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(54) Title: PROTEASOMAL ACTIVITY

(57) Abstract

The present invention relates to modulation and/or regulation of proteasomal activity. The invention provides assay methods for identifying compounds which inhibit viral replication and pathogenesis, for identifying inhibitors of the nuclease–inhibitory function of the human immunodeficiency virus *TAT* protein, for identifying inhibitors of the protease–inhibitory function of the human immunodeficiency virus *TAT* protein, for identifying compounds which change the concentrations of regulatory proteins by modulating the rate of proteasomal destruction of specific mRNAs, and methods for generating resistance to bacterial or viral infection damage. The invention provides assays, kits for carrying out said assays, compounds identified by said assays, together with amino acid sequences encoding said compounds and medicinal compositions derived therefrom. Where legally permissible the invention also provides methods of treatment based on the modulation/regulation of proteasomal activity.

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PROTEASOMAL ACTIVITY

Background on the Proteasome

It is a truism that we are not the people we used to be just a few months ago. This is because most of the cells in our body have a relatively short life span; our bodies make new cells as existing cells age and die. The processes of cell 'birth' are increasingly well-known. Those of cell decay and death, although equally fundamental, have been much less well understood. As cells turn over more rapidly than the whole organism, so cellular components turn over more rapidly than the cells themselves. In the mature cell, the rate at which proteins are synthesised is matched by the rate at which they are broken down (proteolysis). This simultaneous combination of synthesis and degradation allows cells to respond to changes in their environment.

The proteolytic system decreases the concentrations of specific proteins and their attendant activities. The specificity of this process is achieved by a highly selective mechanism, mediated by large, multi-subunit cytosolic complexes, the proteasomes [1]. Proteasomes can also degrade ribonucleic acid, and are particularly effective against viral and cytokine mRNAs [2].

To date most attempts to control cell turnover have centred on the synthetic side of the cell cycle, on the assumption that degradation was relatively uncontrolled and lacked specificity. Recent studies have clearly shown that this is not the case, and that the proteasome with its complex regulation is an important potential target for chemotherapy in a number of well-defined clinical conditions.

Structure and Function of the Proteasome

The major proteolytic activity in the cell is the 26S proteasome, a key regulatory protease which controls physiological processes as diverse as the cell cycle [3], apoptosis [4], oncoprotein degradation [5], gene expression [6], the inflammatory response [6] and bulk proteolysis [7]. The proteasome consists of a catalytic core (20S) to which is attached regulatory complexes [8]. In humans, the cylindrical catalytic core comprises 28 subunits—two copies each of seven different α-type and seven different β-type subunits—assembled to form a stack of four seven-membered rings with the general structure $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. This stack contains internal chambers harbouring the proteolytic threonine residues. Regulatory complexes (19S), each containing 6 ATPases and at least 9 other subunits, are attached to the proteasome complex. An 11S regulatory complex substitutes for the 19S regulator [9] when the proteasome fragments antigens for MHC Class I presentation. Importantly, selective interchange of both core and regulatory subunits also occurs in response to various physiological stimuli (for example, exposure to interferon-γ) [4, 10,11].

The Proteasome Cycle

The diverse functions of the proteasome depend on multiple catalytic sites which include chymotrypsin-like, trypsin-like and peptidylglutamylpeptide hydrolysing activities. Unlike other proteolytic systems, however, these proteasomal activities are energy-dependent.

Ubiquitin is a protein of 76 amino acid residues. Its function is as a 'tag' for proteins which are to be degraded by the 26S proteasome. This 'tagging' involves the covalent attachment of multiple chains of ubiquitin to the protein. Ubiquitin is first activated by a ubiquitin-activating enzyme (E1). Ubiquitin-conjugating enzymes (E2s), with the help of ubiquitin-protein ligases (E3s), then join several (4–12) ubiquitins sequentially to a lysine residue in the target protein, leading to the formation of multi-ubiquitin chains [12].

Multi-ubiquitinylated proteins are first recognised by subunits of the 19S regulator [13] which presents the protein chain for processing within the 20S cylindrical core. Other 19S regulator subunits can recognise non-ubiquitinylated proteins. Ubiquitinylated and non-ubiquitinylated proteins cannot enter the proteolytic chambers of the 20S particle without being unfolded in a complex process requiring the chaperone functions of the 19S regulator [14]. Concomitant with unfolding and degradation, multi-ubiquitin chains are removed from target proteins by proteasome-associated ubiquitin carboxy-terminal hydrolases (UCHs); the ubiquitin is recycled [15].

