Methods and pharmaceutical compositions for the treatment of Netherton syndrome

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ABSTRACT

The present invention relates to methods and pharmaceutical compositions for the treatment of Netherton syndrome. In particular, the present invention relates to a method for the treatment of Netherton syndrome (NS) in a subject in need thereof comprising administering the subject with a therapeutically effective amount of at least one inhibitor of elastase.

DESCRIPTION

METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF NETHERTON SYNDROME

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of Netherton syndrome.

BACKGROUND OF THE INVENTION:

The epidermis is a stratified epithelium that prevents from dehydration, excludes toxins and microbes, protects from mechanical injury, and participates in immune responses. The main barrier is provided by the stratum corneum, the outermost epidermal layer composed of multiple sheets of terminally differentiated keratinocytes (mummified corneocytes) embedded in a lipid matrix (Fuchs, 2007; Simpson et al, 2011).

Netherton Syndrome (NS, OMIM 256500) is a rare autosomal recessive skin disease characterized by extensive skin desquamation, inflammation, allergies, atopic manifestations and hair shaft defects (Netherton, 1958). NS is caused by LEKTI deficiency due to loss-of-function mutations in SPINK5 (Chavanas et al, 2000) resulting in KLK hyperactivation and aberrantly increased epidermal proteolysis (Borgono et al, 2007; Deraison et al, 2007; Egelrud et al, 2005). Currently, there is no specific therapy for NS but only palliative treatments for management of skin infections, reduction of itching and pain. Spink5−/− mice recapitulate NS disease (Descargues et al, 2005; Hewett et al, 2005; Yang et al, 2004) and are used as prototype for other common skin diseases such as atopic dermatitis. A key finding is that, similarly to what has been observed in subjects with NS, Spink5−/− mice epidermis displays unopposed Klk5 and Klk7 protease activities (Descargues et al, 2005). According to the current state-of-the-art hypothesis, pro-KLKS are synthesized and activated in the stratum granulosum and active KLK enzymes are rapidly complexed with LEKTI, thus preventing premature degradation of desmosomes at the stratum corneum/stratum granulosum interface (Borgono et al, 2007; Deraison et al, 2007; Ovaere et al, 2009). KLK-LEKTI complexes diffuse to the outer stratum corneum where the acidic microenvironment causes the release of active KLKs which cleave cemodesmosomal proteins in the most superficial layers of the stratum corneum. This ensures the finely-tuned regulation of the desquamation process. Two scenarios aim to explain the modes of -

proKLK activation in skin. It was assumed that proKLK5 was auto-activated and that mature KLK5 activates other KLK zymogens (Deraison et al, 2007). However, recent work established that proKLK5 activation involves the transmembrane protease matriptase, which also activates proKLKJ, a downstream target (Sales et al, 2010). Howver the role of elastase 2 has never been suggested in overdesquamating and inflammatory skin in NS.

SUMMARY OF THE INVENTION:

1. A method for the treatment of Netherton syndrome (NS) in a subject in need thereof comprising administering the subject with a therapeutically effective amount of at least one inhibitor of elastase.

2. The method of claim 1 wherein the inhibitor of elastase is selected from the group consisting of small organic molecules, polypeptides, aptamers and antibodies.

3. The method of claim 1 wherein the inhibitor of elastase is an inhibitor of KLK5.

4. The method of claim 1 wherein the inhibitor of elastase is an inhibitor of KLK5.

5. The method of claim 1 wherein the inhibitor of elastase is an inhibitor of KLK5.

6. The method of claim 1 wherein the inhibitor of elastase is used in combination with an inhibitor of KLK5.

7. The method of claim 1 wherein the inhibitor of elastase and the inhibitor of KLK5 are to be used simultaneous or sequentially within a given time.

8. The method of claim 7 wherein the inhibitor of KLK5 is administered first and then the inhibitor of elastase is administered to the subject.
The present invention relates to methods and pharmaceutical compositions for the treatment of Netherton syndrome. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

Netherton syndrome (NS) is a severe type of ichthyosis caused by mutations in SPINK5 encoding the serine protease inhibitor LEKTL. The disease is characterized by aberrant epidermal proteolysis leading to extensive stratum corneum detachment, allergies, chronic inflammation, and atopic manifestations. Lekti-deficient mice (Spink5-/-) recapitulate skin abnormalities of NS and die within a few hours after birth because of severe skin barrier defect secondary to protease hyperactivities in the epidermis. The inventors show that ablation of the kallikrein-related peptidase 5 gene (Klk5) in Lekti-deficient newborn mice rescues severe skin inflammation, prevents over-desquamation and epidermal hyperplasia, restores skin barrier function and whisker growth. This shows that elimination of Klk5 hyperactivity in the absence of Lekti is sufficient to reverse the severe barrier defect and to restore normal skin function at birth, supporting a key role of Klk5 in skin homeostasis. However, transplantation of Spink5-/-Klk5-/- skin onto immune-competent animals leads to skin inflammation involving Klk7/14 and elastase proteolytic activities, suggesting that therapeutic approaches for NS should target these proteases as well as KLK5.

Accordingly, a first aspect of the present invention relates to a method for the treatment of Netherton syndrome (NS) in a subject in need thereof comprising administering the subject with a therapeutically effective amount of at least one inhibitor of elastase.

As used herein the term "elastase" has its general meaning in the art and refers in particular to leukocyte elastase and epidermal elastase (Ela2). Elastase (EC 3.4.21.36.) is a member of a group of enzymes termed "serine proteases" which are characterised by the reactivity of a serine residue in the active site of the enzyme. Elastase is a very broad range proteolytic enzyme, its substrates include various extracellular matrix proteins, such as elastin, fibronectin and collagen as well as adhesive molecules like ICAM-I and junctional cadherins. The term generally refers to mature elastase proteins with elastase activity as well as immature elastase proteins, including immature proelastase proteins (also referred to herein as elastase proproteins) and immature preproelastase proteins (also referred to herein as elastase preproproteins).

