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(54) Abstract Title: **Compositions containing serine protease inhibitors**

(57) A compound which is a serine protease inhibitor selected from camostat, a metabolite of camostat, gabexate, nafamostat or sepimostat and pharmaceutically acceptable salts thereof, for the treatment or prevention of a condition caused, or exacerbated by, activity of skin kallikrein enzymes in a mammal, e.g. atopic dermatitis or Netherton's disease.

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The Therapeutic Use of Serine Protease Inhibitors

Field of the Invention

The present invention relates to the use of a serine protease inhibitor for the treatment of a condition caused or exacerbated by activity of skin kallikrein enzymes in a mammal and, for example, for the treatment of atopic dermatitis and Netherton's disease.

Background of the Invention

Atopic dermatitis (AD), sometimes referred to as atopic eczema, is a chronic, relapsing condition which is characterised by pruritis, erythema, dry skin and inflammation. The pathogenesis of AD is still not fully understood. It is generally recognised that perturbations in the skin barrier, due to genetic influences, render the skin more permeable to extrinsic challenges in the form of aeroallergens, chemicals and micro-organisms. This ongoing challenge, in combination with other genetic factors, results in an allergic response characterised by priming of antigen-presenting cells (dendritic cells, Langerhans cells), elevated levels of IgE, activation of resident cells (keratinocytes, mast cells, fibroblasts) and an influx of inflammatory cells into the sites of challenge (T cells, eosinophils). The perturbation in the skin barrier also results in water loss, producing very dry skin.

Another consequence of a defective skin barrier is that the pathogen *Staphylococcus aureus* is found to colonise the skin lesions of more than 90% of AD patients, while being present in only 5% of normal subjects. The density of bacteria has been shown to correlate with the severity of lesion.

Prevalence of the disorder varies widely, but has been estimated to be as high as 20% among children in some western countries. AD is frequently seen in families with a history of atopic diseases (asthma, allergic rhinitis and AD).

Current treatments typically involve a number of approaches, e.g. (i) skin hydration, including use of moisturisers and emollients, (ii) the use of drugs to reduce or modulate the immune response, such as steroids (glucocorticoids) and immunosuppressants (e.g. cyclosporine A, tacrolimus and pimecrolimus), and (iii) elimination of contributory factors such as irritants, allergens, emotional stress factors and infectious agents. Although the drugs can effectively deal with acute phases of the disorder, there are questions over their long-term use,

due to potentially severe side-effects. Oral antibiotics are often used to treat superinfections, although the general use of antibiotics, especially topically, is generally discouraged due to the risk of increasing antibiotic resistance in bacteria.

Netherton's disease is a severe autosomal recessive skin disorder which is caused by mutations in the SPINK5 gene which encodes the serine protease inhibitor LEKTI. This disorder is characterised by ichthyosiform erythroderma, atopy (atopic dermatitis and very high IgE levels) and trichorrhexis invaginata. The disease is often fatal in infants, due to complications with dehydration and enteropathy and an inability to diagnose the disease until the symptoms become too severe. The disease is often confused with atopic dermatitis or erythrodermic psoriasis, non-bullous congenital ichthyosiform erythroderma or other infantile erythrodermas. However, three distinctive features of Netherton's disease have been identified: (1) premature secretion of lamellar body contents; (2) lamellar body-derived extracellular lamellae and stratum corneum lipid membranes extensively separated by foci of electron-dense material; and (3) disturbed transformation of the lamellar body-derived lamellae into mature lamellar membrane structures.

Camostat is 4-(2-(2-(dimethylamino)-2-oxoethoxy)-2-oxoethyl)phenyl 4-guanidinobenzoate. Camostat mesilate (FOY-305) is known as an oral inhibitor of multiple serine proteases and has been used for many years in the clinical therapy of pancreatitis and post-operative reflux eosophagitis.

The metabolite of camostat is chemically 2-(4-(4-guanidinobenzoyloxy)phenyl)acetic acid. It (FOY-251) is a known serine protease inhibitor which is believed to have therapeutic effect in the treatment of pancreatitis and post-operative reflux eosophagitis by virtue of being the active metabolite of camostat.

Nafamostat is chemically 6-carbamimidoylnaphthalen-2-yl 4-guanidinobenzoate. Nafamostat mesilate (FUT-175) is known as an intravenous inhibitor of multiple serine proteases and has been used for several years in the clinical therapy of pancreatitis, disseminated intravenous coagulation and prevention of blood coagulation during extracorporal circulation.

Sepimostat is chemically 6-carbamimidoylnaphthalen-2-yl 4-(4,5-dihydro-1H-imidazol-2-ylamino)benzoate. Sepimostat mesilate (FUT-187) is known as an oral active inhibitor of multiple serine proteases and has been evaluated as a possible drug for pancreatitis and other gastrointestinal conditions.

Gabexate is chemically ethyl 4-(6-guanidinohexanoyloxy)benzoate. Gabexate mesilate (FOY) is known as an intravenous inhibitor of multiple serine proteases and has been used for many years in the clinical therapy of pancreatitis and disseminated intravenous coagulation.

EP0893437 describes certain guanidine derivatives, including camostat and camostat metabolite, nafamostat and gabexate, as inhibitors of tryptase. Amongst the speculative potential indications for tryptase inhibitors, AD is mentioned. However, the document focuses on other indications and there is no evidence for the usefulness of the agents when administered topically to the skin for local effect.

WO2006/108643 discloses the use of salts of camostat for the treatment of cystic fibrosis or chronic obstructive pulmonary disease.

Reference is made to topical administration of camostat mesilate for treatment of recessive dystrophic epidermolysis bullosa, in Ikeda *et al* (1988) *J Am Acad Dermatol*, **18**, 1246-52.