UCHs are members of a large family of ubiquitin-specific cysteine proteases, many of which are free in the cytosol [16]. proteins are continuously ubiquitinylated and de-ubiquitinylated in the cell in an editing process controlled by E3s and cytosolic uchs. multi-ubiquitinylated proteins failing the inspection process and remaining multi-ubiquitinylated, such a oxidation-damaged proteins or proteins of viral origin, are degraded by the 26s proteasome. this degradation process can also be activated or inhibited by large (130-250 kda) cytosolic regulatory proteins [17].

To summarise:

Proteasomes are large multi-unit protease complexes that play a key role in the control of cellular processes by selectively degrading intracellular proteins.

Most of the proteins removed by these proteases are tagged for destruction by ubiquitinylation.

Tagged proteins are recognised by elements within the 19S proteasomal regulator, unfolded, de-ubiquitinylated and translocated to the internal chamber of the 20S proteasomal core complex for partial or complete hydrolysis by an array of

proteolytic functionalities.

The process may be subject to further control by cytosolic regulators.

The Proteasome as a Therapeutic Target

Can inhibitors of proteasomal activity be selective in their actions when the proteasome is a universal component of the protein turnover mechanism?

Such selectivity has been shown in an animal model of rheumatoid arthritis in which the boronate MG341, a compound with good oral potency and long half-life but which is a relatively non-specific serine protease inhibitor, produced substantially complete remission from clinical symptoms over a 30-day period [18]. The same compound was also efficacious in a model of allergic skin reaction [18]. It is logical therefore to expect that compounds directed against proteasomal targets with more specific functions will be equally or more selective, not least because of the recent recognition that forms of the complex differ qualitatively and quantitatively from one tissue to another.

The active form of the protein NF-κB is required for the expression of a large number of genes involved in immune and inflammatory responses; these genes encode inflammatory and chemotactic cytokines, haematopoietic growth factors, cell adhesion molecules, antibodies, class I MHC molecules and cytokine receptors, as well as the key enzymes nitric oxide synthetase and cyclooxygenase-2. NF-κB is normally inactive in the cytosol; it is activated by the proteasome pathway in response to a variety of pathogenic stimuli, including viruses, bacteria, radiation, oxidants and inflammatory cytokines. Inhibitors directed against this specific proteasomal function should therefore provide therapeutic benefit [18] in a wide range of inflammatory and allergic diseases such as rheumatoid arthritis, inflammatory bowel diseases, and asthma.

The cellular immune system acts naturally to defend the body from foreign proteins. This aggressive response is the major reason why transplanted tissues suffer rejection. It is also inappropriately elicited by 'self' proteins in autoimmune disease (multiple sclerosis, lupus erythrematosus, etc.) This response is initiated by the degradation of non-recognised proteins into short antigenic peptide fragments by 20S/11S proteasome complexes, and by the presentation of these on the cell surface with the almost ubiquitous MHC-I antigen. Inhibitors of the formation of these antigens would, therefore, be potentially useful in tissue transplantation and in the treatment of autoimmune conditions.

The cycle of reactions involved in cell division is controlled by cyclin-dependent protein kinases. The activity of particular kinase complexes during different phases of the cell cycle is regulated by the amount of specific proteins in the cell, this amount

being controlled by the balance between synthesis and proteasome-dependent degradation [19]. In hyperproliferating cells inhibition of proteasomal catalytic function leads to cessation of cell growth and may induce apoptosis. It is reasonable therefore to expect that appropriate inhibitors may have utility in the treatment of a variety of cancers and non-malignant hyperproliferative conditions such as psoriasis and restenosis.

Proteasome Inhibitors

Compounds known for some time and used to inhibit proteolytic activity, mostly in experimental systems include peptide aldehydes, boronic esters, and lactacystin and related compounds (reviewed in [20]). The former compounds with their poor pharmacokinetic properties or low specificities have weak prospects as potential therapeutic agents. The naturally-occurring lactacystins are of more interest but are general and irreversible inhibitors. These compounds were identified by their capacity to inhibit the proteolytic function of isolated 20S proteasomal subunits, the least discriminatory measure of compound activity.