As used herein, the term "inhibitor of elastase" refers to any molecule modulating elastase production and/or action in such a way that elastase production and/or action is attenuated, reduced, or partially, substantially or completely prevented or blocked. Typically, elastase inhibitors can be small organic molecules, antibodies, oligonucleotides, polypeptides or peptides. In some embodiments, the inhibitor of elastase is a small organic molecule. Inhibitors of elastase are well known in the art:

- Peter Norman Crystalline form of a neutrophil elastase inhibitor, 6-methyl-5-(l- methyl-1 H-pyrazol-5-yl)-N-[(5-(methylsulfonyl)pyridin-2-yl)methyl]-2-oxo-1-[3- (trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide p-toluene sulfonate - is it AZD-9668?: WO-2007/129963 Expert Opinion on Therapeutic Patents Feb 2011, Vol. 21, No. 2, Pages 277-280;

In some embodiments, the inhibitor is selected from the group consisting of molecules described in WO2004041309. A neutrophil elastase inhibitor is, for example, 6-[2-(4-Cyano-phenyl)-2H-pyrazol-3-yl]-5- methyl-3-oxo-4-(3-trifluoromethyl-phenyl)-3,4-dihydro- -pyrazine-2-carboxylic acid ethylamide (WO 2007/129963). In some embodiments, the inhibitor of elastase is selected from the group consisting of macrocyclic compounds as described in WO 2013130358. In some embodiments, the neutrophil elastase inhibitor is selected from the group consisting of AZD-9668, ONO-5046, MR-889, L-694,458, CE-1037, GW-31161, TEI-8362, ONO-6818, AE-3763, FK-706, ICI-200,880, ZD-0892 and ZD-8321 (See, for example, Expert Opinion on Investigational Drugs, July 2002, Vol. 11, No. 7: Pages 965-980). In some embodiments the inhibitor of elastase is selected from the group consisting of dihydropropirimide multimers as described in WO 2006136857. In some embodiments, the inhibitor of elastase is selected from the group consisting of compounds described in WO2008036379. In some embodiments, the inhibitor of elastase is selected from the group consisting of:
In some embodiments, the inhibitor of elastase is selected from the group consisting of 2-pyridone derivatives as described in WO2005026123. In a particular embodiments, the inhibitor of elastase is selected from the group consisting of:

N-[4-(methylsulfonyl)benzyl]-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

5-[4-(hydroxymethyl)phenyl]-6-methyl-N-[4-(methylsulfonyl)benzyl]-2-oxo-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

5-furan-3-yl-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

6'-methoxy-2-methyl-N-[4-(methylsulfonyl)benzyl]-6-oxo-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

5-(2-methoxypyrimidin-5-yl)-6-methyl-N-[4-(methylsulfonyl)benzyl]-2-oxo-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6'-methoxy-2-methyl-N-[4-(methylsulfonyl)benzyl]-6-oxo-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

5-(2-methoxy-pyrimidin-5-yl)-6-methyl-N-[4-(methylsulfonyl)benzyl]-2-oxo-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

5-[4-(acetylamino)phenyl]-6-methyl-N-[4-(methylsulfonyl)benzyl]-2-oxo-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6'-methyl-2-oxo-5-(1H-pyrro1-1-3-yl)-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

5-furan-2-yl-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

6-methyl-2-oxo-5-thiophen-3-yl-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

6-methyl-2-oxo-5-thiophen-2-yl-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

5-(3,5-dimethyl-isoxazol-4-yl)-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

5-(2,4-dimethoxy-pyrimidin-5-yl)-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

5-(2,4-dioxo-1,2,3,4-tetrahydro-pyrimidin-5-yl)-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

6-methyl-5-[5-methyl-1,3,4]oxadiazol-1-yl)-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

6-methyl-2-oxo-5-(5-propyl-[1,3,4]oxadiazol-2-yl)-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

{5-[5-(4-methanesulfonyl-benzylcarbamoyl)-2-methyl-6-oxo-1-[3-trifluoromethylphenyl]1,2-dihydropyridine-3-carboxylic acid ethyl ester;

5-(5-cyanomethyl-[1,3,4]oxadiazol-2-yl)-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

5-(5-amino-[1,3,4]oxadiazol-2-yl)-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;

