) activity or expression for use in a method for treating or preventing cardiovascular fibrosis in a subject in need thereof.
METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT
OF CARDIOVASCULAR FIBROSIS

FIELD OF THE INVENTION:
The present invention relates to methods and pharmaceutical compositions for the
treatment of cardiovascular fibrosis.

BACKGROUND OF THE INVENTION:
Hypertension and heart failure are major health problems worldwide. Patients with
hypertensive heart disease and heart failure develop cardiovascular fibrosis that significantly
reduces heart function. Typically, fibrosis develops when the body's natural wound-healing
process goes awry. Under normal conditions of wound healing, specialized cells known as
fibroblasts become activated by transforming to myofibroblast. The myofibroblasts then
undergo proliferation causing increased synthesis of collagen protein in the extracellular
matrix composed predominantly of type I collagen and to a lesser extent type III collagen
(normal wound healing process). What is initially an adaptive process, perhaps meant to
enhance tensile strength, can progress to maladaptive (pathologic) conditions when the
"healing" process persists with the development of diffuse and heterogeneous myocardial
fibrosis characterized by a matrix made of increased collagen deposits and myofibroblast
numbers. Thus, rationally designed antifibrotic therapies are highly desirable. The use of
agents to block the renin-angiotensinaldosterone and sympathetic nervous systems has been
shown to inhibit (and sometimes even reverse) cardiovascular fibrosis and to improve the
clinical course of patients with cardiac dysfunction. However, drugs aiming at direct
inhibition or reduction of cardiovascular fibrosis are not yet available, largely because the
underlying basis of cardiovascular fibrosis is unclear.

SUMMARY OF THE INVENTION:
The present invention relates to an inhibitor of Neutrophil Gelatinase-Associated
Lipocalin (NGAL) activity or expression for use in a method for treating or preventing
cardiovascular fibrosis in a subject in need thereof.

DETAILED DESCRIPTION OF THE INVENTION:
The present invention relates to an inhibitor of Neutrophil Gelatinase-Associated Lipocalin (NGAL) activity or expression for use in a method for treating or preventing cardiovascular fibrosis in a subject in need thereof.

The terms "subject," and "patient," used interchangeably herein, refer to a mammal, particularly a human who has been previously diagnosed with cardiovascular fibrosis or who is at risk for having or developing cardiovascular fibrosis. Typically, the subject may suffer from a cardiovascular disease leading to cardiovascular fibrosis such as hypertension or may suffer from a cardiovascular disease resulting from cardiovascular fibrosis such as heart failure. In a particular embodiment, cardiovascular fibrosis of the patient is induced by aldosterone. The aim of the invention is to reduce cardiovascular fibrosis in the patient.

As used herein the term "cardiovascular fibrosis" refers to the fibrosis that could affect the cardiovascular tree (whole or partly) including heart (i.e. cardiac fibrosis) or blood vessels (i.e. vascular fibrosis).

As used herein, the term "NGAL" has its general meaning in the art and refers to the Neutrophil Gelatinase-Associated Lipocalin as described in Schmidt-Ott KM. et al. (2007) (Schmidt-Ott KM, Mori K, Li JY, Kalandadze A, Cohen DJ, Devarajan P, Barasch J. Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol. 2007 Feb;18(2):407-13. Epub 2007 Jan 17. Review.). NGAL was shown to exist both as a 25-kDa monomer and a 45-kDa disulfide-linked homodimer, and it may also be covalently complexed with neutrophil gelatinase (also known as matrix metalloproteinase 9, MMP-9) via an intermolecular disulphide bridge as a 135-kDa heterodimeric form.

An "inhibitor of NGAL activity" has its general meaning in the art, and refers to a compound (natural or not) which has the capability of reducing or suppressing the activity of NGAL. For example the compound may block the interaction of NGAL with the NGAL binding ligands, or may bind to NGAL in manner that NGAL losses its capacity to interact with gelatinase, and thus altering gelatinase activity. Typically, said inhibitor is a small organic molecule or a biological molecule (e.g. peptides, lipid, antibody, aptamer...).

In a particular embodiment, the activity of NGAL can be reduced using a "dominant negative." To this end, constructs which encode, for example, defective NGAL polypeptide,
can be used in gene therapy approaches to diminish the activity of NGAL on appropriate target cells. For example, nucleotide sequences that direct host cell expression of NGAL in which all or a portion of the DNA binding domain is altered or missing can be administered to the subject (either by in vivo or ex vivo gene therapy methods known in the art).