Summary of the Invention

According to a first aspect of the present invention, a serine protease inhibitor selected from camostat, a metabolite of camostat, nafamostat, sepimostat, gabexate and a pharmaceutically acceptable salts thereof, are useful for the treatment or prevention of atopic dermatitis or Netherton's disease in a mammal. More generally, such a compound is useful for the treatment or prevention of a condition caused or exacerbated by activity of skin kallikrein enzymes in a mammal, by administration of the serine protease inhibitor, e.g. in a pharmaceutical composition also comprising a pharmaceutically acceptable carrier or excipient.

Uses and compositions of the invention are expected to be useful in veterinary applications (i.e. wherein the mammal is a domestic or livestock mammal e.g. cat, dog, horse, pig etc). However, the principal expected use or method is in pharmaceutical applications (i.e. wherein the mammal is a human).

References to "treatment" and "prevention" include treatment and prevention of symptoms as well as any underlying disorder, if appropriate. Netherton's disease is a disorder caused by a genetic defect and it is not suggested that the therapy described herein can cure a genetic defect, although it is expected to cure, prevent or improve symptoms.

Detailed Description of the Invention

In one embodiment, the serine protease inhibitor is selected from camostat and pharmaceutically acceptable salts thereof, e.g. the mesilate. In another embodiment, the serine protease inhibitor is selected from a metabolite of camostat and pharmaceutically acceptable salts thereof, e.g. the mesilate. Camostat, camostat metabolite and methods of preparing them are described, e.g. in US4021472 and US4224342 (which documents are herein incorporated by reference in their entirety).

In a further embodiment, the serine protease inhibitor is selected from nafamostat and pharmaceutically acceptable salts thereof, e.g. the mesilate. Nafamostat and methods of preparing it are described, e.g. in US4454338 (which document is herein incorporated by reference in its entirety).

In yet another embodiment, the serine protease inhibitor is selected from sepimostat and pharmaceutically acceptable salts thereof, e.g. the mesilate. Sepimostat and methods of preparing it are described, e.g. in US4777182 and US4820730 (which documents are herein incorporated by reference in their entirety).

In yet a further embodiment, the serine protease inhibitor is selected from gabexate and pharmaceutically acceptable salts thereof, e.g. the mesilate. Gabexate and methods of preparing it are described, e.g. in US3751447 (which document is herein incorporated by reference in its entirety).

The term "pharmaceutically acceptable salt" refers to a salt, for example an acid addition salt or, in certain cases, salts of an organic and inorganic base such as carboxylate, sulphonate and phosphate salt. All such salts are within the scope of this invention, and references to serine protease inhibitors described herein include the salt forms of the compounds. Examples of

pharmaceutically acceptable salts are provided in Berge *et al.*, 1977, "Pharmaceutically Acceptable Salts," *J. Pharm. Sci.*, **66**, 1-19.

The salts of the present invention can be synthesized from the parent compound that contains a basic or acidic moiety by conventional chemical methods such as methods described in *Pharmaceutical Salts: Properties, Selection, and Use*, P. Heinrich Stahl (Editor), Camille G. Wermuth (Editor), ISBN: 3-90639-026-8, August 2002. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, non-aqueous media such as ether, ethyl acetate, ethanol, isopropanol or acetonitrile are used.

Examples of acid addition salts include salts formed with an acid selected from acetic, 2,2-dichloroacetic, adipic, alginic, ascorbic (e.g. L-ascorbic), L-aspartic, benzenesulphonic, benzoic, 4-acetamidobenzoic, butanoic, (+)-camphoric, camphor-sulphonic, (+)-(1*S*)-camphor-10-sulphonic, capric, caproic, caprylic, cinnamic, citric, cyclamic, dodecylsulphuric, ethane-1,2-disulphonic, ethanesulphonic, 2-hydroxyethanesulphonic, formic, fumaric, galactaric, gentisic, glucoheptonic, D-gluconic, glucuronic (e.g. D-glucuronic), glutamic (e.g. L-glutamic), α-oxoglutaric, glycolic, hippuric, hydrobromic, hydrochloric, hydriodic, isethionic, lactic (e.g. (+)-L-lactic, (±)-DL-lactic), lactobionic, maleic, malic, (-)-L-malic, malonic, (±)-DL-mandelic, methanesulphonic (mesilate), naphthalenesulphonic (e.g.naphthalene-2-sulphonic), naphthalene-1,5-disulphonic, 1-hydroxy-2-naphthoic, nicotinic, nitric, oleic, orotic, oxalic, palmitic, pamoic, phosphoric, propionic, L-pyroglutamic, salicylic, 4-amino-salicylic, sebacic, stearic, succinic, sulphuric, tannic, (+)-L-tartaric, thiocyanic, toluenesulphonic (e.g. *p*-toluenesulphonic), undecylenic and valeric acids, as well as acylated amino acids and cation exchange resins.

The serine protease inhibitors described herein may exist as mono- or di-salts.

If the serine protease inhibitor described herein is anionic, or has a functional group which may be anionic (e.g., -COOH may be -COO^-), then a salt may be formed with a suitable cation. Non-limiting examples of suitable inorganic cations include alkali metal ions such as Na^+ and K^+, alkaline earth

metal cations such as Ca^{2+} and Mg^{2+}, and other cations such as Al^{3+}. Non-limiting examples of suitable organic cations include ammonium ion (i.e., NH_4^+) and substituted ammonium ions (e.g., NH_3R^+, $NH_2R_2^+$, NHR_3^+, NR_4^+).

Examples of some suitable substituted ammonium ions are those derived from ethylamine, diethylamine, dicyclohexylamine, triethylamine, butylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine, benzylamine, phenylbenzylamine, choline, meglumine, and tromethamine, as well as amino acids, such as lysine and arginine. An example of a common quaternary ammonium ion is $N(CH_3)_4^+$.

Where the serine protease inhibitors described herein contain an amine function, these may form quaternary ammonium salts, for example by reaction with an alkylating agent according to methods well known to the skilled person. Such quaternary ammonium compounds are within the scope of the invention.