5-(5-amino-[1,3,4]thiadiazol-2-yl)-6-methyl-2-oxo-1-[3-trifluoromethylphenyl]-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
methanesulfonyl-benzylamide;
5- (6-ethylamino-[3,4]oxadiazol-2-yl)-6-methyl-2-oxo-l-(3-trifluoromethylphenyl)-1,2-dihydro-pyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
5-(5,N,N-dimethylamino-[3,4]oxadiazol-2-yl)-6-methyl-2-oxo-l-(3-trifluoromethylphenyl)-1,2-dihydro-pyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
6- methyl-N-[4-(methylsulfonyl)benzyl]-2-oxo-5-pyrazin-2-yl-1 - [3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;
6-methyl-5-oxazol-2-yl-2-oxo- 1 -(3-trifluoromethylphenyl)-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
6-methyl-5-(1-methyl- 1 H-imidazo l-2-yl)-2-oxo-1 -(3-trifluoromethylphenyl)-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
6-methyl-2-oxo-5-(1 H-pyrazo l-1-4-yl)-1 -(3-trifluoromethylphenyl)-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
6-methyl-N-[4-(methylsulfonyl)benzyl]-2-oxo-5-pyrimidin-2-yl-1 - [3-(trifluoro- methyl)phenyl]-1,2-dihydropyridine-3-carboxamide;
6-methyl-5-(2-methyl-2H-pyrazol-3-yl)-2-oxo- 1 -(trifluoromethylphenyl)-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
6-methyl-5-(3-methylisoxazol-4-yl)-N-[4-(methylsulfonyl)benzyl]-2-oxo-l-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;
5-(3,5-dimethylisoxazol-4-yl)-N-[4-(isopropylsulfonyl)benzyl]-6-methyl-2-oxo-l-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;
6-methyl-5-(3,5-dimethylisoxazol-4-yl)-N-[4-(ethylsulfonyl)benzyl]-2-oxo-l-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;
6-methyl-5-(3,5-dimethylisoxazol-4-yl)-N-[4-(cyclopropylsulfonyl)benzyl]-2-oxo-l-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;
l-(3-cyanophenyl)-5-(3,5-dimethylisoxazol-4-yl)-6-methyl-N-[4- (methylsulfonyl)benzyl]-2-oxo-l,2-dihydropyridine-3-carboxamide;
l-(3-chlorophenyl)-5-(3,5-dimethyl-isoxazol-4-yl)-6-methyl-2-oxo-l,2-dihydro-pyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide; 5-(3,5-dimethyl-isoxazol-4-yl)-6-methyl-2-oxo-l,2-dihydro-pyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
5-(5-isopropyl-[1,3,4]oxadiazo l-2-yl)-6-methyl-2-oxo-l -(3-trifluoromethylphenyl)-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
5 -(5-methoxymethyl-[1,3,4]oxadiazo l-2-yl)-6-methyl-2-oxo-l -(3-trifluoromethylphenyl)-1,2-dihydropyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
5-(4,5-dimethyl-4H-[1,2,4]triazol-3-yl)-6-methyl-2-oxo-1 -(3-trifluoromethylphenyl)-1,2-dihydro-pyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
5-(5-methoxymethyl-[1,3,4]oxadiazo l-2-yl)-6-methyl-2-oxo-l -(3-trifluoromethylphenyl)-1,2-dihydro-pyridine-3-carboxylic acid 4-methanesulfonyl-benzylamide;
N-[4-(isopropylsulfonyl)benzyl]-6-methyl-5-(5-methyl-[1,3,4]oxadiazo l-2-yl)-2-oxo-l-[3-(trifluoromethyl)phenyl]-1,2-dihydro-pyridine-3-carboxamide;
N-[4-(ethylsulfonyl)benzyl]-6-methyl-5-(5-methyl-[1,3,4]oxadiazo l-2-yl)-2-oxo-l-[3-(trifluoromethyl)phenyl]-1,2-dihydro-pyridine-3-carboxamide;
N-[4-(cyclopropylsulfonyl)benzyl]-6-methyl-5-(3,4-oxadiazol-2-yl)-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-5-(5-methyl-1,3,4-oxadiazol-2-yl)-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-5-(3,4-oxadiazol-2-yl)-2-oxo-1-(3-trifluoromethylphenyl)-1,2-dihydro-6-methyl-5-(3,4-oxadiazol-2-yl)-2-oxo-N-[4-(methylsulfonyl)benzyl]-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-5-(5-methyl-1,3,4-oxadiazol-2-yl)-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-5-(2-methyl-1,3-oxazol-4-yl)-N-[4-(methylsulfonyl)benzyl]-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-5-(2-amino-thiazol-4-yl)-6-methyl-2-oxo-N-[4-(methylsulfonyl)benzyl]-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-5-(2,5-dimethyl-1,3-oxazol-4-yl)-6-methyl-N-[4-(methylsulfonyl)benzyl]-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-5-(2-amino-5-methyl-thiazol-4-yl)-6-methyl-2-oxo-N-[4-(methylsulfonyl)benzyl]-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-5-(2-hydroxymethyl-5-methyl-thiazol-4-yl)-6-methyl-2-oxo-N-[4-(methylsulfonyl)benzyl]-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-2-oxo-5-(1H-tetrazol-5-yl)-1-(3-trifluoromethylphenyl)-1,2-dihydro-5-(4-methyl-oxazol-2-yl)-2-oxo-1-(3-trifluoromethylphenyl)-1,2-dihydro-5-(4,5-dimethyl-oxazol-2-yl)-2-oxo-N-[4-(methylsulfonyl)benzyl]-2-oxo-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-5-(1-ethylpyrrolidin-2-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(1-naphthylmethyl)-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(2-morpholin-4-ylethyl)-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-2-oxo-5-phenyl-N-(1-phenylethyl)-1-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-2-oxo-5-phenyl-N-(2-phenylethyl)-1-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(1,3-benzodioxol-5-ylmethyl)-2-oxo-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(2-chloro-4-fluorobenzyl)-2-oxo-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(2-thienylmethyl)-2-oxo-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(2-cyclohex-1-en-1-ylethyl)-2-oxo-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(4-phenoxybenzyl)-2-oxo-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro-6-methyl-N-(2,5-dimethyl-3-furyl)methyl]-2-oxo-5-phenyl-N-[3-(trifluoromethyl)phenyl]-1,2-dihydro.
N-{2-[4-[(aminosulfonyl)phenyl]ethyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;}

