Abstract: A protease inhibitor or protease inhibitor-protease complex for use in treatment of inflammation and/or its sequelae, wherein the inflammation involves inflammatory cells that have the capacity to release proteases and for treatment of a condition in a subject characterised by or involving an increased number or proportion of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, relative to the corresponding number or proportion of said cells in a healthy subject, wherein the protease inhibitor is Aα@Ab5 of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of the inflammatory or other cells, or alternatively wherein the protease inhibitor is Aα@Ab5 of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of the inflammatory or other cells or has no effect on apoptosis of the inflammatory or other cells.
before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
PROTEASE INHIBITORS AND THEIR THERAPEUTIC APPLICATIONS

TECHNICAL FIELD OF THE INVENTION

The present invention relates to protease inhibitors and their use in new or modified therapeutic applications. The present invention also relates to complexes of protease inhibitors and proteases, and use of the complexes in new or modified therapeutic applications.

BACKGROUND OF THE INVENTION

Several protease inhibitors are already available for therapeutic use. These include serum-derived, recombinant and transgenic preparations of alpha 1-antitrypsin, also known as alpha 1-proteinase inhibitor, alpha 1-protease inhibitor or alpha 1-antiprotease, synthetic inhibitors that block neutrophil elastase, and recombinant secretory leucoprotease inhibitor. Therapeutic strategies using protease inhibitors have been designed to inhibit proteases in vivo and hence reduce protease-mediated tissue damage.

It is known that some proteases, also known as proteinases, peptidases or proteolytic enzymes, such as elastase, are pro-apoptotic. It has been generally accepted that protease inhibitors, including alpha 1-antitrypsin, have an anti-apoptotic effect by inhibiting protease-mediated apoptosis. For example, WO 00/51624 describes a method of treating a subject suffering from a disease characterised by excessive apoptosis by administering a serine protease inhibitor. According to WO 00/51624, increased levels or apparent induction of apoptosis is associated with a number of diseases including cancer, autoimmune diseases including rheumatoid arthritis, neurodegenerative diseases, myocardial infarction, stroke, sepsis, ischemia-reperfusion injury, toxin induced liver injury, and AIDS (see Kidd, V.J., Annu Rev Physiol, 1998, 60, 533; List, P.J.M., et al., Arterioscler Thromb Vasc Biol, 1999, 19, 14; Jabs, T., Biochem Pharmacol, 1999, 57, 231; Deigner, H. P., et al., Curr Med Chem, 1999, 6, 399). The apoptosis appears to be mediated by oxygen free radicals [O] which have been implicated in various disorders including atherosclerosis, diabetes, sepsis, Alzheimer's disease, arthritis, muscular dystrophy, cancer, Downs syndrome, multiple sclerosis, HIV infection and other inflammatory diseases (Morel, J.B. and Dangle, J.L., Cell Death Differ, 1997, 4, 671; Beal, M.F., Curr Opin Neurobiol, 1996, 6, 661). In the examples of WO 00/51624, it is
demonstrated that the serine protease inhibitor alpha 1-antitrypsin has an anti-apoptotic effect on both rat cerebral granule neuronal cells and primary rat brain granule cells. The synthetic serine protease inhibitor CE-2072 also has an anti-apoptotic effect on rat cerebral granule neuronal cells. That teaching is confirmed in Ikari et al (J. Biol. Chem., 2001, 276, 11798-11803), which demonstrates that the serine protease inhibitors alpha 1-antitrypsin, alpha 1-antichymotrypsin and alpha 2-macroglobulin are anti-apoptotic factors for human smooth muscle cells. The mechanism of the anti-apoptotic effect involves the protection of the tissues and extracellular matrix from degradation by cell-derived proteinases. The anti-apoptotic effect was found to be proportional to the proteinase inhibitory activity.

SUMMARY OF THE INVENTION

The present invention is based on the observation that some protease inhibitors but not others have a pro-apoptotic effect on inflammatory cells.

The present invention relates to a protease inhibitor for use in treatment of a condition in a subject characterised by or involving an increased number or proportion of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, relative to the corresponding number or proportion of said cells in a healthy subject.

In one embodiment of the invention the protease inhibitor is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells.

In another embodiment of the invention the protease inhibitor has substantially no effect on apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, or is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells.

The invention also provides the use of a protease inhibitor for the manufacture of a medicament for use in a treatment as defined above.
The invention further provides a method of treatment, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a protease inhibitor as defined above.

In a further aspect the present invention provides an isolated complex or complexes comprising a protease inhibitor and a protease. The protease and protease inhibitor of the complex may be present in native or preferably in modified form. Such complexes may provide a more potent medicament than a protease inhibitor alone. The present invention also provides such complexes for use as a medicament, and in particular for use in a treatment as defined above. The invention further provides a method of treatment as defined above, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of such a complex. This embodiment is based on the observation that a complex comprising protease inhibitor and protease has a greater pro-apoptotic effect on neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, than either protease inhibitor or protease alone.

**BRIEF DESCRIPTION OF THE FIGURES**

Figures 1a-1e show the light microscopy appearance of human blood neutrophils aged in culture.

Figure 1a shows the appearance of the cells at 0 hours at x 63 magnification. Normal mature healthy neutrophils with intact cell membranes and multi-lobed nuclei can be seen. Figure 1b shows the appearance of the cells at 9 hours at x 54 magnification. The majority of the neutrophils appear normal but a minority have progressed to early stages of apoptosis.

Figure 1c shows the appearance of the cells at 24 hours at x 63 magnification. Many of the neutrophils have characteristic features of early stage apoptosis, including cell size shrinkage and nuclear chromatin condensation. Figure 1d shows the appearance of the cells at 48 hours at x54 magnification. In the absence of clearance mechanisms, some neutrophils have progressed to secondary necrosis as indicated by loss of cell membrane integrity, rupture of cell membranes, discharge of cell contents and release of cytoplasmic constituents.

Figure 1e shows the appearance of the cells induced to undergo primary necrosis by heating at 56°C for 30 minutes, at x 54 magnification.
Figures 2a-2d show electron micrographs of human blood neutrophils aged in culture. Figure 2a shows the appearance of a neutrophil at 0 hours at x 7,500 magnification. The neutrophil is a normal mature healthy cell with a typical multi-lobed nucleus.

Figure 2b shows a neutrophil at 24 hours at x 5,000 magnification. The neutrophil is at an early stage of apoptosis and has condensed nuclear chromatin. The nuclear and cell membranes and cytoplasmic lysosomal granules are intact.

Figure 2c shows a neutrophil at 48 hours at x 5,000 magnification. The neutrophil is at a later stage of apoptosis showing nuclear fragmentation and breakdown of the nuclear membrane. The cell membrane and cytoplasmic lysosomal granules are still intact.

Figure 2d shows a neutrophil at 72 hours at x 7,500 magnification. The neutrophil has progressed to secondary necrosis. The cell membrane has deteriorated allowing cytoplasmic contents to spill into the extracellular space.

Figure 3 shows the effect of elastase on neutrophil survival. The percentage of normal neutrophils and the percentage of apoptotic neutrophils (early and late stages) for samples with and without elastase are shown at 0, 9, 18 and 24 hours. Data are given as means ± SEM (n=3); * denotes p≤0.05, *** denotes p≤0.001 compared to without elastase; paired samples t-test.

Figure 4 shows a comparison of the proportion of viable and non-viable neutrophils in cultures treated with and without elastase. The percentage of non-viable neutrophils and the percentage of viable neutrophils for samples with and without elastase are shown at 0, 9, 18 and 24 hours.

Figure 5 shows the effect of protease inhibitors on elastase induced neutrophil apoptosis. The percentage of normal neutrophils and the percentage of apoptotic neutrophils (early and late stages) for samples without elastase, with elastase only, with elastase and alpha 1-antitrypsin and with elastase and aprotinin are shown at 0, 9, 18 and 24 hours. Data are given as means ± SEM (n=3); ** denotes p≤0.01, *** denotes p≤0.001 and # compared to neutrophils with elastase only; paired samples t-test.
Figure 6 shows a comparison of the effects of inhibitors alone and inhibitors in combination with elastase on neutrophil apoptosis. The percentage of normal neutrophils and the percentage of apoptotic neutrophils (early and late stages) for samples without elastase or inhibitor, with elastase only, with elastase and alpha 1-antitrypsin, with alpha 1-antitrypsin only, with elastase and aprotinin and with aprotinin only are shown at 0, 9, 18 and 24 hours. Data are given as means ± SEM (n=3); * denotes p≤0.05, ** denotes p≤0.01 compared to neutrophils with elastase and inhibitor; # denotes p≤0.05, ## denotes p≤0.01 compared to neutrophils without elastase or inhibitor; paired samples t-test.

Figure 7 shows the effects of G-CSF and GM-CSF on neutrophil survival. The percentage of normal neutrophils and the percentage of apoptotic neutrophils (early and late stages) for samples with G-CSF, with GM-CSF and a control without colony stimulating factors at 0, 9, 18 and 24 hours. Data are given as means ± SEM (n=3); * denotes p≤0.05, ** denotes p≤0.01, *** denotes p≤0.001 compared to control neutrophils without colony stimulating factors; paired samples t-test.

Figure 8 shows a comparison of the effects of G-CSF and GM-CSF alone and in combination with elastase on neutrophil survival. The percentage of normal neutrophils and the percentage of apoptotic neutrophils (early and late stages) for samples with elastase only, with G-CSF only, with elastase and G-CSF, with GM-CSF only, with elastase and GM-CSF, and a control without colony stimulating factors are shown at 0, 9, 18 and 24 hours. Data are given as means ± SEM (n=3); * denotes p≤0.05, *** denotes p≤0.001 compared to neutrophils with CSF only; # denotes p≤0.05 compared to neutrophils with elastase only; paired samples t-test.

Figure 9 shows a comparison of the effects of G-CSF and GM-CSF in combination with alpha 1-antitrypsin and elastase on neutrophil survival. The percentage of normal neutrophils and the percentage of apoptotic neutrophils (early and late stages) for samples with alpha 1-antitrypsin only, with elastase and alpha 1-antitrypsin, with elastase, G-CSF and alpha 1-antitrypsin, with elastase, GM-CSF and alpha 1-antitrypsin and a control are shown at 0, 9, 18 and 24 hours. Data are given as means ± SEM (n=3); * denotes p≤0.05 compared to neutrophils with elastase and alpha 1-antitrypsin; paired samples t-test.
Figure 10 shows a comparison of the effects of G-CSF and GM-CSF in combination with aprotinin and elastase on neutrophil survival. The percentage of normal neutrophils and the percentage of apoptotic neutrophils (early and late stages) for samples with aprotinin only, with elastase and aprotinin, with elastase, G-CSF and aprotinin, with elastase, GM-CSF and aprotinin and a control are shown at 0, 9, 18 and 24 hours. Data are given as means ± SEM (n=3); * denotes p<0.05 compared to neutrophils with elastase and alpha 1-antitrypsin; paired samples t-test.