Specific salts of camostat, nafamostat, sepimostat or gabexate that may be mentioned include the hydrogen succinate, succinate, phosphate, acetate, hydrogen tartrate, glycolate, hippurate, 1-hydroxy-2-naphthoate, adipate and glutarate salts. The mesilates are preferred.

It will be appreciated that the serine protease inhibitors described herein may exist in a number of different geometric isomeric, and tautomeric forms and references to compounds of the formula (I) include all such forms.

It will also be appreciated that where the serine protease inhibitors described herein contain one or more chiral centres, they may exist in the form of two or more optical isomers, references to serine protease inhibitors described herein include all optical isomeric forms thereof (e.g. enantiomers, epimers and diastereoisomers), either as individual optical isomers, or mixtures (e.g. racemic mixtures) of two or more optical isomers.

The optical isomers may be characterised and identified by their optical activity (i.e. as + and - isomers, or *d* and *l* isomers) or they may be characterised in terms of their absolute stereochemistry using the "R and S" nomenclature developed by Cahn, Ingold and Prelog, see *Advanced Organic Chemistry* by Jerry March, 4th Edition, John Wiley & Sons, New York, 1992, pages 109-114, and see also Cahn, Ingold & Prelog (1966) *Angew. Chem. Int. Ed. Engl.*, **5**, 385-415.

It will be appreciated that optical isomers may be separated by a number of techniques which are well known to the person skilled in the art, for example, chiral chromatography (chromatography on a chiral support).

References to serine protease inhibitors described herein include references to these compounds as solids in either amorphous or crystalline form, including all polymorphic forms. Crystalline forms may be prepared by recrystallisation of the compounds from appropriate solvents. Amorphous forms may be prepared e.g. by spray drying a solution of the compounds. Examples of polymorphic forms include solvates (e.g. hydrates), complexes (e.g. inclusion complexes or clathrates with compounds such as cyclodextrins, or complexes with metals of the compounds), and pro-drugs of the compounds.

Without wishing to be limited by theory, the serine protease inhibitors described herein are believed to exert their therapeutic effect by inhibition of skin kallikreins, particularly skin kallikreins 5, 6, 7, 8, 13 and 14 (e.g. hK5, hK6, hK7, hK8, hK13 and/or hK14), e.g. skin kallikreins 5, 7, 8 and 14 (e.g. hK5, hK7, hK8 and hK14). Hence the serine protease inhibitors described herein are expected to be useful in treating conditions caused or exacerbated by skin kallikrein activity.

The human tissue kallikrein (KLK) gene family localises as a cluster to chromosome 19q13.4 and encodes 15 secretory serine proteases (hK1-hK15; Yousef and Diamandis (2001) Endocr. Rev. **22**, 184-204) which have been suggested to function as an enzymatic cascade pathway in many tissues (Yousef and Diamandis (2002) Biol Chem **383**, 1045-1057). Several kallikreins have been identified histochemically in the stratum corneum (SC), the stratum granulosum and the skin appendages and are herein referred to as skin kallikreins.

Examples of skin kallikreins include hK5 (previously known as SC trypsin-like enzyme SCTE; Brattsand and Egelrud (1999) *J. Biol. Chem.* **274**, 30033-30040; Yousef and Diamandis (1999) J. Biol. Chem. **274**, 37511-37516), hK7 (previously known as SC chymotrypsin-like enzyme SCCE; Hansson *et al* (1994) *J. Biol. Chem.* **269**, 19420-19426) as well as hK6, hK8 and hK13 (Komatsu et al (2005) *Br. J. Dermatol.* **153**, 274-281).

Such skin kallikreins have been predicted to be secretory serine proteases which are responsible for skin desquamation through an enzymatic cascade pathway (Komatsu, N *et al* (2005) *J. Invest. Dermatol.* **125**, 1182-1189). One such enzyme pathway is believed to be cell signalling via the cleavage/activation of proteinase-activated receptors (PARs). The hypothesis that the human tissue kallikrein (hK) family of proteinases regulates PAR signalling was investigated and indicated that certain kallikreins (e.g. hK5, hK6 and hK14) represent important endogenous regulators of the PARs and that different hKs can have differential actions on PAR_1, PAR_2 and PAR_4 (Oikonomopoulou *et al* (2006) *J. Biol. Chem.* **281**, 32095-32112).

It is therefore believed that abnormalities in the skin desquamation enzymatic cascade pathway may result in certain skin disorders such as atopic dermatitis (for example, caused by overexpression of skin kallikreins). It has been reported that increased epidermal expression of hK7 has been found in chronic lesions of atopic dermatitis (Hansson *et al* (2002) *J. Invest. Dermatol.* **118**, 444-449). Further studies have shown an increase in the number of cells expressing multiple skin kallikreins in atopic dermatitis which could contribute to the pathogenesis (Komatsu *et al* (2005) *Br. J. Dermatol.* **153**, 274-281). Serine protease inhibitors which are capable of inhibition of skin kallikreins are therefore likely to be of therapeutic benefit in skin disorders such as atopic dermatitis.

Interaction of skin kallikreins with potential inhibitors has been examined and found that multiple KLKs (e.g. hK5, hK6, hK13 and hK14) were potently inhibited by differing domains of lympho-epithelial Kazal-type-related inhibitor (rLEKTI(1-6), rLEKTI(6-9') and rLEKTI(9-12)) and hK5 was inhibited by rLEKTI(12-15) (Borgono *et al* (2007) *J. Biol. Chem.* **282**, 3640-3652). LEKTI and its fragments do not inhibit hK1 revealing a selectivity in kallikrein inhibition. Such findings indicate a likelihood that multiple KLKs may participate in desquamation through cleavage of desmogelin and regulation by LEKTI. Netherton's disease is caused by mutations in the SPINK5 gene which encodes the serine protease inhibitor LEKTI. Without being limited by theory, it is believed that skin kallikrein inhibition is restricted in patients with Netherton's disease because regulation of desquamation by LEKTI is correspondingly restricted. Serine protease inhibitors which are capable of inhibition of skin

kallikreins are therefore likely to be of therapeutic benefit in skin disorders such as Netherton's disease by compensating for the restricted activity of LEKTI.