N-6-methyl-2-oxo-N-[4-([1H-pyrazol-1-yl]benzyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-N-[5-fluoro-4H-1,3-benzodioxin-8-yl]methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[2-(tetrahydro-2H-pyran-4-y]ethyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[3-([1H-pyrazol-1-yl]propyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[1-methyl-1H-pyrazol-4-yl]methyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-N-[2-(tetrahydro-2H-pyran-4-y]ethyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-N-[2-(tetrahydro-2H-pyran-4-y]ethyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[lH-pyrazol-1-yl]propyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-N-[2-(tetrahydro-2H-pyran-4-y]ethyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[3-([1H-pyrazol-1-yl]propyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[1-phenyl-1H-pyrazol-4-yl]methyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[1-phenyl-1H-pyrazol-4-yl]methyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

N-6-methyl-2-oxo-5-phenyl-N-[1-phenyl-1H-pyrazol-4-yl]methyl]-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;

6-methyl-2-oxo-5-phenyl-N-[(6-fluoro-4H-1,3-benzodioxin-8-yl)methyl]-6-methyl-2-oxo-5-phenyl-1-[3-(trifluoromethyl)phenyl]-1,2-dihydropyridine-3-carboxamide;


In some embodiments, the inhibitor of elastase is a polypeptide. In some embodiments, the inhibitor of elastase is the human monocyte elastase inhibitor (herein referred to as human monocyte EI or human EI). In some embodiments, the inhibitor of elastase is human elafin as described in WO 2011107505. Elafin comprises an amino acid sequence as set forth by SEQ ID NO: 1.

**Figure imgf000012_0001**

In some embodiments, the inhibitor of elastase is a polypeptide. In some embodiments, the inhibitor of elastase is the human monocyte elastase inhibitor (herein referred to as human monocyte EI or human EI). In some embodiments, the inhibitor of elastase is human elafin as described in WO 2011107505. Elafin comprises an amino acid sequence as set forth by SEQ ID NO: 1.
In some embodiments, the inhibitor of elastase is selected from the groups consisting of cyclic depsipeptides as described in WO 2009024527. In some embodiments, the inhibitor of elastase is a peptide as described in WO 2010091893. In some embodiments, the inhibitor of elastase is an elastase antibody that prevents the binding of elastase to its targets, thus, diminishing or preventing reactions mediated by elastase. As used herein, "antibody" includes both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and mono- and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. The antibody may be a human or non human antibody. A non human antibody may be humanized by recombinant methods to reduce its immunogenicity in man. Typically, monoclonal antibodies are prepared according to conventional methodology. Monoclonal antibodies may be generated using the methods of Kohler and Milstein (Nature, 256:495, 1975). As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules.

Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby incorporated by reference. The above U.S. Pat. Nos. 5,585,089 and 5,693,761, and WO 90/07861. A "humanized" antibody retains a similar antigenic specificity as the original antibody. However, using certain methods of humanization, the affinity and/or specificity of binding of the antibody may be increased using methods of "directed evolution", as described by Wu et al. / Mol. Biol. 294: 151, 1999, the contents of which are incorporated herein by reference. Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the see, contents of which are incorporated herein by reference. These animals have been genetically modified such that there is a functional deletion in the production of endogenous (e.g., murine) antibodies. The animals are further modified to contain all or a portion of the human germ-line immunoglobulin gene locus such that immunization of these animals will result in the production of fully human antibodies to the antigen of interest. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMab mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human -immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (KAMA) responses when administered to humans. In vitro methods also exist for producing human antibodies. These include phage display technology (U.S. Pat. Nos. 5,565,332 and 5,573,905) and in vitro stimulation of human B cells (U.S. Pat. Nos. 5,229,275 and 5,567,610). The contents of these patents are incorporated herein by reference. In another embodiment, the antibody according to the invention is a single domain antibody. The term "single domain antibody" (sdAb) or "VHH" refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains. Such VHH are also called "nanobody®". According to the invention, sdAb can particularly be llama sdAb.

In some embodiments, the inhibitor of elastase is an elastase aptamer that prevents the binding of elastase to its targets, thus, diminishing or preventing reactions mediated by elastase. 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. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consist of conformationally constrained antibody variable regions displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods.

An inhibitor of production can be any molecule negatively affecting the synthesis, processing or maturation of elastase. The inhibitors considered according to the invention can be, for example, an inhibitor of elastase expression. An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. In a preferred embodiment of the invention, said inhibitor of gene expression is a siRNA, an antisense oligonucleotide or a ribozyme. For example, anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of elastase mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of elastase, 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 elastase can be synthesized, e.g., by conventional phosphodiester techniques. 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, elastase 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 elastase gene expression is specifically inhibited (i.e. RNA interference or RNAi). 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 typically cells expressing elastase. Typically, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequences. Viral vectors
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.

The bioavailability of the inhibitor of elastase according to the invention can also be ameliorated by using conjugation procedures which increase the half-life of the molecule in the human body, for example linking the molecule to poly ethylenyl col, as described in the PCT Patent Application WO 92/13095.

The therapeutically effective amounts of the active molecule will be a function of many variables, including the type of molecule used, any residual cytotoxicity exhibited by the molecule, the route of administration, the clinical condition of the subject. A "therapeutically effective amount" is such that when administered, the inhibitor of elastase results in decreased skin inflammation. The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factors, including the molecule pharmacokinetic properties, the route of administration, subject conditions and characteristics (sex, age, body weight, health size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled in the art, as well as in vitro and in vivo methods of determining the effect of the molecule in an individual. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Typically, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the symptomatic adjustment of the dosage to the subject to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, typically from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

Typically the inhibitor of elastase is combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form pharmaceutical compositions. The term "Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, or encapsulating material or formulation auxiliary of any type. 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 vegetable 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. -

In the pharmaceutical compositions of the present invention, the inhibitor of elastase can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports. 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.