Alternatively, targeted homologous recombination can be utilized to introduce such deletions or mutations into the subject's endogenous NGAL gene. The engineered cells will express non-functional NGAL polypeptides.

In one embodiment the inhibitor of NGAL activity may consist in an antibody (the term including antibody fragment) that can block NGAL activity.

Antibodies directed against the NGAL can be raised according to known methods by administering the appropriate antigen or epitope to a host animal selected, e.g., from pigs, cows, horses, rabbits, goats, sheep, and mice, among others. Various adjuvants known in the art can be used to enhance antibody production. Although antibodies useful in practicing the invention can be polyclonal, monoclonal antibodies are preferred. Monoclonal antibodies against NGAL can be prepared and isolated using any technique that provides for the production of antibody molecules by continuous cell lines in culture. Techniques for production and isolation include but are not limited to the hybridoma technique, the human B-cell hybridoma technique and the EBV-hybridoma technique. Alternatively, techniques described for the production of single chain antibodies can be adapted to produce anti-NGAL single chain antibodies. Inhibitor of NGAL activity useful in practicing the present invention also include anti-NGAL antibody fragments including but not limited to F(ab')2 fragments, which can be generated by pepsin digestion of an intact antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab and/or scFv expression libraries can be constructed to allow rapid identification of fragments having the desired specificity to NGAL.

Humanized anti-NGAL antibodies and antibody fragments therefrom can also be prepared according to known techniques. "Humanized antibodies" are forms of non-human (e.g., rodent) chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (CDRs) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity and capacity. In some instances, framework region (FR) residues of the human immunoglobulin
are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Methods for making humanized antibodies are described, for example, by Winter (U.S. Pat. No. 5,225,539) and Boss (Celltech, U.S. Pat. No. 4,816,397).

Then after raising antibodies directed against the NGAL as above described, the skilled man in the art can easily select those inhibiting NGAL activity.

In another embodiment the Inhibitor of NGAL activity is an aptamer directed against NGAL. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by Exponential enrichment (SELEX) of a random sequence library. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods. Then after raising aptamers directed against the NGAL as above described, the skilled man in the art can easily select those blocking NGAL activity.

In still another embodiment, the Inhibitor of NGAL activity may be a small organic molecule. Typically, the small organic molecule may target the ligand binding site as described in Grzyb J. et al. (Journal of Plant Physiology 163 (2006) 895—915) and Flower DR. et al. (Biochimica et Biophysica Acta 1482 (2000) 9-24) and thus impends the binding between NGAL and its ligands.
An "inhibitor of NGAL expression" refers to a natural or synthetic compound that has a biological effect to inhibit or significantly reduce the expression of the gene encoding for NGAL.

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

Small inhibitory RNAs (siRNAs) can also function as inhibitors of expression for use in the present invention. NGAL gene expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that NGAL gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known. All or part of the phosphodiester bonds of the siRNAs of the invention are advantageously protected. This protection is generally implemented via the chemical route using methods that are known by art. The phosphodiester bonds can be protected, for example, by a thiol or amine functional group or by a phenyl group. The 5' and/or 3' ends of the siRNAs of the invention are also advantageously protected, for example, using the technique described above for protecting the phosphodiester bonds. The siRNAs sequences advantageously comprises at least twelve contiguous dinucleotides or their derivatives.

As used herein, the term "siRNA derivatives" with respect to the present nucleic acid sequences refers to a nucleic acid having a percentage of identity of at least 90% with erythropoietin or fragment thereof, preferably of at least 95%, as an example of at least 98%, and more preferably of at least 98%.