Camostat mesilate is known to inhibit plasma kallikrein, however, it is also known not to inhibit other kallikreins, e.g. renal kallikrein (see Bonner *et al* (1987) *Arzneimittelforschung* **37**, 535-7). Thus it is clear that the ability to inhibit one kallikrein does not indicate or suggest the ability to inhibit another.

Moreover, in at least some embodiments, the activity of the active agent may be enhanced through an antibacterial action. In particular, it has been found that gabexate, camostat and nafamostat are inhibitors of growth of *S. aureus* and that nafamostat is bacteriocidal for *S. aureus*. This bacterium, as mentioned above, is an exacerbatory factor in AD and Netherton's disease. Apparently, this surprising anti-bacterial effect of these agents has not previously been reported. It has also been found that gabexate, camostat and nafamostat are capable of inhibiting the activation of human keratinocytes by *S. aureus*. Activated keratinocytes produce IL-8 and other inflammatory cytokines which exacerbate underlying inflammatory skin conditions.

Accordingly, the invention may permit a useful synergy of therapeutic approach by targeting endogenous skin kalllikreins and having anti-bacterial activity.

By the term 'atopic dermatitis' or 'AD' is meant a chronic relapsing inflammatory skin disease characterised by intense pruritis and cutaneous hyperreactivity associated with elevated serum levels of IgE and eosinophils.

By the term "Netherton's disease" is meant a severe autosomal recessive skin disorder characterised by ichthyosiform erythroderma, atopy (atopic dermatitis and very high IgE levels) and trichorrhexis invaginata. Most patients experience recurrent or persistent bacterial infections.

In a preferred embodiment, the present invention is for the treatment or prevention of atopic dermatitis.

The ability of a given serine protease inhibitor to inhibit skin kallikreins can be determined using the "Skin kallikrein inhibition assay" given in the Examples below.

By "inhibition of skin kallikrein" or "skin kallikrein inhibitor" is meant a compound that exhibits an IC50 value of less than 100 micromolar in the skin

kallikrein inhibition assay, preferably less than 20 micromolar, especially less than 1 micromolar.

In one embodiment, the serine protease inhibitor is formulated for topical or oral administration. It is preferred that the serine protease inhibitor is formulated for topical administration to the skin and it may be administered to a patient in an amount such that from 0.01 mg to 10 g, preferably from 0.1 mg to 1 g active ingredient is delivered per m^2 of the area being treated. Amongst other advantages of topical administration, it is contemplated that an antibacterial effect of the agents may be more readily realised by this route of administration than by other routes.

When administered topically, the agents preferably have a local effect. When administered topically, the agents preferably have little or no systemic effect. When administered topcally for local effect, the agents should not require any systemic effect to be useful in therapy. The topical formulation will preferably maximise surface exposure and minimise systemic exposure to the active ingredient.

Typically, the total amount of inhibitor is from 0.0001 to 10 wt%, suitably from 0.001 to 1.0 wt% based on the total weight of the formulation.

The topical formulation may, for example, take the form of a gel, ointment, cream, lotion or oil. Other example presentations include impregnated dressings, pastes, dusting powders, sprays, transdermal devices etc.

A suitable gel typically comprises a hydrophilic polymer such as cross-linked polyethylene glycol, cross-linked starch or polyvinyl pyrrolidone. An ointment, cream or lotion typically contains an aqueous phase and an oleaginous phase in admixture. They may generally be characterised as oil-in-water emulsions or water-in-oil emulsions. Alternatively, the formulation may be entirely oleaginous, consisting of an oleaginous base or oil such as fractionated coconut oil, sesame oil, avocado oil, corn oil or primrose oil, and may be thickened with white soft paraffin or other suitable thickener.

The formulation may additionally contain one or more emollients, emulsifiers, thickeners and/or preservatives, particularly when it is a cream or ointment.

Emollients suitable for inclusion in creams or ointments are typically long chain alcohols, for example a C8-C22 alcohol such as cetyl alcohol, stearyl alcohol and cetearyl alcohol, hydrocarbons such as petrolatum and light mineral oil, or acetylated lanolin. The total amount of emollient in the formulation is preferably about 5 wt% to about 30 wt%, and more preferably about 5 wt% to about 10 wt% based on the total weight of the formulation.

The emulsifier is typically a nonionic surface active agent, e.g., polysorbate 60 (available from ICI Americas), sorbitan monostearate, polyglyceryl-4 oleate and polyoxyethylene(4)lauryl ether. Generally the total amount of emulsifier is about 2 wt% to about 14 wt%, and more preferably about 2 wt% to about 6 wt% by weight based on the total weight of the formulation.

Pharmaceutically acceptable thickeners, such as VeegumK (available from R. T. Vanderbilt Company, Inc.), and long chain alcohols (i.e. C8-C22 alcohols such as cetyl alcohol, stearyl alcohol and cetearyl alcohol) can be used. The total amount of thickener present is preferably about 3 wt% to about 12 wt% based on the total weight of the formulation.

Preservatives such as methylparaben, propylparaben and benzyl alcohol can be present in the formulation. Other examples of preservatives are phenoxyethanol and chlorocresol. The appropriate amount of such preservative(s) is known to those skilled in the art.

Optionally, an additional solubilizing agent such as benzyl alcohol, lactic acid, acetic acid, stearic acid or hydrochloric acid can be included in the formulation. If an additional solubilizing agent is used, the amount present is preferably about 1 wt% to about 12 wt% based on the total weight of the formulation.