In some embodiments, it may be desirable to administer the inhibitor of elastase in admixture with a topical pharmaceutically acceptable carrier. The topical pharmaceutically acceptable carrier is any substantially nontoxic carrier conventionally usable for topical administration of pharmaceuticals in which the inhibitor of elastase will remain stable and bioavailable when applied directly to skin. For example, carriers such as those known in the art effective for penetrating the keratin layer of the skin into the stratum corneum may be useful in delivering the inhibitor of elastase to the area of interest. Such carriers include liposomes, inhibitor of elastase can be dispersed or emulsified in a medium in a conventional manner to form a liquid preparation or mixed with a semi-solid (gel) or solid carrier to form a paste, powder, ointment, cream, lotion or the like. Suitable topical pharmaceutically acceptable carriers include water, buffered saline, petroleum jelly (vaseline), petrolatum, mineral oil, vegetable oil, animal oil, organic and inorganic waxes, such as microcrystalline, paraffin and ozocerite wax, natural polymers, such as xanthanes, gelatin, cellulose, collagen, starch, or gum arabic, synthetic polymers, alcohols, polyls, and the like. The carrier can be a water miscible carrier composition. Such water miscible, topical pharmaceutically acceptable carrier composition can include those made with one or more appropriate ingredients outset of therapy.

According to the invention, the inhibitor of elastase can be administered to an individual prior to, simultaneously or sequentially with other therapeutic regimens (e.g. multiple drug regimens) or agents, in a therapeutically effective amount. In a particular embodiment, the inhibitor of elastase is used in combination with an inhibitor of kallicrein, in particular in combination with an inhibitor of KLK5. In some embodiments, the inhibitor of elastase and the inhibitor of KLK5 are to be used simultaneously or sequentially within a given time. Typically, the inhibitor of KLK5 is administered first and then the inhibitor of elastase is administered to the subject.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.
Scanning and transmission electron microscopy. For SEM, neonates were decapitated and their heads were fixed in 4% formaldehyde in PBS pH 7.4 for 24 hrs with PBS and stained with 0.1 % toluidine blue O in PBS for 1 h. Mice were photographed following destaining in PBS (2 wks after grafting on C57B1/6 recipient mice). Spink5+/−, Klk5+/− and Spink5−/−, Klk5−/− mice skin 8 wks after grafting on C57B1/6 recipient mice were resolved by gel zymography using casein as an in-gel substrate. Areas of proteolytic activity are visible as clear bands against a dark background. (b) Spink5+/− and Spink5−/−, Klk5−/− samples showed higher proteolytic activities. The 20-23 kDa band can be partially inhibited by chymostatin (an inhibitor of chymotrypsin-like proteases) and the 28 kDa band is completely inhibited by N-Methoxy succinyl-Ala-Ala-Pro-Val-chloromethyl ketone (an elastase inhibitor). (c-e) Quantification of proteolytic activities using fluorometric substrates that are specifically cleaved by different proteases. Activities were assayed using GPR-Rhodamine-110 for trypsin-like (c), Suc-Leu-Leu-Val-Tyr-AMC for chymotrypsin-like (d) and BODIPY-FL Elastin for elastase activity (e). All these activities were higher in Spink5−/− and Spink5−/−, Klk5−/− in grafted mice skin as compared to wt. *, p<0.05 comparing genotypes as indicated, ns, non significant.

Figure 2 Klk5 ablation does not prevent pro-allergic and inflammatory signals in Spink5+/−, Klk5+/− grafted skin. (a, b) Relative mRNA expression of pro-inflammatory and pro-allergic cytokines analyzed by quantitative RT-PCR in RNA extracted from wt, Spink5+/−, Klk5+/−, and Spink5−/−, Klk5−/− mice skin 8 wks after grafting on C57B1/6 recipient mice. Spink5+/− and Spink5−/−, Klk5−/− mice skin show higher expression of pro-allergic and pro-inflammatory markers than wt grafted skin with the exception of IL-17. TSLP, TNF-α, and IL-6 were significantly elevated in Klk5+/−. (c) Toluidine blue staining to detect infiltration of mast cells (stained purple). An increased number of mast cells were detected in Spink5−/− and Spink5−/−, Klk5−/− mice skin but also in Klk5−/− % wks after grafting on C57B1/6 recipient mice. Scale bar: 100µm. **, p<0.01 comparing genotypes as indicated.

EXAMPLE:
Material & methods:
Materials. All chemicals were obtained from Sigma or Merck. Rabbit polyclonal antibodies against Dsg1 were obtained from Santa Cruz (sc-20114) and TSLP from R&D systems (AF555).
Animal handling. All experiments with animals were carried out according to our Institutions Guidelines and EU legislation.
Generation of the Klk−/−. Mouse embryonic stem cells with targeted deletion in the Klk5 gene were obtained from KOMP (http://www.komp.org) and used to derive chimeric mice with diploid aggregation chimeras (Hardman et al, 1998). Chimeric mice were found to be 100% transmitters and gave birth to Klk5+/− mice which were intercrossed to obtain Klk5−/− mice.

Genotyping. Genomic DNA was isolated from mouse tails using Nucleospin (Macherey-Nagel) and subjected to PCR using GoTaq polymerase (Promega).

RNA isolation and reverse transcription. Total RNAs from mouse tissues were extracted with RNaseasy (Qiagen) and treated with DNase according to manufacturer’s instructions. The quality and quantity of RNA were determined by agarose electrophoresis and spectrophotometry. Reverse transcription was carried out with 1µg of total RNA with MMLV reverse transcriptase (Invitrogen). Real-time PCR. cDNAs in 25 µl total volume were amplified with MESA GREEN (Eurogentech). The sequences of gene-specific primers are given in Table S1. Gene expression was normalized against Hprt.