As used herein, "percentage of identity" between two nucleic acid sequences, means the percentage of identical nucleic acid, between the two sequences to be compared, obtained
with the best alignment of said sequences, this percentage being purely statistical and the
differences between these two sequences being randomly spread over the nucleic acid acids
sequences. As used herein, "best alignment" or "optimal alignment", means the alignment for
which the determined percentage of identity (see below) is the highest. Sequences comparison
between two nucleic acids sequences are usually realized by comparing these sequences that
have been previously align according to the best alignment; this comparison is realized on
segments of comparison in order to identify and compared the local regions of similarity. The
best sequences alignment to perform comparison can be realized, beside by a manual way, by
using the global homology algorithm developed by SMITH and WATERMAN (Ad. App.
Math., vol.2, p:482, 1981), by using the local homology algorithm developed by
NEDDLEMAN and WUNSCH (J. Mol. Biol, vol.48, p:443, 1970), by using the method of
similarities developed by PEARSON and LIPMAN (Proc. Natl. Acd. Sci. USA, vol.85,
p:2444, 1988), by using computer software using such algorithms (GAP, BESTFIT, BLAST
P, BLAST N, FASTA, TFASTA in the Wisconsin Genetics software Package, Genetics
Computer Group, 575 Science Dr., Madison, WI USA), by using the MUSCLE multiple
To get the best local alignment, one can preferably used BLAST software. The identity
percentage between two sequences of nucleic acids is determined by comparing these two
sequences optimally aligned, the nucleic acids sequences being able to comprise additions or
deletions in respect to the reference sequence in order to get the optimal alignment between
these two sequences. The percentage of identity is calculated by determining the number of
identical position between these two sequences, and dividing this number by the total number
of compared positions, and by multiplying the result obtained by 100 to get the percentage of
identity between these two sequences.

shRNAs (short hairpin RNA) can also function as inhibitors of expression for use in
the present invention.

Ribozymes can also function as inhibitors of expression for use in the present
invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific
cleavage of RNA. The mechanism of ribozyme action involves sequence specific
hybridization of the ribozyme molecule to complementary target RNA, followed by
endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that
specifically and efficiently catalyze endonucleolytic cleavage of NGAL mRNA sequences are
thereby useful within the scope of the present invention. Specific ribozyme cleavage sites
within any potential RNA target are initially identified by scanning the target molecule for
ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and
GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides
corresponding to the region of the target gene containing the cleavage site can be evaluated
for predicted structural features, such as secondary structure, that can render the
oligonucleotide sequence unsuitable.

Both antisense oligonucleotides and ribozymes useful as inhibitors of expression can
be prepared by known methods. These include techniques for chemical synthesis such as, e.g.,
by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules
can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA
molecule. Such DNA sequences can be incorporated into a wide variety of vectors that
incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase
promoters. Various modifications to the oligonucleotides of the invention can be introduced
as a means of increasing intracellular stability and half-life. Possible modifications include
but are not limited to the addition of flanking sequences of ribonucleotides or
deoxyribonucleotides to the 5’ and/or 3’ ends of the molecule, or the use of phosphorothioate
or 2’-0-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides, siRNAs, shRNAs and ribozymes of the invention may be
delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any
vehicle capable of facilitating the transfer of the antisense oligonucleotide, siRNA, shRNA or
ribozyme nucleic acid to the cells and preferably cells expressing NGAL. Preferably, the
vector transports the nucleic acid to cells with reduced degradation relative to the extent of
degradation that would result in the absence of the vector. In general, the vectors useful in the
invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived
from viral or bacterial sources that have been manipulated by the insertion or incorporation of
the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequences. Viral
vectors are a preferred type of vector and include, but are not limited to nucleic acid
sequences from the following viruses: retrovirus, such as moloney murine leukemia virus,
harvey murine sarcoma virus, murine mammary tumor virus, and rous sarcoma virus;
adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr
viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a
retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-
essential genes have been replaced with the gene of interest. Non-cytopathic viruses include
retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic
viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, 1990 and in Murry, 1991).

Preferred viruses for certain applications are the adenoviruses and adeno-associated (AAV) viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. Actually 12 different AAV serotypes (AAV1 to 12) are known, each with different tissue tropisms (Wu, Z Mol Ther 2006; 14:316-27). Recombinant AAV are derived from the dependent parvovirus AAV2 (Choi, VW J Virol 2005; 79:6801-07). The adeno-associated virus type 1 to 12 can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species (Wu, Z Mol Ther 2006; 14:316-27). It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

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

In a preferred embodiment, the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a heterologous promoter.

The inhibitor of NGAL activity or expression may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

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

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

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or
dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The inhibitor of NGAL activity or expression of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred
methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The inhibitor of NGAL activity or expression of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used.

The present invention also relates to a method for screening a plurality of candidate compounds for use as a drugs for the prevention and treatment of cardiovascular fibrosis comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit NGAL activity or expression and (b) and positively selecting the candidate compounds capable of inhibiting said NGAL activity or expression.
Typically, the candidate compound is selected from the group consisting of small organic molecules, peptides, polypeptides or oligonucleotides. Other potential candidate compounds include antisense molecules, siRNAs, or ribozymes.