Optionally, the formulation can contain a humectant such as glycerin and a skin penetration-enhancer such as butyl stearate, urea or DMSO.

It is known to those skilled in the art that a single ingredient can perform more than one function in a cream. For example, cetyl alcohol can serve both as an emollient and as a thickener.

Where the formulation or medicament is a cream, it typically consists of an oil phase and a water phase mixed together to form an emulsion. Preferably, the cream comprises an oil-in-water emulsion. Preferably, the amount of water

present in a cream of the invention is about 45 wt% to about 85 wt% based on the total weight of the cream.

One exemplary formulation is a cream which comprises an emulsifying ointment (e.g. around 30 wt%) comprising white soft paraffin, emulsifying wax and liquid paraffin made to 100% with purified water and containing preservative (e.g. phenoxyethanol). This formulation may also be buffered to the required pH (e.g. with citric acid and sodium phosphate). The concentration of active may typically be between 0.001 and 1.0 wt%.

Where the formulation or medicament is an ointment, it typically comprises a pharmaceutically acceptable ointment base such as petrolatum, polyethylene glycol 400 in combination with polyethylene glycol 3350 (available from Union Carbide) or bases such as propylene glycol dicaprylocaprate, linoleoyl macrogolglycerides or caprylic/capric triglycerides (available from Gattefossé). The base may be an oil, such as fractionated coconut oil, sesame oil, avocado oil, corn oil or primrose oil. The amount of ointment base present in an ointment of the invention is preferably about 60 wt% to about 95 wt% based on the total weight of the ointment.

Oral presentations, e.g. as capsules or tablets, may involve formulating the active ingredient with conventional orally acceptable excipients such as binders, glidants, lubricants, disintegrants, diluents and the like. Active ingredient may, for example, be granulated, formulated and compressed into tablets or granulated, formulated and filled into capsules.

An oral presentation of camostat (as mesilate) is available under the brand name Foipan. An iv formulation of gabexate is available under the brand name FOY. An iv formulation of nafamostat is available under the brand name Futhan.

The serine protease inhibitor may be administered in conjunction with further medicaments, such as conventional therapies for the treatment or prevention of inflammatory skin conditions, for example antibiotics, anti-inflammatory agents such as steroids (such as hydrocortisone, clobetasone butyrate, betamethasone valerate, hydrocortisone butyrate, clobetasol propionate, fluticasone propionate, mometasone furoate and dexamethasone), non-steroidal anti-inflammatory drugs, macrolide immunosuppressants (such as

cyclosporine A, tacrolimus and pimecrolimus), leukotriene antagonists and phosphodiesterase inhibitors. The serine protease inhibitor may be administered in conjunction with metalloprotease inhibitors.

These further treatments may be administered by any convenient route or dosage regimen. Topical (typically to the skin) and oral routes are preferred.

Combination treatments may be administered simultaneously, sequentially or separately, by the same or by different routes. In one example embodiment the further medicament may be administered orally. In another example embodiment the further medicament may be administered topically, e.g. in a combined preparation with the serine protease inhibitor.

For example, the further medicament may be an antibiotic substance which is bacteriocidal (e.g. having anti-bacterial activity against *S. aureus*) and which is administered orally or topically. The further medicament may be a metalloprotease inhibitor which can reduce pro-inflammatory matrix metalloprotease activity on the skin.

The following Examples illustrate the invention.

Example 1

Inhibition of human skin kallikreins by camostat, gabexate and nafamostat

The inhibitory activities of camostat (Wako Chemicals GmbH, Neuss, Germany), gabexate (Tocris Bioscience, Bristol, UK; BIOMOL International LP, Exeter, UK; Wako Chemicals GmbH) and nafamostat (BIOMOL International LP) were tested against a range of recombinant human kallikreins (R&D Systems Europe Ltd, Abingdon, UK). For use, kallikreins 7 and 14 were activated with thermolysin (R&D Systems) followed by the addition of EDTA as described by the manufacturer; control incubations confirmed that under the assay conditions described no residual thermolysin activity was detectable. Kallikrein 8 was activated with endoproteinase Lys-C (Sigma-Aldrich Co Ltd, Poole, UK) as described by the manufacturer; control incubations demonstrated that under the assay conditions described no residual Lys-C activity was detectable. Assay mixtures (0.1 ml) ± compound (dissolved in water) were as follows: kallikrein 5 (3.5 ng per assay), 90 mM sodium phosphate buffer pH 8.0, 0.045% Brij 35, 0.1 mM Boc-Val-Pro-Arg-AMC (Bachem GmbH, Weil am Rhein, Germany); kallikrein 7 (15 ng per assay): 45 mM Tris-HCl pH 8.5, 0.135M sodium chloride,

0.045% Brij 35, 10 µM Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 (R&D Systems); kallikrein 8 (10 ng per assay): 45 mM Tris-HCl pH 8.5, 0.135M sodium chloride, 0.045% Brij 35, 0.1 mM Boc-Val-Pro-Arg-AMC; and kallikrein 14 (0.8 ng per assay); 45 mM Tris-HCl pH 8.0, 0.135M sodium chloride, 0.045% Brij 35, 0.1 mM Boc-Val-Pro-Arg-AMC. Control incubations lacked either compound or enzyme. Assays were incubated at 37ºC/1h and stopped with 0.1 ml 0.5M acetic acid. Because of background fluorescence a series of nafamostat controls was also done in which the enzyme was added after the acetic acid. The extent of substrate cleavage was assessed fluorimetrically using 390 nm excitation/460 nm emission (kallikreins 5, 8 and 14) or 320 nm excitation/405 nm emission (kallikrein 7). Percentage inhibition values were calculated after correction for controls without enzyme and the compound concentration eliciting a 50% decrease in enzyme activity under assay conditions (the IC_{50} value) was determined by curve fitting (XLfit, IDBS Ltd).

The data in Table 1 show that skin kallikreins 5, 7, 8 and 14 were all inhibited by the three compounds.