Transepidermal water loss (TEWL). At least three different measurements were taken for each mouse and averaged and at least three mice from each genotype were used. Measurements were performed with the EP1 evaporimeter (ServoMed) as described (Descargues et al, 2005). Results were reported as fold increase over the wt control in order to exclude day-to-day variations in TEWL values depending on independent environmental conditions e.g. humidity.

Toluidine blue staining of neonates. The method of polar lipid removal was used (Tanaka et al, 2009). Neonates were euthanized and dehydrated by sequential incubation in 25%, 50% and 75% methanol in PBS (1 min per step) and finally in 100% methanol. Then, neonates were rehydrated by incubation in the same methanol solutions but in reverse order, washed with PBS and stained with 0.1 % toluidine blue O in PBS for 1 h. Mice were photographed following destaining in PBS (2 washes for 10 min and 10 min, respectively). Histology. Skin tissues were fixed in 4% formaldehyde in PBS pH 7.4 for 24 hrs and, then, embedded in paraffin. 5 µm sections were cut with a microtome, Hematoxylin/eosin/safranin (HES) and toluidine blue staining were performed on paraffin-embedded sections using standard histological techniques. Immunohistochemistry. Skin tissues were embedded in OCT and cryosectioned to 5µm. The sections were fixed in acetone for 10 min, air-dried for 5 min, rehydrated with PBS for 5 min and the endogenous peroxidase activity was blocked with peroxidase blocking solution for 8 min at room temperature (Dako). The slides were incubated in PBS containing 0.3% BSA for blocking and 0.1% Triton X-100 for membrane permeabilisation for 5 min at room temperature. The antibody against Dsg1 or TSLP was used at a 1:200 dilution and washed with PBS containing 0.3% BSA. Following incubation with the anti-rabbit (K4011, Dako), slides were incubated with the chromogen solution (Dako).
formaldehyde in PBS for 24 hrs, then, washed twice with PBS and dehydrated in a series of ethanol solutions 25%, 50%, 70%, and 100% (10 min each). Finally, ethanol was replaced with 100% acetone (2 washes, 10 min each). The samples were dried, covered with gold and observed in a Field-Emission Scanning Electron Microscope (JEOL, 6300). For TEM, skin from neonates was excised with 4 mm skin biopsy.

punches and fixed in a 2% glutaraldehyde and 4% formaldehyde solution in PBS pH 7.4 at 4°C. Then, samples were processed and stained as described (Descargues et al, 2005).

In situ zymography. Skin cryosections (5 μm thick) were mounted on glass slides, rinsed with 2% Tween 20 in PBS and incubated overnight at 37°C with 10 μg ml⁻¹ BODIPY FL casein (Life Technologies) or 100 μg ml⁻¹ BODIPY FL elastin (Life Technologies) in 50 mM Tris-HCl, pH 8.0. Sections were rinsed with PBS and visualized with a Leica TCS SP5 AOBS confocal laser scanning microscope (CLSM). Data were analyzed using ImageJ.

Fillipin staining. Fillipin stock solution (2.5 mg/ml in DMSO) was stored at -20°C. Before staining cryosections were washed with PBS for 5 min and incubated with fillipin (diluted with PBS to 50 μg/ml) for 30 min at room temperature and in the dark, then, washed with PBS for 10 min mounted with aqueous medium (Dako) and visualized with CLSM with excitation and emission wavelengths 405 and 480 nm, respectively.

Nile red. Nile red was dissolved in acetone and stored at -20°C and, prior to use, diluted to 5 μg/ml in 75% glycerol in water. A drop of this solution was placed on each section and visualized with CLSM with excitation at 488 nm and emission at 520 and 600 nm.

Skin grafting. Total dorsal skin from newborn was transplanted onto C57BL/6 mice using the skin flap technique (Barrandon et al, 1988) and analyzed 8 wks later. Results and discussion:

Here, we aimed to delineate whether KLK5 (Klk5 for mouse) is a central mediator of aberrant proteolysis in NS epidermis and whether its ablation is sufficient to reverse the NS-like symptoms manifested in Lekti-deficient (Spink5+/−) mice. For this, we have generated Klk5+/− mice on a C57BL/6 background. Because of neonatal lethality of Spink5−/− mice, Klk5−/−Spink5−/− animals were intercrossed in order to generate Spink5−/− Klk5−/− double knockout mice. All animals were identified with a PCR-based genotyping strategy.

Spink5−/− mice develop desquamating lesions within 1 hour from birth and die shortly (<5 hrs) after. They also display vibrissae defects with either complete lack of whiskers or rare and disorganized ones (Descargues et al, 2005; Hewett et al, 2005; Yang et al, 2004). In striking contrast, newborn Spink5+/− Klk5+/− mice displayed no apparent phenotype, no stratum corneum ‘stratum granulosum separation and were overall indistinguishable from wild-type (wt) mice, except that they grew shorter whiskers. Neonates from all genotypes were fed normally as milk could be visually detected in their stomach, and had normal weight (1.20-1.40 g for all genotypes) at birth. The microstructure of Spink5−/− Klk5−/− whiskers was nearly identical to wt and completely different from the short, thin, and disorganized whiskers seen in Spink5−/− mice.