Testing whether a candidate compound can inhibit NGAL activity or expression can be determined using or routinely modifying assays known in the art. For example, the method may involve contacting cells expressing NGAL with the candidate compound, and measuring the NGAL mediated activity, and comparing the cellular response to a standard cellular response. Typically, the standard cellular response is measured in absence of the candidate compound. A decrease cellular response over the standard indicates that the candidate compound is an inhibitor of NGAL activity. In another embodiment the invention provides a method for identifying a ligand which binds specifically to NGAL. For example, a cellular compartment, such as a membrane or a preparation thereof, may be prepared from a cell that expresses a molecule that binds NGAL. The preparation is incubated with labelled NGAL and complexes of ligand bound to NGAL are isolated and characterized according to routine methods known in the art. Alternatively, the NGAL interacting polypeptide may be bound to a solid support so that binding molecules solubilized from cells are bound to the column and then eluted and characterized according to routine methods. In another embodiment, a cellular compartment may be prepared from a cell that expresses a molecule that binds NGAL such as a molecule of a signalling or regulatory pathway modulated by NGAL. The preparation is incubated with labelled NGAL in the absence or the presence of a candidate compound. The ability of the candidate compound to bind the binding molecule is reflected in decreased binding of the labelled ligand.

The candidate compounds that have been positively selected may be subjected to further selection steps in view of further assaying its properties on cardiovascular fibrosis. For example, the candidate compounds that have been positively selected may be subjected to further selection steps in view of further assaying its properties on animal models for cardiovascular fibrosis.

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

FIGURES:
Figure 1. Inactivation of NGAL prevents aldo-induce dexpression of fibrotic markers in the heart. The aldo/salt induction (NAS) of cardiac proteins involved in extracellular matrix remodeling process such as Collal (Fig 1 A) or periostin (Fig IB) (Wil-Type) was prevented by the absence of NGAL expression in vivo (NGAL-Knock-out). The ratio of cardiac Coll/Col3, a marker of cardiac stiffness, is increased by aldo/salt challenge (NAS), an effect blunted in the absence of NGAL in NGAL-KO mice (Fig 1C). 6-8 mice per condition; *, p<0.05, ANOVA analysis, Newman and Keuls test.

Figure 2 Inactivation of NGAL prevents perivascular fibrosis: Cardiac perivascular fibrosis, as determined by Sirius red staining, was increased by NAS in WT mice but blunted by NGAL inactivation in NGAL KO mice (panel A : representative image; panel B : quantification). 6-8 mice per condition; *, p<0.05, ANOVA analysis, Newman and Keuls test.

Figure 3 Inactivation of NGAL prevents expression of fibrotic markers in the aorta: Panel A: Collagen I expression is induced by aldo-salt challenge (WT NAS versus basal). NGAL inactivation (NGAL-KO) blunted the increase in Coll (NAS versus basal). Panel B: aldo-salt challenge induced increased Coll/elastin ratio, a marker of arterial stiffness (basal versus NAS, WT mice). NGAL inactivation blunted the effect of NAS (basal versus NAS, NGAL-KO mice). 6-8 mice per condition; *, p<0.05, ANOVA analysis, Newman and Keuls test.

Figure 4 Inactivation of NGAL prevents increased N terminal pro-collagen petide in the plasma. Procollagen I N-terminal propeptide (PINP) is a marker of collagen turnover. Aldo-salt challenge induced increased circulating plasma N terminal pro-collagen peptide (basal vs NAS, Wild-type mice). This is prevented when NGAL expression is blunted (basal versus NAS, NGAL-KO mice). 6 mice per condition; *, p<0.05, ANOVA analysis, Newman and Keuls test.

Figure 5. Upon aldo-salt challenge, NGAL inactivation (NGAL KO mice) blunts the increase of mRNA expression of inflammatory markers (MCP1, CD68, CD80, CD86) in the heart (panel A) as well as osteopontin, a marker of inflammation in the aorta (panel B). The increase in plasma C Reactive Protein (panel C) induced by aldo-salt challenge is also blunted in NGAL KO mice. Sham: control mice, Aldo-salt: nephrectomy
aldo salt treatment, KO: NGAL knockout mice. mean±SEM, n=6-8; * p<0.05, ** p<0.01, *** p<0.001

Figure 6. NGAL is an aldo/MR target in DCs (A) and macrophages (B). A:DCs were obtained from male SD rat bone marrow stem cells and differentiated during 6 days with GM-CSF. DCs were incubated with aldo (10nM), spironolactone (5µM), aldo plus spironolactone, or vehicle for 24hr. * p< 0.05 by ANOVA analysis (n=4); B: peritoneal macrophages from WT mice were stimulated or not with aldo (10nM +/- RU28318 (MR antagonist)). NGAL behaves similarly as MCP1 (monocy chemoattractant protein).