More recently developed inhibitors are indanone-containing peptides [21], glyoxals [22] and further boronic acids [23].

The relationships between the 20S proteasome, the 20S + 19S (26S) complex, the 11S (PA28) complex, the MHC class I, cell division and general cellular proteolysis are well established. The relationships between proteasomal complexes and RNA are often considered to be non-specific. However, 20S proteasomes contain small RNA [24,25] and interfere with protein synthesis [26,27]. Recent work has shown that proteasomes contain an endonuclease activity which selectively degrades RNAs [28]. This selectivity is expressed against RNAs with AU-rich elements [29]. Such sequences are found in retroviral RNAs. The nuclease activity is associated with subunits zeta and iota in the 20S proteasome [2].

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The proteasome has an essential antiviral role *in vivo*. Viral proteins synthesised in infected cells are partially degraded by the proteasome [1-3]. Peptides so generated are bound to MHC class-I molecules and presented on the cell surface where they are recognised by cytotoxic T-lymphocytes. Viruses have developed mechanisms which enable them to subvert this process [4].

HIV RNA codes for a number of proteins which interfere with proteasome function. *Vpu* and *Env* act together to increase the proteasome-mediated degradation of CD4, important for adequate functioning of helper T cells [5]. *Nef* binds to the B-subunit HsN3 [6] although the function of this binding remains unknown. This subunit is also the binding site for *Tax-1*, a protein encoded by HTLV [7].

Viral proteins in naïve cells are degraded by the proteasome—probably by the 20S complex alone, without ubiquitinylation. If this degradation is blocked by inhibitors, p24 Gag proteins accumulate in the cytoplasm and more proviral DNA is synthesised [8]. *Tat* binds to the 20S proteasome, and strongly inhibits the proteolytic activity, as well as blocking the formation of the 20S-11S complex [9].

Thus, *in vivo*, *Tat* occurs in the naïve cell either as a result of synthesis *de novo* or passage from neighbouring infected cells, a process facilitated by the presence in its sequence of an RGD domain which allows binding to cell surface proteins and consequent cell entry. *Tat* binds to the TAR of the viral RNA, protects the RNA from degradation by the proteasomal endonuclease and allows effective transcription to proceed. In addition, *Tat* binds to the proteasome and blocks the proteolytic destruction of viral proteins, thereby preventing presentation of viral antigens on the cell surface. Inhibition of *Tat* binding to the proteasome or of subsequent *Tat*-associated events will therefore allow the proteasome to degrade essential viral proteins and to present peptides derived from these on MHC class-I molecules for cellular destruction by cytotoxic lymphocytes.

HTLV-I *Tax* protein associates with the HsN3 subunit. *Tax* may then act as an anchor for processing of IκB (to which Tax also binds) and thus be driver of the mechanism whereby NF-κB is activated by this virus [7]. The Hepatitis B virus *X* protein *HBX* binds to the subunit XAPC7 and this interaction is important for *HBX*-mediated transactivation in vivo [10]. Furthermore, the Human Papilloma Virus E7 oncoprotein binds to both the retinoblastoma tumour suppressor protein, Rb, and to the S4 ATPase, a subunit of the 19S regulator of the proteasome; this binding is linked to the proteolysis of Rb by the proteasome [11]. Another 19S ATPase, SUG1 (S8), is stimulated by specific mRNA sequences [12]. Typical examples of the sequences of these viral proteins which affect proteasomal function are shown by way of example in Table 1. [SEQ ID Nos. 1,2,3].

Table 1

HTLV-1 TAX PROTEIN

260 AA
MW 28666

MVVVAAAPNP ADGTPKVLLL SGQPASAAGA PAARLPLMVP AQRGASPEAA SGGLPQARKR QRLTHLSPEE KALRRKLKNR VAAQTARDRK KARMSELEQQ VVDLEEENQK LLLENQLLRE KTHGLVVENQ ELRQRLGMDA LVAEEEAEAK GNEVRPVAGS AESAALRLRA PLQQVQAQLS PLQNISPWIL AVLTLQIQSL ISCWAFWTTW TQSCSSNALP QSLPAWRSSQ RSTQKDPVPY QPPFLCQWGR HQPSWKPLMN [SEQ ID No. 1]