Toluidine blue dye permeability assay shows that deletion of Klk5 in Lekti-deficient mice restores normal epidermal function since only a few patches of Klk5−/− Klk5−/− mice exhibit significantly lower transepidermal water loss (TEWL) compared to Spink5−/− . The TEWL values for Spink5−/− Klk5−/− are only slightly higher than those determined for the wt and the improvement of skin integrity in the Spink5−/− Klk5−/− is confirmed as animals do not lose weight after 210 minutes at 37°C. Usually, defective skin barrier results in the development of compensatory mechanisms which involve hyperkeratosis (thickening of the cornified layer) and acanthosis (thickening of the living layers) as observed in various ichthyosis syndromes in humans and mice, including Spink5−/− (Descargues et al, 2005). Neither acanthosis nor hyperkeratosis were observed in skin sections from Spink5−/− Klk5−/− and, most importantly, no microscopic separation of the stratum corneum. A characteristic feature of the Spink5−/− phenotype is the aberrantly increased proteolysis in skin especially in the upper layers of the epidermis (Descargues et al, 2005; Hewett et al, 2005; Sales et al, 2010; Yang et al, 2004), which is responsible for the separation of the stratum corneum from the stratum granulosum. The overall proteolytic activity was visualized in skin sections by in situ zymography using quenched fluorescent casein and elastin substrates. Very high proteolytic activities were detected in Spink5−/− and the caseino lytic activity diffused throughout the epidermis extending to the underlying dermis (Sales et al, 2010). We show the same pattern for elastino lytic activity in Spink5−/− skin. The overall caseino lytic activity in the epidermis of -

newborn Klk5−/− was not significantly reduced compared to the wt. In contrast, genetic ablation of Klk5 had a tremendously suppressing effect on the overall caseino lytic activity in Spink5−/− Klk5−/− skin. This effect of Klk5 ablation on Lekti-deficient background is significantly more pronounced than the one caused by elimination of matriptase in Lekti-deficient mice (Sales et al, 2010). Spink5−/− mice skin shows increased and extended elastino lytic activity. On the contrary, elastino lytic activities were markedly reduced in Klk5−/− and Spink5−/− Klk5−/− epidermis which provides the first in vivo proof that Klk5 contributes to pro-ELA2 activation, as proposed by in vitro studies (Bonnart et al, 2010).

Next, we examined the skin tissue expression of the desmosomal protein desmoglein 1 (Dsg1), since Dsg1 degradation is a major feature of the NS phenotype (Descargues et al, 2005). The crucial role of Dsg1 in epidermal barrier function and structure was shown by studies of the autoimmune disease pemphigus foliaceus in which anti-Dsg1 antibodies cause skin
A recent study revealed that DsgI deficiency results in severe dermatitis, multiple allergies and metabolic wasting (Samuelov et al, 2013). DsgI was also identified as the target of the serine protease exfoliative toxin A that causes skin blistering in the staphylococcal scalded-skin syndrome (Amagai et al, 2000). DsgI is increased in Klk5−/− epidermis compared to wt mice and severely reduced in Spink5−/−. In Spink5−/− Klk5−/−, DsgI is restored to levels equivalent to the wt skin. This result is compatible with the reduced overall proteolytic activity detected in Spink5−/− Klk5−/− epidermis combined with complete absence of spontaneous stratum corneum detachment.

The amounts of cholesterol and of neutral and polar lipids were visualized by filipin and Nile red staining, respectively. Both stains revealed linear lipid structures corresponding to the intercellular spaces. Few cholesterol deposits were seen in the SC of wt and Klk5−/−. In Spink5−/−, a pearl-like lipid distribution is observed that is more obvious with filipin. This staining pattern is observed in skin sections from NS patients (Bonnart et al, 2010). Both stains revealed that this abnormal pearl-like pattern is partially alleviated in the epidermis of Spink5−/− Klk5−/− suggesting that the corneocyte lipid envelope is partially restored by elimination of Klk5. Moreover, Spink5−/− have increased cholesterol in the living layers of their epidermis which is reduced in Spink5−/− Klk5−/− epidermis. Filaggrin integrity is also restored in Spink5−/− Klk5−/− skin.

The macroscopic appearance of Spink5−/− Klk5−/− skin is strikingly different from that of SpinkS−/−. SpinkS−/− Klk5−/− mice look normal and show no redness indicating that skin inflammation associated with Lekti-deficiency can be remarkably inhibited by sole elimination of Klk5. Consistently, we found that several pro-allergic and pro-inflammatory cytokine-encoding mRNAs were markedly reduced in Spink5−/− Klk5−/−. As were markedly reduced in Spink5−/− Klk5−/− skin. KLK5 hyperactivity in the absence of LEKTI has been implicated in sustaining the proinflammatory signaling in NS epidermis via the PAR2-NF-KB axis (Briot et al, 2009) and/or through abnormally enhanced processing of the cathelicidin precursor that yields antimicrobial and pro-inflammatory peptides (Yamasaki et al, 2007; Yamasaki et al, 2006). TSLP (thymic stromal lymphopoietin) a major pro-Th2 cytokine which is highly up-regulated in SpinkS−/− mice, (Briot et al, 2009), was highly diminished, as verified by immunohistochemistry. TNF-α, IL-β, IL-6, and IL-18 cytokines, which are also enhanced in SpinkS−/−, were found significantly down-regulated in SpinkS−/− Klk5 skin. SpinkS−/− mice also reproduce acanthosis and features of incomplete differentiation seen in NS patients, such as persistence of nuclei in corneocytes of the stratum corneum (parakeratosis) (Descargues et al, 2005; Fartasch et al, 1999; Hausser and Anton-Lamprecht, 1996). The apparent normalization of SpinkS−/− skin was further examined by transmission electron microscopy (TEM). TEM revealed that in contrast to wt animals, nuclei were present in the stratum corneum of whereas no nuclei were present in the restored stratum corneum of Spink5−/− Klk5−/− animals. This provides evidence that Klk5 ablation is sufficient to induce normal corneocyte differentiation in the Lekti-deficient context. The abnormal low electron-density vesicle structures apparent in Fig. 4 are typically observed in Spink5−/− (Yang et al, 2004) and in the skin of NS patients (Muller et al, 2002) but are absent in Spink5−/− Klk5−/−. TEM also showed stratum corneum/stratum granulosum separation in Spink5−/− whereas no cleavage is seen in skin ultrasections from Spink5−/− Klk5−/− and wt controls. Importantly, a very compact stratum corneum structure with intact desmosomes was seen in Spink5−/− Klk5−/− mice as opposed to Spink5−/− animals in which the split of desmosomes is obvious. Notably, the structure of desmosomes in both the Klk5−/− and the SpinkS−/− Klk5−/− appears different from that of the wt skin in that it is by far more compact and dense. In Klk5−/−, we observed a higher number of desmosomes compared to the wt, consistent with decreased epidermal proteolysis and higher DsgI staining. These TEM results showed no desmosomal cleavage in Spink5−/− Klk5−/− which is consistent with the absence of skin barrier defect.