EXAMPLE 1:

Material and methods:

Experimental studies

The use of animals was in accordance with the guidelines of the European Community and approved by our Institutional Animal Care and Use Committees. NGAL knock out mice (NGAL KO on C57B16 background, kindly provided by Tak W. Mak, Toronto, Canada) were studied. Control or NGAL KO mice (9 weeks old) were uninephrectomized with xylazine/ ketamine anesthesia. The treated mice (nephrectomy/Aldo/salt) received 200 µg/kg BW/day Aldo, delivered subcutaneously via ALZET® osmotic mini pumps (Charles River Laboratories, L’Arbresle, France) with 1% sodium chloride to drink for 4 weeks. The control mice were uninephrectomized and maintained on standard chow and water. Hearts and aortas were rinsed in cold PBS, weighted, cut into 2 parts (transversal cut). The base was included in tissue-tek (Sakura Finetek France, Villeneuve d’ Ascq, France) and frozen in liquid nitrogen-cooled isopentane for morphological studies. All samples were frozen in liquid nitrogen, and kept at -80°C. Mouse plasma samples were collected using lithium-heparin Microvette® CB300 (Sarstedt, Marnay, France)

Western blot

Proteins were extracted in SDS 1% buffer containing anti-tyrosine phosphatases (Sigma-Aldrich) and anti-proteases (Roche) reagents. Protein concentration was estimated according to the Pierce method. Briefly, proteins (20 µg) were denatured 7 min at 95°C, separated by SDS-PAGE electrophoresis, and electrotransferred onto nitrocellulose
membranes. Membranes were incubated overnight (4°C) with antibodies against Coll, Col13, periostin, (Periostin: Abeam (1:1000), Collagen type I: Sigma (1:500), Collagen type III: Sta Cruz Biotechnology (1:500), Elastin: Abeam (1:500)). Membranes were then rinsed with TBS-T and incubated with the appropriate secondary antibody (HRP-conjugated rabbit anti goat immunoglobulins (DAKO, Trappes, France-, ref P0449). Chemiluminescent signal was produced using ECL+ solution (Amersham, Chalfont St. Giles, UK) and detected with a LAS-4000 luminescent image analyzer (Fuji, Courbevoie, France). Relative densitometry was determined using the computer software Multi Gauge.

Sirius Red
Cardiac cryostat sections (4 μm) were stained with the collagen specific Sirius red staining (0.5% in saturated picric acid). Sections (4 to 5 per animal, at X10) were observed in microscope and quantified with ImageJ (V 1.43) software (NIH).

Plasma Procollagen I N-terminalpropeptides analysis
Procollagen I N-terminal propeptides (PINP) were dosed in the mouse plasma using an Enzyme-linked Immunosorbent Assay Kit (Uscn, Life Science Inc.).

Statistical analyses
Results are provided as mean values ± SEM. The non-parametric Mann Whitney U test was used to assess statistical differences between two experimental groups. Differences among more than two experimental conditions were tested by the ANOVA one-way analysis and Newman and Keuls test; p <0.05 was considered significant.

Rationale and results
Inappropriate aldosterone signaling has been shown to play an important role in the progression of cardiovascular (CV) disease. The receptor of aldosterone, the mineralocorticoid receptor (MR), is a transcription factor of the nuclear receptor family present in the kidney and also in non-epithelial cells (1). Inappropriate MR activation has been shown to promote cardiovascular fibrosis in experimental models (2). The Randomized Aldactone Evaluation Study (RALES) (3), Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) (4) and Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure (EMPHASIS-HF) (5) clinical trials have demonstrated that the addition of the MR antagonists spironolactone or eplerenone to standard care reduced markedly the
overall and cardiovascular mortality in patients with left ventricular systolic dysfunction and mild or severe symptoms of chronic heart failure (HF) or with HF signs after acute myocardial infarction. The beneficial effects of MR antagonists in HF were associated with a reduction of cardiovascular fibrosis, as assessed by circulating biomarkers of cardiac extracellular matrix (6, 7, 8).