HEPATITIS B VIRUS PROTEIN X

154 AA
MW 16583

MAARLYCQLD PSRDVLCLRP VGAESRGRPL SGPLGTLSSP SPSAVPADHG AHLSLRGLPV CAFSSAGPCA LRFTSARCME TTVNAHQILP KVLHKRTLGL PAMSTTDLEA YFKDCVFKDW EELGEEIRLK VFVLGGCRHK LVCAPAPCNF FTSA [SEQ ID No. 2]

HUMAN PAPILLOMA VIRUS E7 ONCOPROTEIN

98 AA
MW 11022

MHGDTPTLHE YMLDLQPETT DLYCYEQLND SSEEEDEIDG PAGQAEPDRA HYNIVTFCCK CDSTLRLCVQ STHVDIRTLE DLLMGTLGIV CPICSQKP [SEQ ID No. 3]

Inhibition of Viral Replication and Pathogenesis

In a first aspect, the invention is designed to identify compounds which will inhibit viral replication and pathogenesis following infection of living cells by the class of viruses whose genomes code for proteins or nucleic acids that bind to the proteasome or cause the binding of host proteins or nucleic acids to the proteasome such that the functioning of the proteasome in cellular surveillance is impeded. The invention consists of an assay which contains proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), viral gene product, and protein or peptide substrates for detection of proteolytic activity by fluorescence, light absorption, luminescence, radioactivity or other physical parameter. Alternatively the assay may contain viral ribo- or deoxyribo-nucleic acid or an oligonucleotide containing sequences recognised by proteasomal subunits or other host proteins binding to proteasomal subunits; nuclease activity is measured spectrophotometrically, fluorometrically, radiometrically or by chemiluminescence. Both types of assay may in addition contain host proteins which bind virally-encoded protein or nucleic acid such that proteasomal proteolytic or nuclease activities are modulated.

Compounds whose effect is to increase proteasomal degradation of viral components or molecules induced by viral infection are potentially of value in the treatment of viral disease.

Inhibitor compounds, inhibitory amino acid sequences, and medicinal compositions derived therefrom (whether peptidic or petidomimetic) useful in the treatment of viral disease in infected patients and obtained or obtainable by the use of the assay or kit are also considered to form part of the present invention. Where legally permissible the invention also provides a method of treatment of viral disease in an infected patient by administration of an inhibitor obtained according to the assay of the invention.

Examples

Assay for the identification, and quantification of the effect, of compounds which interfere with the inhibition of proteasomal nuclease activity by the Hepatitis B Virus X-protein

Preparation of the Substrate Coated Plates

Multiwell (96 or 384 wells per plate) streptavidin coated black plates (for example, Reacti-bind™ Neutravidin™ Coated polystyrene plates, pkg of 5 ref. 15117 from Pierce) are stored dessicated at 4°C.

Before use, each well is rinsed three times with 200µl of wash buffer (25mM-TrisHCl, 150mM-NaCl, 0.05%(v/v) Tween® 20, pH 7.6).

To each well are added 100µl of substrate solution (containing 25pmol of a 31-mer oligoribonucleotide containing one or more AUUUA sequences and tagged with fluorescein at the 5'-end and biotin at the 3'-end). Plates are then incubated for 16h at room temperature before each well is washed with 200µl of wash buffer.

Each well is washed once with 200µl of TBK160 buffer (20mM-TrisHCl, 160mM-KCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4).

Then 50µl of TBK160 (containing compounds to be tested or equivalent vehicle) are added to each well. At this stage the plates are ready for incubation with assay solutions.

Proteasome solution

20S proteasomes are purified by standard methods and stored at 4° in TBK600 (20mM-TrisHCl, 600mM-KCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4, to which is added 5mM-NaN_3 as preservative). This solution is diluted with TBK0 (20mM-TrisHCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4) so that the final concentration is that of TBK160.

HBV X-protein solution

HBV X-protein is obtained by purification from extracts of *Escherichia coli* containing the HBV X-protein gene and appropriate expression vector. It is stored in PsP buffer (50mM-Na phosphate, 100mM-NaCl, 1mM-EDTA, 1mM-dithiothreitol, pH 7.0).

Preincubation

Proteasome and solutions of the HBV X-protein are mixed and incubated at 37° for 20min, such that 50µl of the resulting mixture contain 1pmol of proteasomal protein and 2pmol of HBV X-protein.