DEFINITIONS

Neutrophilia
Neutrophilia denotes an increase in the number of neutrophils or the proportion of neutrophils relative to other cell types in comparison with the corresponding number or proportion in a healthy subject, in an organ, system, secretion, inflammatory infiltrate or exudates, or body fluid of any type.

Increase in cells that are capable of releasing proteases
As used herein, the term includes neutrophilia (see above) and also includes an increase in the number or proportion of any other cells that are capable of releasing proteases, such as inflammatory cells, in comparison with the corresponding number or proportion in a healthy subject, in an organ, system, secretion, inflammatory infiltrate or exudates, or body fluid of any type.

Pro-apoptotic protease inhibitor
The term “pro-apoptotic protease inhibitor” is used herein to denote a protease inhibitor that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells.

Non-apoptotic protease inhibitor
The term “non-apoptotic protease inhibitor” is used herein to denote a protease inhibitor that has substantially no effect on apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, or that is capable of directly or
indirectly reducing, preventing, delaying or suppressing apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells.

5 DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the surprising observation that certain protease inhibitors are capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of cells that are capable of releasing proteases, such as inflammatory cells, whereas other protease inhibitors are capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of the inflammatory cells or have little or no effect on apoptosis of cells that are capable of releasing proteases, such as inflammatory cells. Alpha 1-antitrypsin is an example of a protease inhibitor that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of neutrophils. In particular, alpha 1-antitrypsin is capable of enhancing protease-induced apoptosis of neutrophils. Aprotinin is an example of a protease inhibitor that is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of neutrophils or that has little or no effect on apoptosis of neutrophils.

The present invention provides a new strategy to classify protease inhibitors according to their apoptotic effects in order to select the most appropriate type of inhibitor (with or without pro-apoptotic effects) for use in treatment of different conditions associated with, involving or characterized by inflammation and/or its sequelae, wherein the inflammation involves neutrophils and/or other inflammatory cells that are capable of releasing proteases. This new strategy provides pro-apoptotic inhibitors for use in speeding clearance of inflammatory cells and resolution of inflammation in conditions where removal of inflammatory cells is beneficial, e.g. when inflammation occurs in the absence of infection. It also provides inhibitors without pro-apoptotic effects for use in conditions where it is advantageous to maintain the presence of inflammatory cells, e.g. when inflammation occurs in the presence of infection, in order that beneficial anti-microbial and other functions of inflammatory cells can be maintained while still allowing inhibition of proteases in extra-cellular locations.
This surprising observation enables the design of new and modified therapeutic strategies for treatment of conditions that involve inflammation and/or its sequelae and of conditions characterised by or involving an increased number or proportion of neutrophils and/or other cells that have the capability of releasing proteases, such as inflammatory cells, relative to the corresponding number or proportion of those cells in a healthy subject.

Neutrophilia is generally understood to be an increase in the number of neutrophils or the proportion of neutrophils relative to other cell types in comparison with the corresponding number or proportion in a healthy subject, in an organ, system, secretion, inflammatory infiltrate or exudates, or body fluid of any type. Neutrophilia is a characteristic feature of some but not all types of inflammation. Neutrophilia and/or increases in other types of cells that are capable of releasing proteases, such as inflammatory cells, are involved in many conditions, see the section “Neutrophils and other inflammatory cells” below.

Preferably, a protease inhibitor that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of cells that are capable of releasing proteases, such as inflammatory cells, does not promote apoptosis of other cell types in the surrounding region nor of macrophages, monocytes or other cells that contribute to resolution of inflammation by phagocytosing and clearing apoptotic cells.

**Neutrophils and other inflammatory cells**

Mature neutrophils contain stores of elastase and other neutral proteases in lysosomal granules within their cytoplasm, where the proteases can participate in proteolytic degradation of phagocytosed micro-organisms and other material without damaging the cell itself. However, when neutrophils are activated during inflammation some of their stored proteases are released into the extracellular space. Neutrophils may be activated and the release of proteases may occur before inflammation is evident clinically. If the amount of proteases released exceeds the capacity of protease inhibitors in the extracellular space to fully block their activity, this can result in damage to neighbouring host tissues.

Blocking the activity of excessive amounts of proteases in the extracellular space forms the basis of current therapeutic applications of protease inhibitors, but current therapeutic strategies were designed without the knowledge of the present invention that some but not
other protease inhibitors are also pro-apoptotic to inflammatory cells and consequently also have the ability to promote clearance of inflammatory cells and speed resolution of inflammation. The present invention enables the most appropriate type of inhibitor (with or without pro-apoptotic effects on inflammatory cells) to be selected for treatment of different conditions and this in turn not only leads to new therapeutic applications but also leads to a change of use for some conditions currently treated with protease inhibitors to employ a different type of protease inhibitor from that currently used for therapy.

Other types of cells in addition to neutrophils have the capability of releasing proteases. In particular those cells include inflammatory cells. Those cells may also contain cytoplasmic lysosomal granules. Particular mention may be made of cells, such as basophils and mast cells, that are capable of releasing elastase of the same type as neutrophil elastase (‘granulocyte elastase’) and other proteases, including some specific to those cells. Eosinophils also have lysosomes and can also release proteases including some that are eosinophil specific. It is probable that elastase and its inhibitors may be able to modulate the apoptosis of basophils and mast cells as well as neutrophils, since these cells all produce elastase. In view of the fact that monocytes and macrophages can produce alpha 1-antitrypsin and play an important role in the clearance of inflammation by phagocytosing and degrading apoptotic inflammatory cells, it is unlikely that their apoptosis will be induced by the same proteases and inhibitors that have pro-apoptotic effects on other inflammatory cells. Elastase can stimulate monocytes and macrophages to secrete alpha 1-antitrypsin and binding of the protease-inhibitor complex to a receptor on these cells can signal an increase in alpha 1-antitrypsin biosynthesis. It is possible that this may protect the cells from elastase induced apoptosis. Monocytes and macrophages have receptors for elastase as well as for elastase complexed to alpha 1-antitrypsin. These receptors may facilitate the uptake by monocytes and macrophages of apoptotic cells with surface bound elastase such as neutrophils, basophils or mast cells. Other types of cells, such as cytotoxic lymphocytes and natural killer cells, have the capability of releasing proteases and are involved in inflammation. It is proposed that the types of proteases released by the particular class of inflammatory or other protease-releasing cells and the inhibitors of these proteases are likely to include those that may be capable of modulating the apoptosis of that particular cell type. These mechanisms may play a role in the normal physiologic processes that regulate inflammation.
**Conditions to be treated**

In one aspect of the invention, the protease inhibitor or complex used for treatment is an inhibitor or complex that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, i.e. a pro-apoptotic protease inhibitor or pro-apoptotic complex. Such an inhibitor or complex is preferred for use to speed clearance of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells and resolution of inflammation in conditions where removal of such cells is beneficial to the host e.g. when inflammation occurs in the absence of infection or after elimination of infection when inflammatory cells are no longer needed for antimicrobial host defence functions. Pro-apoptotic inhibitors or pro-apoptotic complexes may be used in treatment of inflammation and/or its sequelae, and in treatment of other conditions characterised by or involving an increased number or proportion of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, relative to the corresponding number or proportion of those cells in a healthy subject.

The pro-apoptotic treatment according to the present invention promotes resolution of neutrophilic inflammation or other inflammatory conditions by increasing the rate of apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, so that they can be more rapidly recognised and removed by mononuclear phagocytes, such as monocytes and macrophages, and other cells that have the capacity to clear apoptotic neutrophils and other types of apoptotic cells. This has the advantage of promoting clearance of neutrophils or other cells as well as helping to suppress extracellular proteolytic tissue damage. This is in direct contrast to previous work which, as indicated above, has shown that in other, non-inflammatory, cell types, protease inhibitors protect against apoptosis. The anti-apoptotic effect has been used for therapeutic purposes, for example, in WO 00/51624, where diseases characterized by excessive apoptosis of tissue cells are treated with protease inhibitors.

A pro-apoptotic protease inhibitor or pro-apoptotic complex according to the present invention may be used for treatment of acute, sub-acute or chronic inflammation in the absence of infection, an allergic disease, an occupational or environmental disease or
disorder, an infection after elimination of the infectious agent where inflammation persists, or another non-infectious disease, disorder or condition involving inflammation or an increase in numbers or proportions of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells.

For example, a pro-apoptotic protease inhibitor or pro-apoptotic complex may be used to treat a rheumatological disease, liver diseases, especially chronic active hepatitis and primary biliary cirrhosis, congestive heart failure, especially following acute myocardial infarction, a neutrophilic dermatosis, ocular diseases such as keratoconjunctivitis or uveitis, or sepsis in the absence of evidence of infection or after elimination of infection by treatment with an antibiotic.

A pro-apoptotic protease inhibitor or pro-apoptotic complex may be used in treatment of a disease, disorder or condition of the respiratory system involving inflammation or an increase in numbers or proportions of the neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells. Examples are treatment of acute or chronic non-infectious inflammatory lung diseases, such as chronic fibrosing lung diseases, including idiopathic pulmonary fibrosis and other idiopathic interstitial pneumonias, collagen vascular diseases involving the lungs and occupational fibrosing lung diseases such as asbestosis; Wegener's granulomatosis; an acute lung injury, such as treatment of severe pneumonia after control of infection, or acute respiratory distress syndrome, or acute lung injury following cardiopulmonary by-pass surgery; treatment of bronchiectasis after control of infection, obliterative bronchiolitis (chronic rejection) in a lung transplant patient after treatment of any infection, chronic obstructive airways disease after treatment of any infection, non-genetic emphysema, emphysema caused by smoking or environmental factors, or emphysema caused by a variant alpha 1-antitrypsin, chronic lung disease especially in premature infants and children, asthma, in particular severe life-threatening asthma or chronic 'non-steroid responsive' asthma, and other allergic diseases such as rhinitis.