Among the molecular mechanisms involved in MR activation, we investigated the activation of Neutrophil Gelatinase-Associated Lipocalin (NGAL), a MR-target we previously identified in a transcriptomic screen (10, 11). Increased systemic and myocardial expression of NGAL has been reported in clinical and experimental heart failure (12, 13, 14). Also known as lipocalin 2 or 24p3, NGAL is a 25-kd glycoprotein belonging to the lipocalin superfamily (9); NGAL may be secreted and can be associated to metalloproteinase 9 (MMP9), increasing its stability and activity (15).

To question whether NGAL may be a mediator of the mineralocorticoid effects in the heart we used mice with constitutive NGAL gene inactivation that were subjected to the aldo/salt challenge. Importantly, we found that NGAL invalidation prevents extracellular matrix remodeling induced by mineralocorticoid challenge in the heart and aorta. The data indicate that NGAL is both a target of MR and an effector of the profibrotic consequences of mineralocorticoid excess in the heart and vessels.

EXAMPLE 2:

We used NGAL knock-out mice to analyze the role of NGAL. Our results indicate that NGAL plays a role in inflammation and may explain the role of NGAL in cardiovascular fibrosis. NGAL gene inactivation blunts the increase in inflammatory markers in the heart (Fig. 5A), as well as aldo-induced increase of osteopontin an inflammatory marker in the aorta (Fig. 5B). Moreover NGAL gene inactivation prevents global inflammation as shown by the prevention of increased in C-Reactive Protein plasma levels (Fig. 5C). Data obtained ex vivo demonstrated that NGAL is expressed in dendritic cells and is upregulated by aldo via MR activation (ie blocked by the MR antagonist spironolactone) (Fig. 6A). Moreover, NGAL is also an aldo/MR target gene in the macrophages (Fig. 6B). Therefore NGAL is a mediator of aldo/MR-mediated recruitment and/or activation of inflammatory cells which in turn is responsible for extracellular matrix remodeling (i.e. the mechanism leading to fibrosis) in the cardiovascular system.
REFERENCES:

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


CLAIMS:

1. A method for treating or preventing cardiovascular fibrosis in a subject in need thereof comprising administering the subject with a therapeutically effective amount of an inhibitor of Neutrophil Gelatinase-Associated Lipocalin (NGAL) activity or expression.

2. The method of claim 1 wherein the subject suffers from a disease selected from the group consisting of hypertension or heart failure.

3. The method of claim 1 or 2 wherein cardiovascular fibrosis of the subject is induced by aldosterone.

4. A method for screening a plurality of candidate compounds for use as a drugs for the prevention and treatment of cardiovascular fibrosis comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit NGAL activity or expression and (b) and positively selecting the candidate compounds capable of inhibiting said NGAL activity or expression.
Figure 1A

**A.**

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![](image5.png)

* p<0.05 vs WT

**B.**

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![](image10.png)

* p<0.05 vs WT
C. Col type I  
Col type III  

![Graph](image)

* * p<0.05 vs WT  

Figure 1C

A.  
Sham  
NAS  

![Images](image)

Wild Type  
NGAL-Knock Out  

Figure 2A
**Figure 2B**

* p<0.05 vs WT

**Figure 3A**

* p<0.05 vs WT
Figure 3B

Aortic Stiffness

Figure 4

Plasma PINP ng/ml

Wild Type | NGAL-Knock Out
---|---
 basal | NAS

* p<0.05 vs WT
Cardiac gene expression

Figure 5A

B) Aortic Osteopontin

Figure B
Figure 6B
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/7088 A61K31/7105 C12N15/113
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 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 practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>wo 2007/028053 A2 (CELL MEDICAL INC X [US]; BAUMBACH W; LIAM R [US]; SHANKAR HARIHARAN [U]) 8 March 2007 (2007-03-08) claims 10, 12, 22, 24</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

Special categories of cited documents:
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*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

Date of the actual completion of the international search
13 December 2013

Date of mailing of the international search report
20/12/2013

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Fax: (+31-70) 340-3016

Authorized officer
Faber, Cerstien
### DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>BOLIGNANO DAVIDE ET AL: &quot;Increased plasma neutrophil gel ati nase-associated lipocalin level s predict mortality in elderly patients with chronic heart failure.&quot;. <strong>REJUVENATION RESEARCH</strong> FEB 2009 LNKD: PUBMED: 19196011, vol. 12, no. 1, February 2009 (2009-02), pages 7-14, XP002687056, ISSN: 1549-1684. <strong>the whole document</strong></td>
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