Nuclease assay

To each well are added 50µl of proteasome/HBV X-protein mixed solution. Plates are then incubated at 37° for a further 20min, before being washed three times with 200µl of TBK160. To each well are then added 50µl of TBK160 and fluorescence is measured in a Labsystems Fluoroscan Ascent FL fluorimeter with appropriate data handling system.

Control assays

Control wells for proteasomal RNase activity contain the same as above but without HBV X-protein. Controls for HBV X-protein RNase activity contain the same as above but without proteasomal protein.

Principle of the assay

Labelled plates incubated with buffer alone will retain all of the fluorescein-derived fluorescence and readings will be high. With proteasomes alone, cleavage of the nucleotide takes place and readings are low because the fluorescein is solubilised and removed by the wash buffer. When HBV X-protein is present, cleavage is inhibited and readings will therefore be higher than with proteasomal protein alone. The extent to which the effect of HBV X-protein is overcome is a measure of the efficacy of test compounds.

Assay for the identification, and quantification of the effect, of compounds which interfere with the inhibition of proteosomal protease activity by the Hepatitis B Virus X-protein

Preparation of Plates

Standard multiwell (96 or 384 wells per plate) plates are used. To each well are added 50µl of buffer (30mM-TrisHCl, 10mM-KCl, 5mM-$MgCl_2$, 0.5mM-dithiothreitol, pH 7.8), containing compounds to be tested or equivalent vehicle. At this stage the plates are ready for incubation with assay solutions.

Proteasome solution

20S proteasomes are purified by standard methods and stored at 4° in TBK600 (20mM-TrisHCl, 600mM-KCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4, to which is added 5mM-NaN_3 as preservative). This solution is diluted with TBK0 (20mM-TrisHCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4) so that the final concentration is that of TBK160.

HBV X-protein solution

HBV X-protein is obtained by purification from extracts of *Escherichia coli* containing the HBV X-protein gene and appropriate expression vector. It is stored in

PsP buffer (50mM-Na phosphate, 100mM-NaCl, 1mM-EDTA, 1mM-dithiothreitol, pH 7.0).

Preincubation
Proteasome and HBV X-protein solutions are mixed and incubated at 37° for 20min, such that 50µl of the resulting mixture contain 1pmol of proteasomal protein and 2pmol of HBV X-protein.

Protease assay
To each well are added 50µl of proteasome/HBV X-protein mixed solution followed by 10µl of 0.11mM fluorescent substrate (Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin, SucLLVY-AMC). Plates are then incubated at 37° for a further 30min, before reaction is stopped by addition of 200µl of stop buffer (0.1M-chloracetic acid, 0.13M-Na acetate, 0.1M-acetic acid, pH 4.3). Fluorescence is measured in a Labsystems Fluoroscan Ascent FL fluorimeter with appropriate data handling system. Results are quantified with reference to standard solutions of aminomethylcoumarin.

Control assays
Control wells for proteasomal protease activity contain the same as above but without HBV X-protein. Controls for HBV X-protein protease activity contain the same as above but without proteasomal protein.

Principle of the assay
Fluorescent product is only released after cleavage of the substrate peptide. Plates incubated with buffer alone will show little or no hydrolysis and readings will be low. With proteasomes alone, cleavage of the substrate takes place and readings are high. When HBV X-protein is present, cleavage is inhibited and readings will therefore be lower than with proteasomal protein alone. The extent to which the effect of HBV X-protein is overcome is a measure of the efficacy of test compounds.

See also the Examples described with respect to the third and fourth aspects of the invention, below. These Examples show Inhibition of the nuclease-inhibitory function of the human immunodeficiency virus *TAT* protein and Inhibition of the protease-inhibitory function of the human immunodeficiency virus *TAT* protein.

References Relating to Inhibition of Viral Replication and Pathogenesis

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In a second aspect, the invention relates to a method for identifying compounds which change the concentrations of cytokines, lymphokines and other regulatory proteins by modulating the rate of proteasomal destruction of the specific messenger ribonucleic acids.