Neutrophilia plays a role in many disorders in which there is no evidence of any infectious agent or any persistent infectious agent. Further, neutrophilia may persist following elimination of infection in patients suffering disorders in which neutrophilia is associated
with infection. The presence or persistence of neutrophilia in the lung may lead to the development or progression of acute lung injury. A pro-apoptotic protease inhibitor or pro-apoptotic complex is useful in the treatment of such disorders and the resolution of the neutrophilia by promoting apoptosis of the neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, and hence their clearance from the site. In the case of an infection, it is preferable to use the pro-apoptotic protease inhibitor or pro-apoptotic complex treatment after treatment of the infection with an antibiotic, for example, after elimination of infection. For example, a pro-apoptotic protease inhibitor or pro-apoptotic complex is suitable for use in the treatment of severe pneumonia after the elimination of infection, sepsis after the elimination of infection, acute lung injury triggered by non-infectious risk factors, acute lung injury following cardiopulmonary by-pass surgery, chronic fibrosing lung diseases, chronic lung disease in premature infants, chronic obstructive Airways disease, severe life-threatening asthma and chronic ‘non-steroid response’ asthma. An acute lung injury triggered by non-infectious risk factors that is particularly suitable for treatment according to the present invention is acute respiratory distress syndrome. Chronic obstructive Airways disease which is not caused by a patient’s deficiency of alpha 1-antitrypsin is especially suitable for treatment according to the present invention.

Chronic neutrophilia occurs in some forms of emphysema. For those forms of emphysema, a therapeutic strategy designed with the main aim of suppressing the adverse effects of chronic neutrophilia is desirable. Accordingly, in one embodiment, the present invention concerns the use of a pro-apoptotic protease inhibitor or pro-apoptotic complex in the treatment of forms of emphysema in which chronic neutrophilia is present, by suppressing the adverse effects of chronic neutrophilia. Forms of emphysema for which the present invention is especially useful are non-genetically induced forms of emphysema. In particular, the present invention finds application in the treatment of environmentally induced or smoking-related emphysema. The invention does not include the use of alpha 1-antitrypsin for treatment of genetic emphysema resulting in a deficiency of alpha 1-antitrypsin, but does include the use for treatment of genetic emphysema resulting in a variant alpha 1-antitrypsin that is less functional than native alpha 1-antitrypsin.
In a further aspect of the invention, the pro-apoptotic protease inhibitor or pro-apoptotic complex is an inhibitor or complex that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of eosinophils. Such an inhibitor may be used in treatment of conditions characterised by or involving an increased number or proportion of eosinophils relative to the corresponding number or proportion of eosinophils in a healthy subject. In particular, a pro-apoptotic inhibitor or pro-apoptotic complex according to the invention may be used in treatment of eosinophilia in association with allergic diseases such as asthma or rhinitis, cryptogenic pulmonary eosinophilia, pulmonary eosinophilia associated with drugs, helminths or bronchopulmonary aspergillosis, Churg Strauss syndrome, or vasculitis. This approach may also be used in treatment of patients with idiopathic pulmonary fibrosis, other idiopathic interstitial pneumonias, other chronic fibrosing lung diseases, or in patients with systemic connective tissue diseases who can have increases in eosinophils as well as neutrophils in the lungs or other affected organs.

In a further aspect of the invention, the pro-apoptotic protease inhibitor or pro-apoptotic complex is an inhibitor or complex that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of basophils or mast cells. Such an inhibitor may be used in treatment of conditions characterised by or involving an increased number or proportion of basophils or mast cells relative to the corresponding number or proportion of basophils or mast cells in a healthy subject. In particular, a pro-apoptotic inhibitor or pro-apoptotic complex according to the invention may be used in treatment of disorders associated with Type I 'immediate' hypersensitivity reactions. Examples are asthma, eczema, hay fever, rhinitis, urticaria and anaphylaxis. Increases in basophils in the blood also occur in myxoedema, hypothyroid conditions, ulcerative colitis and certain types of anaemia. Infiltrates of mast cells in the tissues also occur in rare diffuse cutaneous or systemic mastocytomas. Increases in mast cells also occur in association with fibrotic lesions in the lungs, skin or other organs. Treatment according to the invention may be used to promote the apoptosis and clearance of mast cells or basophils.

In a further aspect of the invention, the pro-apoptotic protease inhibitor or pro-apoptotic complex is an inhibitor or complex that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of lymphocytes. Such an
inhibitor or complex may be used in treatment of conditions characterised by or involving an increased number or proportion of lymphocytes relative to the corresponding number or proportion of lymphocytes in a healthy subject. In particular, a pro-apoptotic inhibitor or pro-apoptotic complex according to the invention may be used in treatment of
granulomatous diseases involving the lungs and/or other systems, for example sarcoidosis, extrinsic allergic alveolitis, berylliosis, silicosis, tuberculosis and other mycobacterial diseases.

In a still further aspect of the invention, the pro-apoptotic protease inhibitor or
pro-apoptotic complex is an inhibitor or complex that is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of malignant cells. Such an inhibitor or complex may be used in treatment of conditions characterised by or involving an increased number or proportion of malignant cells relative to the corresponding number or proportion of malignant cells in a healthy subject. In particular, a pro-apoptotic inhibitor or pro-apoptotic complex according to the invention may be used in treatment of leukaemias, lymphomas, Hodgkin’s lymphoma, and Histiocytosis X also called Langerhan’s cell granuloma. The inhibitor or complex may also be used to reduce the numbers of inflammatory cells or increase local levels or production of protease inhibitors by inflammatory cells in the vicinity of tumours to reduce proteolytic degradation of matrix components, which is thought to facilitate the spread of tumour cells.

A pro-apoptotic inhibitor or pro-apoptotic complex can also be used to counteract the effect of other agents that can prolong the life of inflammatory cells. For example, the cytokines G-CSF and GM-CSF can prolong the life of neutrophils and eosinophils. These mechanisms are operative in many diseases characterised by neutrophilic and/or eosinophilic inflammation, including asthma and acute lung injury. Corticosteroid drugs can also prolong the life of neutrophils and this may contribute to chronic airways inflammation in chronic obstructive airways diseases, non-steroid responsive asthma, and severe asthma in children. Patients with malignancies who develop severe neutropenia when on treatment with cytotoxic drugs are often treated with recombinant G-CSF to increase blood neutrophil levels, but occasionally this can result in development of acute lung injury. A pro-apoptotic inhibitor or pro-apoptotic complex may be used to counteract excessive increases in neutrophils in such patients.
In the above aspects of the invention, the pro-apoptotic protease inhibitor is, for example, alpha 1-antitrypsin.

In another aspect of the invention, the protease inhibitor or complex used for treatment is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, or has substantially no effect on apoptosis of those cells, a non-apoptotic protease inhibitor or non-apoptotic complex. Such an inhibitor or complex finds application in treatment of conditions where it is advantageous to maintain the presence of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells. One example of such a condition is a condition in which inflammation occurs in the presence of infection. In such a condition it is advantageous to maintain the presence of inflammatory cells so that the beneficial anti-microbial function and other physiological functions of those inflammatory cells are maintained while inhibition of proteases in extracellular locations occurs in order to suppress protease-mediated tissue damage. For example, the non-apoptotic protease inhibitor or non-apoptotic complex is preferred for use in treatment of a condition before elimination of an infection, which condition is characterised by or involving inflammation or an increased number or proportion of inflammatory cells that have the capacity to release proteases relative to the corresponding number or proportion of inflammatory cells in a healthy subject. The non-apoptotic protease inhibitor or non-apoptotic complex is generally used before treatment with an antibiotic is started or during treatment with an antibiotic before elimination of the infection.

Examples of conditions that may be treated according to this embodiment of the invention include pneumonias, in particular severe life-threatening pneumonias, and sepsis. In such disorders, there is a risk of development of acute lung injury. The use of a non-apoptotic protease inhibitor or a non-apoptotic complex before antibiotic treatment is started or before the infection has responded to such treatment, maintains the host defence functions of neutrophils and/or other inflammatory cells whilst reducing the risk of protease mediated tissue damage and acute lung injury.
In the above aspects of the invention, the pro-apoptotic protease inhibitor is, for example, aprotinin.

In summary, in the case of an infection, a non-apoptotic protease inhibitor, e.g. aprotinin, or a non-apoptotic complex may be used before treatment with an antibiotic is started or before the infection responds to antibiotic treatment. The use of a non-apoptotic protease inhibitor or non-apoptotic complex results in the maintenance of numbers of inflammatory cells and their host defence functions. At later stages when infection has been eliminated, if neutrophilia or other types of inflammation persist, for example, if acute lung injury persists or progresses, a pro-apoptotic protease inhibitor, e.g. alpha 1-antitrypsin, or pro-apoptotic complex may be used to reduce the number of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, and allow resolution of tissue injury.

In another variant of this application, a protease inhibitor or a protease-protease inhibitor complex that is pro-apoptotic for inflammatory cells such as neutrophils may be used to protect macrophages and/or other cells that are required for phagocytosis and clearance of apoptotic inflammatory cells, by delaying their apoptosis and by stimulating the synthesis of anti-proteases by these cells. Conditions suitable for treatment using this approach are those involving numerical or functional deficiency of macrophages. For example, this approach can be used to treat persistent macrophage deficiency in patients with acute lung injury, which is associated with poor outcome. This approach may also be used to protect macrophages and stimulate their increased intracellular production of protease inhibitors in patients with granulomatous diseases, such as silicosis, berylliosis, extrinsic allergic alveolitis, tuberculosis and other mycobacterial diseases, to improve the stabilisation of cytoplasmic lysosomes and allow more efficient digestion of phagocytosed particles or micro-organisms.

The subject to be treated according to the present invention is generally a human but may be another mammal. For example, the subject may be a companion animal, for example, a dog or a cat; a farm animal raised for hair, hide, meat or milk, for example, a bovine, ovine or porcine animal; a horse; or a zoo animal. For example, horses can develop a form of chronic obstructive airways disease (COPD) known as heaves, and this can affect up to
50% of the animals. Increases in neutrophils in the airways are a feature of the pathology (Dagleish MP, Pemberton AD, Brazil TJ, McAleese SM, Miller HR and Scudamore CL. Kinetics of equine neutrophil elastase release and superoxide anion generation following secretagogue activation: a potential mechanism for antiproteinase inactivation. Vet Immunol Immunopathol 1998; 66: 53-65).

Protease inhibitors

There are several systems of classification and nomenclature of enzymes and their inhibitors in use. The term "protease" is a more up to date term, but the alternative terms "proteinase", "peptidase" and "proteolytic enzyme" are still in common usage. The term "protease" is used herein.