Table 1

Kallikrein	IC_{50} value (µM)		
	Camostat	Gabexate	Nafamostat
5	0.21	6.8	0.22
7	37	62	17
8	0.071	1.9	0.0033
14	1.0	1.3	0.014

Example 2

Inhibition of skin wash serine protease activity by camostat and nafamostat

Skin wash samples from the lesions of AD patients were taken from 1 cm^2 skin defined by a sterile open-ended 20 mm polypropylene cylinder by gentle aspiration with 0.5ml sterile medical saline. The aspirate was transferred to 0.05 ml 0.55 M MOPS buffer pH7.0/55 mM calcium chloride/0.2% Brij 35, cellular debris removed by centrifugation and the samples frozen at -70ºC.

Serine protease activity in 8 such skin wash samples was determined by measuring cleavage of the fluorogenic peptide substrate Boc-Val-Pro-Arg-AMC (Bachem GmbH, Weil am Rhein, Germany). Assay mixtures contained 90 mM Tris-HCl pH 8.0, 0.045% Brij 35, 2.25 mM EDTA, 0.1mM Boc-Val-Pro-Arg-AMC and skin wash sample in a final volume of 0.1ml; control incubations lacked skin wash sample. Reactions were started by the addition of skin wash sample and the fluorescence (390 nm excitation/460 nm emission) monitored continually over a period of 4h taking readings every 10 min; the assay was incubated at 37ºC throughout. Serine protease activity, expressed as fluorescence intensity units (FIU)/min/µl skin wash sample, was calculated from the linear rate of fluorescence increase after correction for controls without skin wash sample. As shown in Table 2, the protease activities in the 8 samples ranged between 0.4 and 5.8FIU/min/µl.

The inhibitory activities of camostat (Wako Chemicals GmbH, Neuss, Germany) and nafamostat (BIOMOL International LP, Exeter, UK) were tested against the same 8 skin wash samples. Assay mixtures, ± compound dissolved in water, contained 45 mM Tris-HCl pH 8.0, 0.135 M sodium chloride, 0.045% Brij 35, 4.5 mM EDTA, 0.1 mM Boc-Val-Pro-Arg-AMC and skin wash sample in a final volume of 0.1 ml; controls lacked either skin wash sample or compound. Reactions were started by the addition of skin wash sample and the fluorescence (390 nm excitation/460 nm emission) monitored continually over a period of 4h taking readings every 10 min; the assay was incubated at 37ºC throughout. Percentage inhibition values were calculated after correction for controls without enzyme and the apparent IC_{50} value for each sample (expressed as the compound concentration eliciting a 50% decrease in activity) was estimated graphically. In a second experiment, the inhibitory activity of nafamostat was retested against 4 of the skin wash samples using a stopped assay. Mixtures, ± compound dissolved in water, contained 45mM Tris-HCl pH 8.0, 0.135 M sodium chloride, 0.045% Brij 35, 4.5 mM EDTA, 0.1 mM Boc-Val-Pro-Arg-AMC and skin wash sample in a final volume of 0.1 ml. As above, controls lacked either skin wash sample or compound. Reactions were started by the addition of skin wash sample, incubated at 37ºC for 4h, stopped with 0.1 ml 0.5 M acetic acid and the extent of substrate cleavage determined

fluorometrically (390 nm excitation/460 nm emission). Percentage inhibition values were calculated after correction for controls without enzyme and the apparent IC_{50} value for each sample (expressed as the compound concentration eliciting a 50% decrease in activity) was estimated graphically. As shown in Table 2, both camostat and nafamostat inhibited the protease activity in skin wash samples with estimated IC_{50} values of 0.08 - 8 µM and 0.04 - 0.8 µM respectively; the IC_{50} values determined for nafomostat using a stopped assay were unchanged.

Table 2

Skin wash sample	Serine protease activity (FIU/min/µl)	Apparent IC_{50} value (µM)		
		Camostat	Nafamostat	Nafamostat (expt. 2)
1	5.8	8	0.8	0.8
2	2.7	0.8	0.1	0.1
3	1.4	0.1	0.06	0.08
4	2.7	0.08	0.04	0.05
5	0.8	0.2	0.08	-
6	0.7	0.7	0.4	-
7	0.6	1	0.6	-
8	0.4	0.1	0.1	-

Example 3

Inhibition of serine protease activity in skin wash samples demonstrated by zymography

Skin wash samples from the lesions of AD patients were taken from 1 cm^2 skin defined by a sterile open-ended 20 mm polypropylene cylinder by gentle aspiration with 0.5 ml sterile medical saline. The aspirate was transferred to 0.05 ml 0.55 M MOPS buffer pH7.0/55 mM calcium chloride/0.2% Brij 35, cellular debris removed by centrifugation and the samples frozen at -70ºC.

Zymographic analysis (Heussen and Dowdle (1980) *Anal. Biochem.* **102**, 196-202) of the proteases in 3 skin wash samples was done using pre-cast 10% gelatin zymogram gels (Invitrogen Ltd, Paisley, UK). Three skin wash samples (20 µl) along with 50 ng of human kallikrein 5 (R&D Systems Europe Ltd,

Abingdon, UK) were mixed with 0.25 volume 5x loading buffer (0.2 M Tris-HCl pH 6.8, 40% (v/v) glycerol, 2.5% (w/v) SDS), loaded in duplicate and run at 125V for approximately 1.5h in Tris-glycine buffer containing 0.1% SDS according to the manufacturer's instructions. Gels were washed in 25 mM MOPS buffer pH7.0 containing 2.5% (w/v) Triton X-100 over a period of 1-2h to remove SDS and allow protease renaturation. The gel was cut in half and incubated overnight at 37ºC in 0.1 M MOPS buffer pH 7.0, 5 mM calcium chloride with or without 1 mM camostat (Wako Chemicals GmbH, Neuss, Germany). Zones of clearing were revealed by staining with Coomassie Brilliant Blue R followed by destaining in 40% (v/v) methanol/10% (v/v) glacial acetic acid.