To investigate the skin phenotype in adult Spink5−/− Klk5−/− animals, we grafted skin from wt, Klk5−/−, SpinkS−/−, and Spink5−/− Klk5−/− mice onto C57BL/6 recipient mice. Eight weeks after grafting, macroscopically, the wt and Klk5−/− grafts had normal hair growth, whereas Spink5−/− and Spink5−/− Klk5−/− grafted skin appeared hyperkeratotic, erythematous, scaly, with no hair. Furthermore, histological examination revealed acanthosis and papillomatosis (invagination) of the epidermis, parakeratosis, and hyperkeratosis associated with stratum corneum detachment. In addition, immune infiltrates were detected in the papillary dermis and neutrophils were present in the stratum corneum. Interestingly, enhanced proteolytic activities were detected by gel zymography not only in Spink5−/− but also in Spink5−/− Klk5−/− skin grafts where these activities were only slightly diminished (Fig. 1). Using specific protease inhibitors and substrates, we identified trypsin- and chymotrypsin-like (20-23 kDa) activities, as well as elastase (28 kDa) in skin graft extracts. The 34 kDa band likely corresponds to mast cell β tryptase (Briot et al, 2009). These results show for the first time that elimination of Klk5 is not sufficient to sustain the normal skin phenotype in adulthood in the absence of Lekti. It appears that in the absence of Klk5, other proteases including Kik7/14 and elastase, remain hyperactive in NS skin. Moreover, analysis of inflammation markers and mast cell infiltration in SpinkS−/− and Spink5−/− Klk5−/− grafted skin showed similar levels of TNF-α and IL-β pro-inflammatory as well as the TSLP and IL-4 pro-allergic cytokines (Fig. 2 A), while IL-17A expression and infiltrating mast cells were markedly reduced in Spink5−/−. 
Klk5” (Fig. 2B-C). Taken together these results indicate that Klk5 ablation in Spink5−/− adult skin only slightly suppresses NS-associated inflammation.

Cumulatively, we provide strong in vivo evidence for a key role of KLK5 in NS since its ablation leads to remarkable inhibition of aberrant epidermal proteolysis and inflammation, normal differentiation, and restoration of skin barrier structure and function at birth. These findings indicate that Klk5 inhibitors represent a potential therapeutic strategy for NS. The observation that Klk5−/− mice show no other detectable phenotype suggests that no significant side-effects are expected from systemic inhibition of Klk5. However, this work uncovers the implication of additional proteases likely to involve Klk7/14 and elastase (Ela2 and neutrophil elastase) which can lead to a delayed severe skin phenotype despite Klk5 - ablation. This means that Lekti ablation leads to unopposed activity of additional proteases other than Klk5. Among them, Klk7 and Klk4 are known to be directly inhibited by Lekti in contrast to Ela2. Our results show that Klk7 and Klk4 can be efficiently activated in the absence of Klk5. In this respect, matriptase which has been shown to be an important activator of Klk7 activity (Sales et al, 2010), may also be involved in proKlk4 activation.

Klk7 and Klk4 are produced by keratinocytes. The observed elastinolytic activity could correspond to epidermal elastase (Ela2) and/or neutrophil elastase produced by infiltrating neutrophils. Klk7 and Klk4 can induce desmosomal cleavage and Klk7 can convert pro-IL-β to bioactive IL-β. Klk4 can activate PAR2 and subsequently the NF-KB pathway, leading to TSLP production and TNF-β pathway activation. In the absence of Klk5, Klk4 could activate PAR2 as previously shown in inflammatory skin disorders where both KLK14 and PAR2 are coexpressed (Stefansson et al, 2008). In addition, neutrophil elastase acts as a biased agonist for PAR2 signaling at sites of inflammation (Ramachandran et al, 2011). Tryptase released by activated infiltrating mast cells also participates to increased proteolytic activity as well as inflammation and itching via PAR2 activation. Proteinases contained in neutrophil granules such as Cathepsin G, proteinase 3 and neutrophil elastase which are released at sites of acute inflammation, can also activate pro-ILβ into IL-β and contribute to tissue inflammation and subsequent tissue destruction.

The interactions of elastases and KLK7 with KLK14 are still unknown, and it is possible that these proteases could be implicated in a more complex biological cascade. The disclosure of delayed but significantly enhanced proteolytic activities leading to lethal lung and gastro-intestinal complications and to severe skin inflammation despite Klk5 ablation in Spink5−/− animals indicate that the use of KLK5 inhibitors combined with compounds targeting Klk7/14 and/or elastase activity should be considered for efficient therapeutic approaches for NS.

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.


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CLASSIFICATIONS

International Classification A61P17/00, A61K38/55
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