Proteases are enzymes that degrade proteins by hydrolysing peptide bonds. They are classified, based on their catalytic mechanisms, into serine, aspartic, metallo, threonine and cysteine proteases. They can be subdivided into exopeptidases, enzymes which act near a terminus of the polypeptide chain, and endopeptidases, which cleave internal peptide bonds. Endopeptidases degrade proteins into polypeptides, while the terminal processing of the polypeptides is carried out by exopeptidases.

Neutrophils contain and can release a number of different proteases, for example, elastase, Proteinase 3, Cathepsin G and the Matrix Metalloproteases MMP-8 and MMP-9. Neutrophil elastase and other elastases are serine proteases. ‘Serine proteases’ are endopeptidases so the term ‘serine proteinase’ is also commonly used.


Naturally occurring protease inhibitors are proteins. A protease inhibitor for use according to the present invention may be naturally occurring, synthetic, recombinant, transgenic, a genetic variant or a chemically or otherwise modified form of a naturally occurring inhibitor, or a synthetic chemical molecule. Examples of protease inhibitors are given in WO 00/51624 and in US Patent No. 5,874,585. The ability of a molecule to function as a protease inhibitor may be determined by standard tests, for example, using an appropriate method to determine the catalytic activity of the particular enzyme, then conducting the assay in the presence and absence of the putative inhibitor to determine the effect of that molecule on the kinetics of the enzyme substrate reaction. Fritz H, Seemuller U and
Tschesche H; ‘General review: proteinases and their inhibitors’; in Bergmeyer et al, 1984 *(loc. cit.)* pages 74 to 98, gives a detailed overview of the proteinase inhibitors and the methods for determination of inhibitor concentrations and inhibitor activity.

The apoptotic effect of any protease inhibitor or of any complex in relation to neutrophils and/or other cells that are capable of releasing proteases may be determined as described below. Having identified whether the inhibitor or complex is pro-apoptotic or non-apoptotic, the inhibitor or complex may be used in accordance with the present invention.

The term ‘apoptosis’ is classically used to describe the sequence of morphological changes that occur in individual cells during programmed cell death. The changes in morphology characteristic of apoptosis include decreased cell volume, condensation of nuclear chromatin then nuclear fragmentation and lysis of the nuclear membrane, compaction of cytoplasmic organelles, and increased cell density. Such changes may be detected using light or electron microscopy.

A suitable test system for apoptosis of neutrophils and also of the other cells that are capable of releasing proteases, and in particular inflammatory cells is described in the examples herein. That system used light microscopy and the ‘gold standard’ of electron microscopy. The test is based on information in the literature, for example, Hebert MJ, Takano T, Holthofer, and Brady H R; Sequential morphologic events during apoptosis of human neutrophils: modulation by lipoxygenase-derived eicosanoids. J. Immunol. 1996; 157: 3105-3115), but is modified to define the optimal standardized procedure and time points to best demonstrate whether a substance can accelerate or delay the rate of neutrophil apoptosis.

Other techniques that may be used in assessing apoptosis include confocal, laser and scanning microscopy, flow cytometry, fluorescent DNA dye binding and molecular techniques, which permit detection of apoptosis in formalin-fixed and embedded tissue, including terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick end labelling (TUNEL) and in situ end labelling (ISEL). Further techniques include, for example, evaluation of cell DNA content using flow cytometry; determination of the characteristic ‘ladder’ pattern of DNA fragmentation in agarose gel electrophoresis,
determination of surface exposure of phosphatidyl serine on the cell membrane using Annexin 5; and many other methods.

The test system described in the examples or any other suitable test, for example, as mentioned above, may be used as a method of determining if a protease inhibitor or a complex is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of the neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of the those cells, or has substantially no effect on apoptosis of those cells. Such a method is part of the present invention. The invention also includes the use of a pro-apoptotic protease inhibitor or complex or a non-apoptotic protease inhibitor or complex identified according to the method of the present invention in a method or use of the present invention.

An example of a protease inhibitor that has been found according to the present invention to be capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, is alpha 1-antitrypsin. Alpha 1-antitrypsin, also called alpha 1-protease inhibitor, alpha 1-protease inhibitor or alpha 1-antiprotease, is a 54 kDa glycoprotein that is synthesized primarily by hepatocytes. Synthesis is also possible in monocytes and macrophages. Increased amounts are synthesized by the liver as part of the acute phase response to inflammation.

Alpha 1-antitrypsin for use according to the present invention may be obtained from any appropriate source, for example, it may be obtained from a natural source, or may be recombinant or produced by a transgenic animal. The amino acid sequence of the protein and/or the glycoprotein element may be modified provided the modified molecule retains the functions necessary for the practice of the present invention, for example, protease inhibitory activity and pro-apoptotic activity in relation to neutrophils and/or other cells capable of releasing proteases.

An example of a protease inhibitor that has been found according to the present invention to be capable of directly or indirectly reducing, preventing, delaying or suppressing
apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, or has substantially no effect on apoptosis of those cells is aprotinin. Aprotinin, also called bovine pancreatic trypsin inhibitor or BPTI, is a polypeptide extracted from bovine pancreas. It is a serine proteinase inhibitor that forms tight and stoichiometric complexes with its target enzymes under the conditions usually used in inhibition assays. It is available commercially in highly purified form (from Bayer AG and others). As described above in relation to alpha 1-antitrypsin, aprotinin may be obtained from any appropriate source and may be modified.

The mechanism by which a protease inhibitor or complex is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of cells that are capable of releasing proteases, such as inflammatory cells, or of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of cells that are capable of releasing proteases, such as inflammatory cells, or has no effect on apoptosis of cells that are capable of releasing proteases, such as inflammatory cells, is not known. However, while not being bound by the following, hypotheses for the mechanism of action are presented below:

In the case of the pro-apoptotic protease inhibitors, it is possible that the increased numbers of apoptotic cells, for example the increased levels of apoptotic neutrophils, observed in the presence of alpha-1 antitrypsin may be due to an ability of the inhibitor to increase the rate at which spontaneous apoptosis occurs. The inhibitor may affect the rate of apoptosis directly or it may affect the rate by some interactive effect i.e. indirectly. For example the inhibitor may enhance the pro-apoptotic effect of proteases on apoptosis of neutrophils, or it may enhance or counteract the effect of other agents, for example certain cytokines, that can increase or reduce apoptosis of neutrophils. A further possibility is that the inhibitor is inducing the onset of apoptosis at an earlier time point than normal, for example by triggering a pathway that switches on the process or by antagonizing a mechanism that represses the process.

In the case of the non-apoptotic protease inhibitors, for example aprotinin, it is possible that the inhibitor has no effect on the apoptosis of inflammatory cells or is able to prolong the life span of inflammatory cells by reducing the rate of apoptosis either directly or indirectly.
For example, aprotinin may directly or indirectly reduce apoptosis of neutrophils or it may reduce, prevent, delay or suppress protease-mediated apoptosis of neutrophils.

However, the mechanism of action is not important for the practice of the invention. The capability of a protease inhibitor or complex for directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, or for directly or indirectly reducing, preventing, delaying or suppressing apoptosis of neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells, or for having little or no effect on apoptosis of the those cells may be determined by routine experimentation as described herein.

**Complexes**

According to another embodiment, the present invention provides for isolated complexes comprising a protease inhibitor and a protease. The protease inhibitor and protease of the complex may be in natural or native form or in modified form. A complex according to the invention may be used as a medicament or in a method of treatment as described above. In particular, a complex according to the invention may be used as a medicament to modulate apoptosis. A complex according to the invention may also be used for the manufacture of a medicament for the treatment of conditions associated with neutrophilia or other types of inflammation. In particular, the complexes according to the invention may find application as a more potent pro-apoptotic therapy than a pro-apoptotic inhibitor alone for treatment of conditions associated with neutrophilia. Preferably, the protease inhibitor in the complex is a serine protease inhibitor. An especially preferred protease inhibitor is alpha 1-antitrypsin.

The invention also provides for the manufacture of isolated complexes comprising a protease inhibitor and a protease.

The amount of the protease inhibitor or complex which is required to achieve a therapeutic effect will, of course, vary with the particular protease inhibitor, the route of administration, the subject under treatment, and the particular disorder or disease being treated.
In the various treatments according to the present invention the protease inhibitor or complex is generally used in the form of a pharmaceutical composition in admixture with a pharmaceutically suitable carrier. Remington's Pharmaceutical Sciences by E. W. Martin is a standard text on pharmaceutical formulations.

The present invention has applications in a wide range of medical conditions, see above, in particular those associated with, involving or characterized by increases in neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells. Those conditions involve many different organs and systems. The preferred routes of administration will vary depending on the pathology of the different diseases. For example, for some of the respiratory tract and lung disorders, introduction of the protease inhibitor or complex via the respiratory tract, for example, by aerosol, instillation or inhalable powder may be most appropriate. However, there is systemic involvement in some respiratory disorders so for those conditions, and the wide range of other disorders, any suitable route and means of administration may be used.

Examples of suitable routes of administration include, for example oral, rectal, parenteral e.g. intravenous, intramuscular, or intraperitoneal, mucosal e.g. buccal, sublingual, nasal, subcutaneous or transdermal administration, including administration by inhalation. The pharmaceutical compositions may be adapted accordingly.

For oral administration, a composition may be formulated as a liquid or solid, for example as a solution, syrup, suspension or emulsion, as a tablet, capsule or lozenge.

A liquid formulation will generally comprise a suspension or solution of the compound in suitable aqueous or non-aqueous liquid carrier(s) for example water, ethanol, glycerine, polyethylene glycol or an oil. The formulation may also contain a suspending agent, preservative, flavouring or colouring agent.

A composition in the form of a tablet may be prepared using any suitable pharmaceutical carrier(s). Examples of such carriers include magnesium stearate, starch, lactose, sucrose and microcrystalline cellulose.
A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, powders, granules or pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, cellulosics, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule.

Compositions for oral administration may be designed to protect the active ingredient against degradation as it passes through the alimentary tract, for example by an outer coating of the formulation on a tablet or capsule.

Typical parenteral compositions, including compositions for subcutaneous administration, comprise a solution or suspension of the compound or physiologically acceptable salt in a sterile aqueous or non-aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilised and then reconstituted with a suitable solvent prior to administration.