Increases in endothelial cellular adhesion factors—endothelial-leukocyte adhesion molecule-1 (E-selectin), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)—are important in a range of common inflammatory diseases including ischaemia, reperfusion injury, asthma, transplantation, inflammatory bowel disease, rheumatoid arthritis and atherosclerosis. These increases are the result of a stimulation of transcription of the specific genes by the protein NFκB. NFκB occurs in the cytosol as an inactive complex with the regulatory protein IκB, and passes into the nucleus after proteasomal degradation of IκB. IκB is tagged for degradation by ubiquitinylation after phosphorylation of two specific serine residues stimulated by extracellular cytokines. The concentration of circulating cytokines is determined by the amount of specific messenger RNA in the originator cells. An increase in the proteasomal degradation of this mRNA will lead to a decrease in the cytokine concentration in the blood and consequently decreases in free NFκB and expression of cellular adhesion proteins. Compounds which modulate proteasomal nuclease activity may therefore have utility in the treatment of inflammatory disease.

The proteasome exhibits an endonuclease activity which has specificity for the RNA substrate [1]. This has now been characterised [2]—proteasomal RNase is highly effective against AU-rich elements containing two or more AUUUA sequences [SEQ ID No. 4] in the 3'-UTR of the mRNA. AUUUA multimers have an essential role in decreasing the stability and translational efficiency of cytokine mRNAs [3]. Insertion of multimeric AUUUA destabilises β-globin mRNA [4-6], is essential for glucocorticoid stimulation of turnover of interferon-β mRNA [7] and is responsible for inhibition of the translation of TNFα-mRNA [8].

TNFα induces phosphorylation of two specific serines of IκB-α (which is also constitutively phosphorylated). Inhibition of this phosphorylation is anti-inflammatory because NFκB, the factor needed for increased expression of adhesion molecules is not released from its complex with IκB-α [9]. In addition to its effects on the synthesis adhesion molecules, NFκB affects expression of IL-1, IL-6 and TNF-α [10]. Cytokines also increase the production of reactive oxygen species [11, 12] which may be important regulators of NFκB [reviewed in 13]. Supported by many anti-oxidants inhibiting cytokine-induced phosphorylation of IκB-α [10, 11,14–17].

Proteasomal Destruction of mRNA

Cytokine messenger RNAs differ from the majority of other mRNAs in having several AUUUA sequences in their UTR. This sequence is recognised by the 20S

proteasome. This permits the development of a novel method for identifying compounds which change the amount of specific mRNAs without having an effect on the general population of mRNAs.

The invention consists of an assay which contains proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), a synthetic oligonucleotide with a 3'-region containing one or more AUUUA recognition sequences and with or without specific proteins which regulate nuclease activity through binding to one or more of the subunits of the 20S proteasome or of the 19S or 11S complexes. Nuclease activity is measured spectrophotometrically, fluorometrically, radiometrically or by chemiluminescence.

Compounds are identified by changes in the rate of the cleavage of the oligonucleotide by the proteasomal endonuclease. This may be because the compound binds to the AUUUA sequence or sequences in the oligonucleotide, to one or more of the 20S proteasome or of the 19S or 11S complexes, or to the regulatory protein.

Compounds, amino acid sequences and medicinal compositions derived therefrom (whether peptidic or peptidomimetic) useful in the treatment of inflammatory disease and obtained or obtainable by the use of the assay or kit are also considered to form part of the present invention.

Where legally permissible the invention also provides for a method of treatment of a patient having an inflammatory disease by administration of a compound obtained according to the assay of the invention.

References Proteasomal Destruction of mRNA

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The Human Immunodeficiency Virus (HIV) is the causative agent of the Acquired ImmunoDeficiency Syndrome (AIDS). Sufferers from AIDS usually die from infection with opportunistic organisms normally readily resisted in the uninfected population. In the Western world, AIDS is currently treated with a 'triple therapy' of a nucleoside reverse transcriptase inhibitor, a non-nucleoside reverse transcriptase inhibitor and a protease inhibitor. This triple therapy has been developed because of the virus' remarkable ability to mutate rapidly and hence become resistant to monotherapy. Although the disease in the West is apparently under control with the triple therapy, there is good reason to expect that this will prove to be a honeymoon period, and that resistance and increasing long-term side-effects will create a demand for therapies based on other approaches. The continuing spread of AIDS in both homosexual and heterosexual populations, particularly in Africa and Southeast Asia, is also a major and urgent reason for novel therapies.