Protease activity, revealed as zones of clearing, was detected in all three skin wash samples at a mass comparable with that of the skin kallikrein, kallikrein 5. One mM camostat inhibited all the protease activities in the skin wash samples as well as human kallikrein 5 activity.

Example 4

Inhibition of Kallikrein 5 - mediated keratinocyte activation

Activated keratinocytes produce IL-8, a proinflammatory chemokine. Serine proteases can cause the activation of keratinocytes through cleavage of protease activated receptors (L Hou *et al* (1998) *Immunology.* **94**, 356-362). Kallikrein 5 may be evaluated for its effects on IL-8 productuion by keratinocytes. Normal human skin epidermal keratinocytes (TCS Cellworks, Botolph Claydon, Buckingham, UK) are maintained as per instructions. Proliferating cultures are trypsinised, harvested, treated with a trypsin inhibitor and resuspended in keratinocyte growth medium (TCS Cellworks) at approximately 5,000 cells/well, to provide monolayers in 96 well cell culture plates. Cells are incubated overnight at 37°C in 5% CO_2 to allow recovery, the spent medium aspirated from the wells, and the cells washed with phosphate buffered saline (Sigma) and replaced. The cells are incubated at 37°C in 5% CO_2 for a further 18hr with fresh keratinocyte growth medium containing human recombinant kallikrein 5 (R&D Systems Europe Ltd. Abingdon, UK) with or without 50 µM camostat (Wako Chemicals GmBH, Neuss Gemany) or 50 µM nafamostat (BIOMOL International LP).

The supernatants are removed from each well and the concentration of IL-8 is determined using a human IL-8 enzyme-linked immunosorbent assay (ELISA) development kit from R&D systems (Catalog Number: DY208) using the manufacturers instructions.

Results of experiments (camostat only) are shown in Table 3A. Results of further experiments (camostat and nafamostat) are shown in Table 3B.

Table 3A

Human kallikrein 5 (ug/ml)	IL-8 (pg/ml) ±s.d.	
	vehicle	camostat 50uM
Un-stimulated	280±41	287±34
1.25	1200±111	511±64
0.625	821±165	328±6
0.3125	781±25	339±51
0.156	500±31	313±30
0.078	439±10	294±3
0.039	336±7	279±31

Table 3B

Human kallikrein 5 (µg/ml)	IL-8 (pg/ml) ±s.d.		
	vehicle	camostat 50µM	nafamostat 50µM
unstimulated	256±40	184±31	300±6
1.0	986±89	303±9	438±9
0.5	810±18	261±8	424±61
0.25	626±185	274±15	398±38
0.125	471±131	228±24	295±9
0.0625	333±140	219±40	290±12
0.0312	366±36	181±57	318±46
0.0156	291±53	190±35	258±12

The experiments show that kallikrein-5 can cause a dose-dependent increase in the secretion of the chemokine IL-8 in keratinocytes and that this increase is inhibited by the serine protease inhibitors camostat and nafamostat.

Example 5

Inhibition of activation of human keratinocytes *by S. aureus*

Activated keratinocytes produce IL-8, a proinflammatory chemokine. Many bacterial products cause the activation of keratinocytes. In this experiment monolayers of human keratinocytes in culture are infected with *S. aureus* and the effect of serine protease inhibitors on the activation of keratinocytes measured via IL-8 production.

The *S. aureus* are prepared by growth in keratinocyte growth medium at 37°C overnight. The absorbance of the culture is measured and adjusted to an optical density at 600 nm (OD_{600}) of 0.1 in keratinocyte growth medium.

Normal human skin epidermal keratinocytes (TCS Cellworks, Botolph Claydon, Buckingham, UK) are maintained in Epidermal Keratinocyte Basal Medium with Keratinocyte Growth Supplement (keratinocyte growth medium) as per instructions. Proliferating cultures are trypsinised, harvested, treated with a trypsin inhibitor and resuspended in keratinocyte growth medium (TCS Cellworks) at approximately 50,000 cells/well, to provide monolayers in 24-well cell culture plates. Cells are incubated overnight at 37°C in 5% CO_2 to allow recovery. The spent medium is aspirated from the wells, and replaced with fresh keratinocyte growth medium ± vehicle or test compound, with 10 µl of the *S. aureus* 8325-4 inoculum added to the appropriate wells. The keratinocytes are incubated at 37°C in 5% CO_2 for a further 18hr. The culture medium is removed from each well and centrifuged at >10,000 x g for 2 minutes and the supernatant removed. The concentration of IL-8 is determined using a human IL-8 enzyme-linked immunosorbent assay (ELISA) development kit from R&D systems (Catalog Number: DY208) using the manufacturers instructions.

Results of the experiments are shown in Table 4A. Results of a subsequent experiment are shown in Table 4B.

Table 4A

Concentration (uM)	IL-8 (pg/ml) Gabexate		Nafamostat	
	Un-stimulated	+ *S. aureus*	Un-stimulated	+ *S. aureus*
Vehicle	34	1089	25	1050
30	42	1343	50	1096
100	40	718	56	72
200	40	69	83	61

Table 4B

Concentration (uM)	Camostat	
	Un-stimulated	+ *S. aureus*
Vehicle	10	790
25	8	734
50	2	975
100	4	916
200	3	582
400	7	135

The results show that gabexate, nafamostat and camostat inhibit the activation of keratinocytes by *S. aureus*.

Example 6

Inhibition of *S. aureus* 8325-4 growth

The effect of the serine protease inhibitors on *S. aureus* growth in conditions optimum for the growth of human keratinocytes in culture was tested.