Compositions for nasal or oral administration may conveniently be formulated as aerosols, drops, gels and powders. Aerosol formulations typically comprise a solution or fine suspension of the active substance in a physiologically acceptable aqueous or non-aqueous solvent and are usually presented in single or multidose quantities in sterile form in a sealed container, which can take the form of a cartridge or refill for use with an atomising device. Alternatively the sealed container may be a unitary dispensing device such as a single dose nasal inhaler or an aerosol dispenser fitted with a metering valve which is intended for disposal once the contents of the container have been exhausted. Where the dosage form comprises an aerosol dispenser, it will contain a pharmaceutically acceptable propellant. The aerosol dosage forms can also take the form of a pump-atomiser. Such compositions may be formulated such that the active substance is distributed in the respiratory tract e.g. the lower respiratory tract and/or in the lungs.
Compositions suitable for buccal or sublingual administration include tablets, lozenges and pastilles, wherein the active ingredient is formulated with a carrier such as sugar and acacia, tragacanth, or gelatin and glycerin.

Compositions for rectal or vaginal administration are conveniently in the form of suppositories (containing a conventional suppository base such as cocoa butter), pessaries, vaginal tabs, foams or enemas.

Compositions suitable for transdermal administration include ointments, gels, patches and injections including powder injections.

A pharmaceutical composition of the invention may be in unit dose form, for example, as a tablet, capsule or ampoule, or may be in the form of a slow release, controlled release or depot preparation.

Combination therapies
It may be advantageous to use treatment with a protease inhibitor or complex according to the present invention in combination with treatment using another therapeutic agent. For example, treatment according to the present invention may be used in addition to the therapeutic agent(s) conventionally used in the treatment of the wide range of different diseases involving, associated with, or characterised by increases in neutrophils and/or other cells that are capable of releasing proteases, such as inflammatory cells.

For example, patients with cystic fibrosis, bronchiectasis, sepsis, pneumonia, other forms of acute lung injury, and lung transplant patients will usually be on treatment with antibiotics. Patients with COPD or asthma will usually be using bronchodilators and may also be on steroids, either inhaled or oral. Patients with rheumatological and other inflammatory diseases involving the connective tissues are likely to be on treatment with steroids or other immunosuppressive or anti-inflammatory drugs. Chronic neutrophilic inflammation is also a feature of the complication of obliterative bronchiolitis (chronic rejection) which frequently develops in lung transplant patient. Patients with that condition will be receiving immunosuppressive drugs such as cyclosporine to prevent rejection, and may receive
methylprednisolone to treat episodes of acute rejection, and also antibiotics of various kinds to treat episodes of infection.

In patients with asthma or COPD, it may be advantageous to combine the therapy according to the invention with therapeutic surfactants or surfactant components, which can assist delivery and also have antimicrobial and antiallergic effects, in aerosols. Combination therapy with instilled therapeutic surfactants may also have advantages for treatment of respiratory distress syndromes.

A protease inhibitor may be combined with the other agent(s) in a single pharmaceutical composition or, more generally, will be administered in a separate composition. Administration may be substantially simultaneous with, or before, or after administration of the other agent.

It may be advantageous to deliver the protease inhibitor or complex in combination with anti-oxidant therapy, for example, an anti-oxidation agent, to protect the inhibitor from in vivo damage by oxidants released during inflammation.

It may also be advantageous to use a protease inhibitor according to the invention in combination therapy with an inhibitor of other protease(s) to protect the inhibitor from proteolytic damage.

In one aspect, the present invention does not include the use, in any of the various embodiments of the invention, of a serine protease inhibitor in the treatment of a disease characterized by excessive apoptosis. The present invention does not include the use, in any of the various embodiments of the invention, of a serine protease inhibitor in the treatment of cancer, autoimmune diseases, neurodegenerative disease, myocardial infarction, stroke, ischemia-reperfusion injury, sepsis or AIDS, characterized by excessive apoptosis.

In another aspect, the present invention does not include the use, in any of the various embodiments of the invention, of a pro-apoptotic serine protease inhibitor in the treatment of a disease characterized by excessive apoptosis. The present invention does not include
the use, in any of the various embodiments of the invention, of a pro-apoptotic serine protease inhibitor in the treatment of cancer, autoimmune diseases, neurodegenerative disease, myocardial infarction, stroke, ischemia-reperfusion injury, sepsis or AIDS, characterized by excessive apoptosis.

In a further aspect, the present invention does not include the use, in any of the various embodiments of the invention, of α1-antitrypsin in the treatment of a disease characterized by excessive apoptosis. The present invention does not include the use, in any of the various embodiments of the invention, of α1-antitrypsin in the treatment of cancer, autoimmune diseases, neurodegenerative disease, myocardial infarction, stroke, ischemia-reperfusion injury, sepsis or AIDS, characterized by excessive apoptosis.

In a further aspect, the present invention does not include the use, in any of the various embodiments of the invention, of an α1-antitrypsin-like agent, including but not limited to oxidation-resistant variants of α1-anti-trypsin, and peptoids with antitrypsin activity in the treatment of a disease characterized by excessive apoptosis. The present invention does not include the use, in any of the various embodiments of the invention, of an α1-antitrypsin-like agent, including but not limited to oxidation-resistant variants of α1-anti-trypsin, and peptoids with antitrypsin activity in the treatment of cancer, autoimmune diseases, neurodegenerative disease, myocardial infarction, stroke, ischemia-reperfusion injury, sepsis or AIDS, characterized by excessive apoptosis.

The one aspect, the present invention provides, in the various embodiments of the present invention, the use of a non-apoptotic serine protease inhibitor in the treatment of sepsis, especially in combination with antibiotic therapy, as described above.

The following non-limiting Examples illustrate the invention.

EXAMPLES

The following general methods were used to perform the studies in the Examples that follow. Terms and abbreviations are consistent with those in current use in the technical field.
GENERAL METHODS

1: Isolation and culture of neutrophils

Human neutrophils were isolated from peripheral blood of normal healthy volunteers. For each set of experiments, a 60 ml volume of venous blood was drawn from each volunteer. Using a glass flask and defibrinating rod, both siliconized to prevent cell adherence, the blood was immediately defibrinated by constant swirling motion to prevent commencement of clotting mechanisms. A 5 ml aliquot of the defibrinated blood was centrifuged at 200 g to obtain autologous plasma, which was then heat inactivated at 56 °C for 20 minutes in a water bath to inactivate complement.

In 15 ml polypropylene tubes, the remaining defibrinated blood was carefully layered in 5 ml aliquots over an equal volume of Polymorphoprep™ separation medium (Nycomed Pharma AS, Norway; product code: 221710), which had been filtered through a 0.22 µm pore size acrodisc filter (Minisart; Sartorius, Germany) prior to use. The tubes containing the layers were then centrifuged at 500 g (Sorval RT6000; DuPont) for 30 minutes at 20°C. The Polymorphoprep™ reagent is constituted from sodium diatrizoate and dextran 500. It has a density of 1.113± 0.001 g/ml, which allows the mononuclear and polymorphonuclear leucocytes to be separated into two distinct bands upon centrifugation. The dextran 500 agglutinates erythrocytes, causing their sedimentation to the bottom of the tube. Tubes were carefully removed from the centrifuge to avoid disturbance of the formed bands. The narrow white band containing neutrophils (below the mononuclear cell band) from each tube was collected and pooled. Neutrophil cells were then washed with 45 ml of 0.5 N minimum essential medium (normal MEM; with 25 mM HEPES without L-Glutamine; Life Technologies, product code: 22370019, which was diluted with normal 0.9% NaCl saline). The neutrophil cells were deposited by centrifugation at 140 g for 5 minutes and washed as before. After washing, the cell pellet was resuspended in a small volume of undiluted MEM and the number of cells per ml was determined using Kimura stained cells in an Improved Neubauer Counting Chamber. The final concentration of cells was adjusted to 1.5 x 10^6 cells per millilitre of culture medium. The culture media consisted of MEM supplemented with 1% heat-inactivated autologous plasma (prepared as described above). The use of autologous plasma rather than foetal calf serum to supplement culture medium, especially in experiments evaluating neutrophil apoptosis is an established method of minimising inadvertent activation of neutrophils. Furthermore, addition of 1% autologous
plasma is sufficient to 'buffer' the neutrophils during culture without enhancing their resistance to protease-induced apoptosis. The culture media was supplemented with 1% antibiotic mixture (Sigma; product code: G-1146), containing 10,000 U penicillin, 10 mg streptomycin and 25 µg amphotericin. Isolated neutrophils resuspended in culture medium with and without added mediators were then incubated in sterile 6ml polypropylene tubes (Falcon, Becton Dickinson) in 1 ml aliquots at 37°C.

2: Cytokines and Inhibitors
Human neutrophil elastase (product code: 324681) and the protease inhibitor alpha 1-antitrypsin (purified from human plasma, product code: 178251) were both purchased in lyophilised form from Calbiochem-Novabiochem Corporation, California, U.S.A; both had a purity of ≥ 95% as determined by SDS-PAGE. The synthetic protease inhibitor, aprotinin, was purchased from Sigma (product code: A1153). The purified neutrophil elastase was provided as specific units of activity per mg of protein. One unit of activity was defined as the amount of enzyme required to hydrolyse 1.0 µmol of MeO-Suc-Ala-Ala-Pro-Val-pNA per minute at 25 °C at a pH of 8.0. The concentration of active elastase in BAL from patients with established ARDS varies from 0.57 ± 0.14 to 1.85 ± 0.39 Units per ml BAL fluid, depending on the stage in injury. Therefore, for this study 1 Unit of elastase activity per ml (molarity = 1.70µM) culture medium was chosen because this dose was comparable to reported in vivo activity levels in the lungs in ARDS. The molarity of the added elastase was calculated to be 1.70µM, but additional endogenous elastase release by neutrophils in culture is estimated at 1µg per million neutrophils and will increase the overall molarity. Therefore, in cultures containing 1.5 × 10⁶ cells/ml the total elastase molarity is estimated to be 1.75µM. The protease inhibitors alpha 1-antitrypsin and aprotinin were added to cultures of isolated neutrophils at concentrations which were five fold in excess of elastase molar equivalents, as it has been demonstrated that using this ratio, elastase activity can be completely abolished by alpha 1-antitrypsin (Fujita et al., 1990). Experiments were also conducted with human recombinant colony stimulating factors, G-CSF (R&D Systems Europe Ltd, product code: 214-CS) and GM-CSF (Biosource International, U.S.A, product code: PHC2014). Concentrations of both mediators were equivalent to 100 U/ml. Those concentrations are optimal for inhibiting neutrophil apoptosis at the required time points.
3: Neutrophil Culture Experimental Protocol

Isolated peripheral blood neutrophils, resuspended at a concentration of $1.5 \times 10^6$ in culture medium, were added to sterile polypropylene tubes (Falcon 2063, Becton Dickinson, U.K) in 1 ml aliquots. Neutrophils were either cultured alone i.e. without any added mediators and thus represented the control preparation for each set of experiments, or neutrophils were cultured in the presence of different combinations of mediators, as detailed below, to study the effects of different treatments.