HIV RNA encodes relatively few proteins that are targets for antiviral chemotherapy. Reverse transcriptase and the aspartyl protease have been the major foci for drug development to date. The protein *Tat*, which comprises 86 amino acids and is crucial to viral replication within the host cell, is an attractive target for inhibition but work on this has been slow because of the lack of a robust high-throughput assay. A few compounds have been developed but have not progressed to the clinic, largely because they are peptidic and have poor bioavailability and pharmacokinetic profiles. These have been developed using assay techniques which rely on the direct interaction of *Tat* with the TAR (transactivation response region) of the viral RNA.

Inhibition of the Nuclease-Inhibitory Function of the Human Immunodeficiency Virus *TAT* Protein

In addition to binding to the TAR region of the viral RNA, *Tat* binds to subunits of the 20S and 19S components of the proteasome. This binding inhibits the proteasomal endonuclease activity and hence permits transcription and replication of the virus. We have developed an assay which allows the identification of compounds which interfere with the action of *Tat* on both RNA and the proteasomal complexes. Previous assay systems do not contain the proteasome or any of its components and therefore cannot detect binding to the proteasome. This novel assay will be of use in the identification of novel compounds with utility in the treatment of AIDS in HIV infected patients.

In a third aspect, the invention provides an assay which consists of a synthetic oligonucleotide with a TAR sequence upstream of two or more AUUUA sequences, a proteasomal preparation having nuclease activity and comprising any or all of the 20S, 19S and 11S components and HIV *Tat* or a polypeptide containing the sequence of amino acids 48 to 57 of HIV *Tat* and/or sequences involved in the binding to the 20S, 19S or 11S proteasomal complexes. Nuclease activity is measured spectrophotometrically, fluorometrically, radiometrically or by chemiluminescence.

Nuclease activity is decreased in the presence of *Tat* or polypeptides which bind to the TAR of the added oligonucleotide or to one or more subunits of the 20S, 19S or 11S proteasomal complexes. Compounds which bind to *Tat*, the added polypeptides, the TAR of the added oligonucleotide or proteasomal subunits mediating the inhibition of nuclease activity such that nuclease activity in the assay system is increased are identified as *Tat* inhibitors and will block replication of HIV in infected cells. Such compounds are potentially of value in the treatment of HIV infection.

The invention also provides a kit which comprises the components mentioned above necessary for carrying out an assay for identifying *Tat* inhibitors. Also the invention provides a method for identifying one or more *Tat* inhibitor(s) by use of the assay or kit. *Tat* inhibitor compounds, inhibitory amino acid sequences, and medicinal compositions derived therefrom (whether peptidic or peptidomimetic) useful in the treatment of AIDS in HIV infected patients and obtained or obtainable by the use of the method, assay or kit are also considered to form part of the present invention. Where legally permissible the invention also provides for a method of treatment of AIDS in an HIV infected patient by administration of a *Tat* inhibitor.

Example

General Example

Assay for detection and quantification of the effect of compounds which interfere with the HIV-*Tat* inhibition of proteasomal nuclease activity

Wells of plates suitable for routine large-scale analysis (96, 384, 1512-well plates, etc.) are coated with streptavidin. A buffer solution containing a synthetic oligonucleotide (e.g. SEQ ID 5, $^{5'}$CUGGUUAGACCAGAUCUGAGCCUGGGAGC UCUCUGGCUAACUAGAGGAUGCAUUUAUUUAUUAUUUUAGCG$^{3'}$, known as TARAU$_4$) modified to have biotin at the 3'-end and fluorescein at the 5'-end, is added together with HIV-*Tat* and compounds for testing. Biotin binds to streptavidin such that the oligonucleotide is bound to each well and remains so after washing. Reaction is initiated by addition of a preparation of 20S proteasomes isolated from human spleen. After a fixed time interval, plates are washed. Nuclease activity cleaves the substrate oligonucleotide such that the fluorescent label is released into the medium and removed by washing. Thus proteasomes will, in the absence of *Tat*,

degrade the oligonucleotide and little fluorescence will be detected still bound to each well after washing. In the presence of *Tat*, nuclease activity will be inhibited and greater fluorescence will remain in each well after washing. Compounds which inhibit the effect of *Tat* are therefore detected by decreases in the fluorescence left in wells after washing in assays containing proteasomes and *Tat*.