To each well of a 24-well cell culture plate are added 0.5 ml Epidermal Keratinocyte Basal Medium with Keratinocyte Growth Supplement (TCS CellWorks) ± vehicle or test compounds. The *S. aureus* inoculum is prepared by growth of *S. aureus* 8325-4 in keratinocyte growth medium at 37°C overnight. The absorbance of the culture is measured and adjusted to an optical density at 600nm (OD_{600}) of 0.1 in keratinocyte growth medium. 10 µl of *S. aureus* 8325-4 inoculum is added to the appropriate wells.

The cultures are incubated for 18-20hrs at 37°C in an atmosphere of 5% CO_2 and the absorbance measured at 620 nm. Growth inhibition was determined by reference to the vehicle control and the minimal inhibitory

concentration determined as the concentration at which there was >90% inhibition of growth.

Results of experiments are shown in Table 5.

Table 5

Compound	MIC (uM)
gabexate	400
nafamostat	50
camostat	200

The experiment shows that gabexate, nafamostat and camostat inhibit the growth of *S. aureus* when grown in conditions optimised for the in vitro growth of human epidermal keratinocytes (nafamostat shows the strongest effect under these conditions).

Example 7

Time-kill analysis following exposure to nafamostat

S. aureus 8325-4 was maintained on tryptone soya agar (Oxoid). Cultures were grown at 37°C in tryptone soya broth (TSB) overnight then diluted with fresh broth to yield a starting inoculum of approximately 4 x 10^6 CFU/ml. Nafamostat was added at a final concentration four- or eightfold above its minimal inhibitory concentration, and the cultures incubated with agitation at 37°C. A parallel culture containing no antibiotic served as a control. Colony counts were determined at intervals by serial dilution onto tryptone soya agar plates (see Table 6).

Table 6

Time hrs	Colony forming units / ml		
	vehicle	250 µM nafamostat	500 µM nafamostat
0	3,690,000	3,690,000	3,690,000
1	20,400,000	44,530	17,200
2	97,000,000	15,730	4,550
4	1,560,000,000	4,300	950
6	2,190,000,000	400	100

This analysis shows that nafamostat can rapidly kill *S. aureus* and has a bactericidal mode of action.

All references referred to in this application, including patents and patent applications, are incorporated herein by reference to the fullest extent possible.

Throughout the specification and the claims which follow, unless the context requires otherwise, the word 'comprise', and variations such as 'comprises' and 'comprising', will be understood to imply the inclusion of a stated integer, step, group of integers or group of steps but not to the exclusion of any other integer, step, group of integers or group of steps.

Abbreviations

AD	atopic dermatitis
AMC	7-amido-4-methylcoumarin
Boc	*t*-butyloxycarbonyl
Dnp	2,4-dinitrophenyl
FIU	fluorescence intensity units
Mca	(7-methoxycoumarin-4-yl)acetyl
MOPS	3-(N-morpholino)ethanesulphonic acid
SC	stratum corneum
SDS	sodium dodecyl sulphate
iv	intravenous

CLAIMS

1. A compound which is a serine protease inhibitor selected from camostat, a metabolite of camostat, gabexate, nafamostat, sepimostat and pharmaceutically acceptable salts thereof, for the treatment or prevention of a condition caused or exacerbated by activity of skin kallikrein enzymes in a mammal.

2. A compound as defined in claim 1, for the treatment or prevention of atopic dermatitis or Netherton's disease in a mammal.

3. A compound according to claim 1 or claim 2, which is camostat or a pharmaceutically acceptable salt thereof.

4. A compound according to claim 1 or claim 2, which is camostat mesilate.

5. A compound according to claim 1 or claim 2, which is camostat metabolite or a pharmaceutically acceptable salt thereof.

6. A compound according to claim 1 or claim 2, which is gabexate or a pharmaceutically acceptable salt thereof.

7. A compound according to claim 1 or claim 2, which is gabexate mesilate.

8. A compound according to claim 1 or claim 2, which is nafamostat or a pharmaceutically acceptable salt thereof.

9. A compound according to claim 1 or claim 2, which is nafamostat mesilate.

10. A compound according to claim 1 or claim 2, which is sepimostat or a pharmaceutically acceptable salt thereof.

11. A compound according to claim 1 or claim 2, which is sepimostat mesilate.

12. A compound according to any preceding claim, to be administered orally or topically.

13. A compound according to any preceding claim, to be administered topically.

14. A compound according to any preceding claim, wherein the serine protease inhibitor is administered in combination with a further agent selected from an antibiotic, an agent which modulates the inflammatory response, an immunosuppressant and a metalloprotease inhibitor.

15. A topical pharmaceutical composition comprising a compound as defined in any of claims 1 to 11, together with a topically acceptable diluent or carrier.

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Application No:	GB0809068.0	**Examiner:**	Dr Jeremy Kaye
Claims searched:	1-15	**Date of search:**	16 September 2008

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-15	JP06192085 A (INADA YUJI & ONO PHARMA CORP.) see abstract
X	1-15	US 5346886 A (LEZDEY ET AL.) see whole document
X	1-15	WO 2005/117955 A1 (ARRIVA PHARMA. INC.) see p.2, l.8 - p.4, l.28
X	1-15	Allergy, Vol.61, 2006, Smith, P. K. et al., "Serine proteases, their inhibitors and allergy", pp.1441-1447 see esp. pp.1444-1445, "Thrapeutic serine protease inhibitors"
X	1-15	Annals Allergy, Vol.69, 1992, Wachter, A. M. et al., "Treatment of atopic dermatitis...", pp.407-414
A	-	US4978534 A (SAITOH) see whole document

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X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
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Worldwide search of patent documents classified in the following areas of the IPC

A61K

The following online and other databases have been used in the preparation of this search report

WPI, EPODOC, TXTE, BIOSIS, MEDLINE, CAPLUS

International Classification:

Subclass	Subgroup	Valid From
A61K	0031/24	01/01/2006
A61K	0031/235	01/01/2006
A61P	0017/00	01/01/2006