Table 1: Experimental design for neutrophil cultures showing the different additives

<table>
<thead>
<tr>
<th>Entry</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry 1</td>
<td>Control (no mediators added)</td>
</tr>
<tr>
<td>Entry 2</td>
<td>G-CSF</td>
</tr>
<tr>
<td>Entry 3</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>Entry 4</td>
<td>neutrophil elastase</td>
</tr>
<tr>
<td>Entry 5</td>
<td>α1-AT</td>
</tr>
<tr>
<td>Entry 6</td>
<td>Aprotinin</td>
</tr>
<tr>
<td>Entry 7</td>
<td>neutrophil elastase + α1-AT</td>
</tr>
<tr>
<td>Entry 8</td>
<td>neutrophil elastase + aprotinin</td>
</tr>
<tr>
<td>Entry 9</td>
<td>neutrophil elastase + G-CSF</td>
</tr>
<tr>
<td>Entry 10</td>
<td>neutrophil elastase + GM-CSF</td>
</tr>
<tr>
<td>Entry 11</td>
<td>neutrophil elastase + G-CSF + α1-AT</td>
</tr>
<tr>
<td>Entry 12</td>
<td>neutrophil elastase + GM-CSF + α1-AT</td>
</tr>
<tr>
<td>Entry 13</td>
<td>neutrophil elastase + G-CSF + aprotinin</td>
</tr>
<tr>
<td>Entry 14</td>
<td>neutrophil elastase + GM-CSF + aprotinin</td>
</tr>
</tbody>
</table>

G-CSF added to cultures at a concentration of 100 U/ml, GM-CSF (100 U/ml), neutrophil elastase (1 U/ml) and the inhibitors, α1-AT and aprotinin were added at five fold molar equivalents of neutrophil elastase.

Aliquots of cell suspension from each tube were removed at 0 hours of culture, and then subsequently at 9 hours, 18 hours and 24 hours after the start of culture, in order to prepare cytocentrifuge slides and determine cell viability counts.
4: Assessment of Neutrophil Morphology by Light Microscopy

To assess morphological changes of neutrophils when aged in culture, cytocentrifuge preparations (slides) of cells were made. Using a 100μl aliquot of culture suspension per slide, cells were spun at 450 rpm for 10 minutes in a cytocentrifuge (Cytospin 3; Shandon, U.K.). The slides were dried overnight and then stained the next day with May-Grünwald Giemsa stain. Using a light microscope (Dialux 20, Leitz, Germany), slides were viewed at a magnification of x40, and the changes in neutrophil morphology over time, under the different culture conditions were noted. The characteristic changes manifested by apoptotic neutrophils include reduction in cell size and more obviously the condensation of nuclear material with the disappearance of the typical normal ‘loose’ multilobed nuclei connected by thin chromatin strands. At a later stage, due to the absence in clearance of apoptotic neutrophils, secondary necrosis commencing with nuclear fragmentation then cell membrane lysis and spillage of intracellular contents occurs. Counts of neutrophils with normal, apoptotic and secondary necrosis features were determined for each of the time points with a minimum of 300 cells counted per slide. The number of normal and apoptotic (including the later stage of secondary necrosis) neutrophils were expressed as a percentage of the total cells.

5: Trypan Blue Viability Test

In addition to morphological assessment of neutrophil ageing, the use of trypan blue staining to further examine the kinetics of programmed cell death on cell viability was determined. A 20 μl aliquot of neutrophil cell suspension was gently but thoroughly mixed with 20μl of 1% trypan blue (made using 0.9% NaCl). Then 10 μl of this mixture containing the stained cells was pipetted onto a glass slide and covered with a cover slip. Using a x40 objective on a light microscope (Dialux 20, Leitz, Germany), slides were read in an organised manner from the top left hand corner across and down in a ziz-zag pattern. Cells were categorized as either (i) viable; if no stain was taken up cell nuclei i.e. they were not stained blue indicating that nuclear cell membrane integrity was intact, or (ii) non-viable; if cell nuclei were blue in appearance, indicative of loss of nuclear membrane integrity. Ultimately ≥100 cells were counted, and the numbers of viable and non-viable cells were expressed as a percentage of the total number of cells.
6: Electron Microscopy (EM)

To preserve material for EM analysis, aliquots from the isolated peripheral blood neutrophil cultures were fixed in a double strength solution of glutaraldehyde (containing 50 ml of 0.1 M sodium cacodylate; 4 ml of 0.1 M hydrochloric acid; 2 ml of 1% calcium chloride solution and 10 ml of 25% aqueous glutaraldehyde) to compensate for the dilutional effect of the culture medium. This is double the strength of the ‘normal’ solution conventionally used for EM, which is diluted by half with distilled water prior to use in tissue fixation. Therefore, for the cell suspensions the volume of double strength glutaraldehyde solution added to the volume of sample used was 1.5:1, respectively. The samples were fixed in this solution for a minimum of 24 hours at 4 °C.

Fixation, dehydration and embedding of cellular material

A second stage fixation with osmium tetroxide was then used, because it offers better preservation of tissue/cells, than can be achieved by using either fixative alone. Therefore, the first stage glutaraldehyde fixative used to preserve the cellular material was removed by centrifuging the samples for 5 minutes at 500 g and discarding the supernatant. Cells were then washed with 10 ml of sodium cacodylate buffer (100 ml of 0.1 M sodium cacodylate; 8 ml 0.1 M hydrochloric acid; 4 ml 1% calcium chloride; 88 ml distilled H₂O), which was removed by centrifugation as before. The pellet of cells was left in 1% osmium tetroxide (made up in 0.1 M sodium cacodylate buffer) for an hour. This second fixative was then carefully discarded and the cells washed in 10 ml of distilled water, which was removed by centrifugation as before. To make handling of the cells easier, to randomise their distribution and to minimise loss of material in subsequent processing steps, cells were resuspended in 2% molten agar (heated at 45 °C). Small ‘jellified’ pellets were created which were then left to semi-solidify at room temperature for 15 minutes. For the fixed cells to be cut into sections thin enough for microscopy, it was necessary to infiltrate the sample with an embedding material that would support the cells and reduce damage caused by the cutting. Most embedding media are not miscible with water and therefore any water present within the sample has to be replaced through a graded series of methanol solutions.

The pellets were firstly dehydrated in 70% methanol for 10 minutes, after which the alcohol was carefully removed by pipette. This step was repeated with 90% methanol and then 100% methanol. The agar pellets containing the samples were then transferred to small circular baskets which were then stacked into the holder tray of the Lynx machine.
(Australian Biomedical Corporation Ltd). This automated processing of samples was used for the final stages of dehydration and the intermediate stage between dehydration and embedding with propylene oxide, which is mutually miscible with methanol and araldite (embedding material). The samples were taken through a series of cylinder tubes, the contents of which are detailed below:

<table>
<thead>
<tr>
<th>Cylinders</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-9</td>
<td>Blank (these stages already completed manually)</td>
</tr>
<tr>
<td>10 &amp; 11</td>
<td>100% methanol (12ml)</td>
</tr>
<tr>
<td>12 &amp; 13</td>
<td>Propylene oxide</td>
</tr>
<tr>
<td>14</td>
<td>¼ (3ml) araldite + ½ (9ml) propylene oxide</td>
</tr>
<tr>
<td>15</td>
<td>½ (6ml) araldite + ½ (6ml) propylene oxide</td>
</tr>
<tr>
<td>16</td>
<td>¾ (9ml) araldite + ¼ propylene oxide</td>
</tr>
<tr>
<td>17</td>
<td>Blank</td>
</tr>
<tr>
<td>18-20</td>
<td>Araldite</td>
</tr>
</tbody>
</table>

After completion of the above stages, samples were transferred into plastic moulds containing the embedding medium, araldite (Agar Scientific Ltd, Essex) an epoxy resin which permits embedded material to be cut into sections as thin as 0.1μm, a necessary prerequisite for electron beam penetration. These were then baked in an oven at 60°C for 24 hours. The polymerised araldite was cooled at room temperature and removed from the moulds before being stored until the cutting process.

1 μm thick Sections for light microscopy analysis
Using an ultramicrotome (Ultracut E, Reichert-Jung), 1μm thick sections of the sample blocks were cut. On average, 4-5 sections from each block were dried onto slides and then stained with toluidine blue stain (1% solution made with 1% sodium tetraborate). They were then viewed using a light microscope (Zeiss, Germany) and the blocks containing good quality samples were further processed as described below for EM analysis.

0.1μm thin sections for electron microscopy analysis
Ultrathin sections of 0.1μm thickness were cut of the appropriate area using the same ultramicrotome used for cutting 1μm sections. These were then stained with 1.5% uranyl
acetate (made up with methanol) and Reynolds lead citrate (Reynolds, 1963). The sections were then viewed under a transmission electron microscope (Hitachi TEM 7000, Japan).

7: Statistical Analysis
All statistical analysis was carried out using SPSS for Windows software. Results are given as mean ± SEM. Differences were analysed by Paired Student’s t-test. A p value of ≤0.05 was accepted as statistically significant.

Example 1: Effect of Neutrophil Elastase on Neutrophil Survival
Suspensions of isolated peripheral blood neutrophils were cultured with and without neutrophil elastase, in accordance with general method 3 described above, over a period of 24 hours and compared at selected time points. Figure 3 shows that the percentage of neutrophils without neutrophil elastase displaying normal morphological appearances progressively declined with time whereas apoptotic neutrophils increased with time. Neutrophils cultured in the presence of neutrophil elastase were similar in morphology to untreated neutrophils at 9 hours of culture. However, by 18 hours, neutrophil elastase treated cells showed a more rapid decrease in survival with mean 86.82% of neutrophils showing apoptotic (early and late) features compared to mean 52.88% in cultures without elastase (p ≤0.001). By 24 hours, neutrophil elastase treated neutrophils were still showing accelerated cell death compared to cultures without neutrophil elastase (p ≤0.05). Using trypan blue exclusion as an indicator of cell viability, assessment of the proportion of viable and non-viable neutrophils was made to further confirm the effects of elastase on reducing neutrophil survival. Figure 4 shows that neutrophils treated with elastase had a more rapid increase in the number of non-viable cells and thus a decrease in viable cells. Viability of control neutrophils without elastase was mean 99.3± 0.48 SEM %, 95.5 ± 1.5%, 93 ± 0% and 86.5 ± 2.5% after 0, 9, 18 and 24 hours of aging compared with mean 99.25±0.48 SEM%, 94.7 ± 1.2%, 70.0 ± 16% and 50.0 ± 26%.

Example 2: Effect of Protease Inhibitors on Elastase-Induced Neutrophil Apoptosis
Suspensions of isolated peripheral blood neutrophils were cultured with and without the protease inhibitors alpha 1-antitrypsin or aprotinin, optionally in the presence of neutrophil elastase, in accordance with general method 3 described above, over a period of 24 hours.
and compared at selected time points. Figure 5 shows that in contrast to the expected findings, α1-AT, (natural elastase inhibitor) combined with neutrophil elastase actually enhanced neutrophil apoptosis. Significantly increased counts of apoptotic neutrophils and consequent decreases in cells with normal morphology were observed at 9 hours compared to neutrophils treated only with neutrophil elastase. By 18 and 24 hours of culture, the majority of neutrophils treated with α1-AT and neutrophil elastase were apoptotic, and levels were still significantly higher in comparison to neutrophils cultured with neutrophil elastase only. The effects of the synthetic protease inhibitor, aprotinin on neutrophil ageing were different to those of α1-AT. Figure 5 shows that at 18 hours, aprotinin inhibited some of the neutrophil elastase induced neutrophil apoptosis, with a trend for the percentage of normal neutrophils to be higher in neutrophil elastase plus aprotinin treated cultures, and the proportion of apoptotic neutrophils to be lower compared with neutrophil elastase only treated cells. By 24 hours, there was no longer a trend for percentage normal neutrophils and the relative proportion of apoptotic cells to be different to neutrophil elastase treated neutrophils. Therefore, aprotinin could not completely prevent neutrophil elastase induced apoptosis as proportions of apoptotic neutrophils were always higher in the aprotinin and neutrophil elastase treated cells compared with control preparations without neutrophil elastase. Figure 6 shows the results of neutrophil culture experiments conducted in the presence of inhibitors alone, to determine if α1-AT by itself i.e. in the absence of neutrophil elastase could promote neutrophil apoptosis, and if aprotinin alone was capable of inhibiting apoptosis. At 9 and 18 hours, α1-AT treated cells had a significantly lower proportion of neutrophils with apoptotic morphology and thus, higher percentages of normal neutrophils compared with α1-AT plus neutrophil elastase treated neutrophils. These differences were significantly different compared to the control, indicating that α1-AT can cause increased neutrophil apoptosis by itself but to a lesser degree than when combined with neutrophil elastase. By 24 hours, like neutrophil elastase and neutrophil elastase plus α1-AT treated cells, most α1-AT treated neutrophils were also apoptotic with significantly higher percentages compared with the control. As seen in figure 6, in particular at 18 and 24 hours, aprotinin significantly reduced neutrophil apoptosis not only compared to the neutrophil elastase plus aprotinin group, but also to controls without neutrophil elastase or inhibitor. This indicates that aprotinin has the ability to prolong neutrophil longevity.
Example 3: Effects of G-CSF and GM-CSF on Elastase-Induced Neutrophil Apoptosis

Suspensions of isolated peripheral blood neutrophils were cultured with and without human recombinant G-CSF or human recombinant GM-CSF, optionally in the presence of neutrophil elastase, in accordance with general method 3 described above, over a period of 24 hours and compared at selected time points. Figure 7 shows that both human recombinant G-CSF and human recombinant GM-CSF were capable of inhibiting neutrophil apoptosis, but the inhibitory effects of G-CSF were greater than those of GM-CSF. This result confirms that the two colony stimulating factors inhibit apoptosis of neutrophils under the specified culture conditions. By 24 hours, a mean 84% of neutrophils treated with G-CSF were normal compared with 61% of GM-CSF treated cells, and 18% of control neutrophils. Figure 8 shows the effects of G-CSF and GM-CSF on neutrophil elastase induced neutrophil apoptosis. At 9 hours, percentages of normal neutrophils in culture with neutrophil elastase and GM-CSF were significantly higher compared to neutrophil elastase alone unlike for G-CSF + elastase. However by 18 hours, proportions of normal neutrophils in GM-CSF + neutrophil elastase cultures were actually lower compared to neutrophil elastase only treated cells, and in G-CSF + neutrophil elastase cultures the percentage of normal neutrophils were significantly higher compared to neutrophil elastase alone. At the later time of 24 hours, it can be seen that there were very few normal neutrophils observed in cultures containing neutrophil elastase. The proportion of normal neutrophils were still significantly lower in the GM-CSF + neutrophil elastase cultures compared to neutrophil elastase only, and the effects of G-CSF and neutrophil elastase on neutrophils are similar to the effects of neutrophil elastase alone. Thus the colony stimulating factors G-CSF and GM-CSF were unable to over-ride the apoptosis inducing effects of neutrophil elastase.

Suspensions of isolated peripheral blood neutrophils were cultured with and without human recombinant G-CSF or human recombinant GM-CSF, optionally in the presence of alpha 1-antitrypsin or aprotinin, and in the presence of neutrophil elastase, in accordance with general method 3 described above, over a period of 24 hours and compared at selected time points. Figure 9 demonstrates that at 9 hours, G-CSF was able to significantly reduce α1-AT + neutrophil elastase induced neutrophil apoptosis unlike GM-CSF. By 18 hours the vast majority of neutrophils were apoptotic in all treated cultures, thus distinction
between different treatments was difficult to make. Overall, these results indicate that the colony stimulating factors did not substantially reduce α1-AT + neutrophil elastase induced neutrophil death. Figure 10 shows that at 9, 18 and 24 hours, GM-CSF significantly reduced neutrophil elastase + aprotinin induced neutrophil apoptosis unlike G-CSF. This effect of GM-CSF is likely to be due to enhancement of aprotinin function because GM-CSF alone could not counteract the effects of elastase as seen in figure 8.

Example 4: Morphological Assessment of Neutrophil Apoptosis by Light Microscopy
In order to identify the different stages of neutrophil apoptosis, for the purpose of this study, it was important to familiarise oneself with the sequence of morphological appearances of neutrophils throughout their life span. Thus, using peripheral blood neutrophils, assessment of spontaneous apoptosis at sequential time points from 0-3 days was undertaken as a learning exercise to identify morphological differences, but also to be able to select time points for optimal appearance of apoptotic neutrophils using the stated culture conditions. The actual time points studied were 0, 3, 6, 9, 12, 18, 24, 48 and 72 hours. Figures 1a to 1e show examples of the appearance of peripheral blood neutrophils by light microscopy. Figure 1a) is at 0 hours of culture, showing that there is little or no evidence of apoptosis and that all cells have morphological features of mature neutrophils with intact cell membranes and multi-lobed nuclei (similar appearances were seen up to 9 hours of culture, see Figure 1b). Figure 1c) is at 24 hours of culture at 37°C when many of the neutrophils show characteristic apoptotic features, namely shrinkage of cell size and condensed nuclear chromatin (detected in increasing proportion from 12 hours of culture onwards). Figure 1d) is at 48 hours of culture, showing that some neutrophils have progressed to secondary necrosis as indicated by loss of cell membrane integrity and discharge cell contents. These neutrophils resemble aliquots of the same original cells induced to undergo primary necrosis by heating at 56°C for 30 minutes (see Figure 1e). Thus by 48 hours of culture in vitro (in the absence of clearance mechanisms), the majority of neutrophils have gone through a sequence of spontaneous apoptotic events, ultimately leading to disintegration by a process of secondary necrosis.

Example 5: Morphological Assessment of Neutrophil Apoptosis by Electron Microscopy
To evaluate apoptotic characteristics of neutrophils in much greater detail as a ‘gold standard’ to confirm the interpretation of the light microscopy appearances, cultures of
isolated peripheral blood neutrophils examined at the above mentioned time points were also evaluated at ultrastructural level. Electron micrographs (Figures 2a to 2d) of the cells definitely confirmed the light appearances showing the sequence of morphological changes undergone by neutrophils when aged in culture: Figure 2a) is at 0 hours, showing normal mature healthy neutrophils with hyper-segmented nuclei without evidence of apoptotic changes. Figure 2b) is at 24 hours of culture, showing an example of a neutrophil in early stages of apoptosis with cell shrinkage and condensed nuclear chromatin, but with nuclear and cell membranes intact, and no evidence of deterioration of cytoplasmic lysosomes and mitochondria. Figure 2c) is at 48 hours of culture, showing an example of a neutrophil at a later stage of apoptosis with nuclear fragmentation and dissolution of the nuclear membrane, but with cytoplasmic organelles and cell membrane still intact. Figure 2d) is at 72 hours of culture, showing an example of a neutrophil which has progressed to secondary necrosis in vitro (due to lack of clearance by phagocytes).
Claims

1. A protease inhibitor for use in treatment of inflammation and/or its sequelae, wherein the inflammation involves inflammatory or other cells that are capable of releasing proteases, wherein the protease inhibitor is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of the inflammatory or other cells.

2. A protease inhibitor for use in treatment of a condition in a subject characterised by or involving an increased number or proportion of inflammatory or other cells that are capable of releasing proteases relative to the corresponding number or proportion of said cells in a healthy subject, wherein the protease inhibitor is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of the inflammatory or other cells.

3. A protease inhibitor as claimed in claim 1 or claim 2, for use in treatment of acute, sub-acute or chronic inflammation in the absence of infection, an allergic disease, an occupational or environmental disease or disorder, an infection after elimination of the infectious agent where inflammation persists, or another non-infectious disease, disorder or condition involving inflammation or an increase in numbers or proportions of the inflammatory or other cells.

4. A protease inhibitor as claimed in claim 3, for use in treatment of a rheumatological disease, liver diseases, especially chronic active hepatitis and primary biliary cirrhosis, congestive heart failure especially following acute myocardial infarction, a neutrophilic dermatosis, ocular diseases such as keratoconjunctivitis or uveitis, or sepsis in the absence of evidence of infection or after elimination of infection by treatment with an antibiotic.

5. A protease inhibitor as claimed in claim 3, for use in treatment of a disease, disorder or condition of the respiratory system involving inflammation or an increase in numbers or proportions of the inflammatory or other cells.
6. A protease inhibitor as claimed in claim 5, for use in treatment of an acute or chronic non-infectious inflammatory lung disease, such as chronic fibrosing lung diseases, including idiopathic pulmonary fibrosis and other idiopathic interstitial pneumonias, collagen vascular diseases involving the lungs and occupational fibrosing lung diseases such as asbestosis; Wegener’s granulomatosis; an acute lung injury, such as treatment of severe pneumonia after control of infection, or acute respiratory distress syndrome, or acute lung injury following cardiopulmonary by-pass surgery; treatment of bronchiectasis after control of infection; obliterative bronchiolitis in a lung transplant patient after treatment of any infection; chronic obstructive airways disease after treatment of any infection; non-genetic emphysema, emphysema caused by smoking or environmental factors, or emphysema caused by a variant alpha 1-antitrypsin; chronic lung disease especially in premature infants and children; asthma, in particular severe life-threatening asthma or chronic ‘non-steroid responsive’ asthma; and other allergic diseases such as rhinitis.

7. A protease inhibitor as claimed in claim 6, for use in treatment of severe pneumonia after the elimination of infection, sepsis after the elimination of infection, acute lung injury triggered by non-infectious risk factors such as acute respiratory distress syndrome, acute lung injury following cardiopulmonary by-pass surgery, chronic fibrosing lung diseases, chronic lung disease in premature infants, chronic obstructive airways disease, in particular chronic obstructive airways disease which is not caused by a patient’s deficiency of alpha 1-antitrypsin, severe life-threatening asthma and chronic ‘non-steroid response’ asthma.

8. A protease inhibitor as claimed in claim 1 or claim 2, wherein the cells are eosinophils, for use in treatment of eosinophilia in association with allergic diseases such as asthma or rhinitis, cryptogenic pulmonary eosinophilia, pulmonary eosinophilia associated with drugs, helminths or bronchopulmonary aspergillosis, Churg Strauss syndrome, vasculitis, idiopathic pulmonary fibrosis, other idiopathic interstitial pneumonias, other chronic fibrosing lung diseases, or systemic connective tissue diseases in patients who have increases in eosinophils as well as neutrophils in the lungs or other affected organs.

9. A protease inhibitor as claimed in claim 1 or claim 2, wherein the cells are basophils or mast cells, for use in treatment of disorders associated with Type I ‘immediate’ hypersensitivity reactions such as asthma, eczema, hay fever, rhinitis, urticaria or
anaphylaxis; myxoedema; hypothyroid conditions; ulcerative colitis; certain types of anaemia; rare diffuse cutaneous or systemic mastocytomas; fibrotic lesions in the lungs, skin or other organs.

10. A protease inhibitor as claimed in claim 1 or claim 2, wherein the cells are lymphocytes, for use in treatment of granulomatous diseases involving the lungs and/or other systems, for example sarcoidosis, extrinsic allergic alveolitis, berylliosis, silicosis, tuberculosis and other mycobacterial diseases.

11. A protease inhibitor as claimed in any one of claims 1 to 10, which inhibitor is alpha 1-antitrypsin.

12. A protease inhibitor for use in treatment of inflammation and/or its sequelae, wherein the inflammation involves inflammatory or other cells that are capable of releasing proteases, wherein the protease inhibitor is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of the inflammatory or other cells or has no effect on apoptosis of the inflammatory or other cells.

13. A protease inhibitor for use in treatment of a condition in a subject characterised by or involving an increased number or proportion of inflammatory or other cells that are capable of releasing proteases relative to the corresponding number or proportion of said inflammatory or other cells in a healthy subject, wherein the protease inhibitor is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of the inflammatory or other cells or has no effect on apoptosis of the inflammatory or other cells.

14. A protease inhibitor as claimed in claim 12 or claim 13, for use in treatment of cystic fibrosis.

15. A protease inhibitor as claimed in claim 12 or claim 13, for use in treatment of an infection characterised by or involving inflammation or an increased number or proportion of inflammatory or other cells that are capable of releasing proteases relative to the corresponding number or proportion of inflammatory or other cells in a healthy subject, said treatment comprising treatment with the protease inhibitor before treatment with an
antibiotic is started or during treatment with an antibiotic before elimination of the infection.

16. A protease inhibitor as claimed in claim 15, wherein the disorder is pneumonia, especially severe life-threatening pneumonia, or sepsis.

17. A protease inhibitor as claimed in any one of claims 12 to 16, wherein the protease inhibitor is aprotinin.

18. A protease inhibitor as claimed in any one of claims 1 to 17, wherein the cells are lymphocytes, basophils, mast cells, or neutrophils.

19. A protease inhibitor as claimed in claim 18, wherein the cells are neutrophils.

20. A protease inhibitor as claimed in any one of claims 1 to 19, for use in combination or supplementary therapy with one or more further therapeutic agents.

21. Use of a protease inhibitor for the manufacture of a medicament for use in a treatment as defined in any one of claims 1 to 20.

22. A method of treatment of inflammation and/or its sequelae, wherein the inflammation involves inflammatory or other cells that are capable of releasing proteases, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a protease inhibitor, wherein the protease inhibitor is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of the inflammatory or other cells.

23. A method of treatment of a condition characterised by or involving an increased number or proportion of inflammatory or other cells that are capable of releasing proteases relative to the corresponding number or proportion of inflammatory or other cells in a healthy subject, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a protease inhibitor, wherein the protease
inhibitor is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of the inflammatory or other cells.

24. A method of treatment of inflammation and/or its sequelae, wherein the inflammation involves inflammatory or other cells that are capable of releasing proteases, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a protease inhibitor, wherein the protease inhibitor is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of the inflammatory or other cells or has no effect on apoptosis of the inflammatory or other cells.

25. A method of treatment of a condition characterised by or involving an increased number or proportion of inflammatory or other cells that are capable of releasing proteases relative to the corresponding number or proportion of inflammatory or other cells in a healthy subject, which method comprises administering to a subject in need of such treatment a therapeutically effective amount of a protease inhibitor, wherein the protease inhibitor is capable of directly or indirectly reducing, preventing, delaying or suppressing apoptosis of the inflammatory or other cells or has no effect on apoptosis of the inflammatory or other cells.

26. A method of treatment as claimed in any one of claims 22 to 25, as defined in any one of claims 1 to 17.

27. An isolated complex comprising one or more protease inhibitors and one or more proteases.

28. A complex as claimed in claim 27, wherein the protease inhibitor is alpha 1-antitrypsin or aprotinin.

29. A complex as claimed in claim 27 or claim 28 for use as a medicament.

30. A protease inhibitor as claimed in any one of claims 1 to 20, use of a protease inhibitor as claimed in claim 21, or a method of treatment as claimed in any one of claims
22 to 26 comprising administering a protease inhibitor, wherein the protease inhibitor is in the form of a complex as claimed in any one of claims 27 to 29.

31. A protease inhibitor for use in treatment of a condition in a subject characterised by or involving an increased number or proportion of malignant cells relative to the corresponding number or proportion of said malignant cells in a healthy subject, wherein the protease inhibitor is capable of directly or indirectly increasing, inducing, promoting, accelerating or augmenting apoptosis of the malignant cells.

Figure 4

% Non-Viable Neutrophils

- Without Elastase
- With Elastase

Time (hours)

% Viable Neutrophils

- Without Elastase
- With Elastase

Time (hours)
Figure 5

% Normal Neutrophils

% Apoptotic Neutrophils (early & late stages)
Figure 8

% Normal Neutrophils

% Apoptotic Neutrophils (early & late stages)
Figure 9

% Normal Neutrophils

% Apoptotic Neutrophils (early & late stages)

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Legend:
- Control
- E-Granulocyte + AT
- E-Granulocyte + GM-CSF + AT
Figure 10

% Normal Neutrophils

% Apoptotic Neutrophils (early & late stages)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K38/57 A61P43/00 A61P35/00 A61P11/00 A61P11/06 A61P37/08

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P

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

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

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 00/51624 A (THE TRUSTEES OF UNIVERSITY TECHNOLOGY CORPORATION) 8 September 2000 (2000-09-08) page 5, line 29 - page 6, line 11 page 11, line 25 - line 31 claims</td>
<td>1-11, 18-23,26</td>
</tr>
<tr>
<td>X</td>
<td>US 6 489 308 B1 (SHAPIRO LELAND) 3 December 2002 (2002-12-03) column 5, line 36 - column 6, line 60 claims column 13, line 32 - column 14, line 45</td>
<td>1-11, 18-23,26</td>
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Patent family members are listed in annex.

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Date of the actual completion of the international search 14 September 2005

Date of mailing of the international search report 27/09/2005

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Authorized officer Böhmerova, E

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<td>X</td>
<td>WO 92/06706 A (LEZDEY, JOHN; WACHTER, ALLAN) 30 April 1992 (1992-04-30) claims page 4, paragraph 6; examples VIII,X</td>
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<td>DE 197 25 014 A1 (BAYER AG, 51373 LEVERKUSEN, DE) 17 December 1998 (1998-12-17) claims page 4, line 24 – line 42</td>
<td>12-21, 24-26</td>
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<td>TREVANI ANALIA S ET AL: &quot;Neutrophil apoptosis induced by proteolytic enzymes&quot; LABORATORY INVESTIGATION, vol. 74, no. 3, 1996, pages 711-721, XP009053825 ISSN: 0023-6837 abstract page 713, left-hand column, paragraph 2</td>
<td>12-21, 24-26</td>
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<tr>
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<td>EP 0 080 279 A (RYAN, JAMES WALTER) 1 June 1983 (1983-06-01) claims page 6, line 18 – line 28; table 1</td>
<td>27, 28</td>
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<tr>
<td>X</td>
<td>EP 1 066 834 A (STIEF, THOMAS, DR) 10 January 2001 (2001-01-10) paragraphs ‘0001!’, ‘0020!; claim 10</td>
<td>27, 29</td>
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<td>AU 3731400 A</td>
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<td>OA 9768 A</td>
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<tr>
<td>US 2003077266 A1</td>
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<tr>
<td>EP 1374891 A</td>
<td>02-01-2004</td>
<td>NONE</td>
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<td>DE 19725014 A1</td>
<td>17-12-1998</td>
<td>AU 8108998 A</td>
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<td>WO 9856916 A1</td>
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