Specific Example:
Preparation of the Substrate Coated Plates

Multiwell (96 or 384 wells per plate) streptavidin coated black plates (for example, Reacti-bind™ Neutravidin™ Coated polystyrene plates, pkg of 5 ref. 15117 from Pierce) are stored dessicated at 4°C.

Before use, each well is rinsed three times with 200µl of wash buffer (25mM-TrisHCl, 150mM-NaCl, 0.05%(v/v) Tween® 20, pH 7.6).

To each well are added 100µl of substrate solution (containing 25pmol of a 31-mer oligoribonucleotide containing one or more AUUUA sequences and tagged with fluorescein at the 5'-end and biotin at the 3'-end). Plates are then incubated for 16h at room temperature before each well is washed with 200µl of wash buffer.

Each well is washed once with 200µl of TBK160 buffer (20mM-TrisHCl, 160mM-KCl, 5mM-$MgCl_2$ and, pH 7.4).

Then 50µl of TBK160 (containing compounds to be tested or equivalent vehicle) are added to each well. At this stage the plates are ready for incubation with assay solutions.

Proteasome solution
20S proteasomes are purified by standard methods and stored at 4° in TBK600 (20mM-TrisHCl, 600mM-KCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4, to which is added 5mM-NaN_3 as preservative). This solution is diluted with TBK0 (20mM-TrisHCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4) so that the final concentration is that of TBK160.

***Tat* solution**
Tat is obtained by purification from extracts of *Escherichia coli* containing the *Tat* gene and appropriate expression vector. It is stored in PsP buffer (50mM-Na phosphate, 100mM-NaCl, 1mM-EDTA, 1mM-dithiothreitol, pH 7.0).

Preincubation
Proteasome and *Tat* solutions are mixed and incubated at 37° for 20min, such that 50µl of the resulting mixture contain 1pmol of proteasomal protein and 2pmol of *Tat*.

Nuclease assay

To each well are added 50µl of proteasome/*Tat* mixed solution. Plates are then incubated at 37° for a further 20min, before being washed three times with 200µl of TBK160. To each well are then added 50µl of TBK160 and fluorescence is measured in a Labsystems Fluoroscan Ascent FL fluorimeter with appropriate data handling system.

Control assays

Control wells for proteasomal RNase activity contain the same as above but without *Tat*. Controls for *Tat* RNase activity contain the same as above but without proteasomal protein.

Principle of the assay

Labelled plates incubated with buffer alone will retain all of the fluorescein-derived fluorescence and readings will be high. With proteasomes alone, cleavage of the nucleotide takes place and readings are low because the fluorescein is solubilised and removed by the wash buffer. When *Tat* is present, cleavage is inhibited and readings will therefore be higher than with proteasomal protein alone. The extent to which the effect of *Tat* is overcome is a measure of the efficacy of test compounds.

The proteasome, a ubiquitous cellular organelle, has an essential antiviral role *in vivo*. Viral proteins synthesised in infected cells are partially degraded by the proteasome [30, 31, 32]. Peptides so generated are bound to MHC class-I molecules are presented on the cell surface where they are recognised by cytotoxic T-lymphocytes. Viruses have developed mechanisms which enable them to subvert this process [33].

HIV RNA codes for a number of proteins which interfere with proteasome function. *Vpu* and *Env* act together to increase the proteasome-mediated degradation of CD4, important for adequate functioning of helper T cells [34]. *Nef* binds to the B-subunit HsN3 [35] although the function of this binding remains unknown. This subunit is also the binding site for *Tax-1*, a protein encoded by HTLV [36].

Viral proteins in naïve cells are degraded by the proteasome—probably by the 20S complex alone, without ubiquitinylation. If this degradation is blocked by inhibitors, p24 Gag proteins accumulate in the cytoplasm and more proviral DNA is synthesised [37]. *Tat* binds to the 20S proteasome, and strongly inhibits the proteolytic activity, as well as blocking the formation of the 20S-11S complex [38].

Thus, *in vivo*, *Tat* occurs in the naïve cell either as a result of synthesis *de novo* or passage from neighbouring infected cells, a process facilitated by the presence in its sequence of an RGD domain which allows binding to cell surface proteins and consequent cell entry. *Tat* binds to the TAR of the viral RNA, protects the RNA from degradation by the proteasomal endonuclease and allows effective transcription to proceed. In addition, *Tat* binds to the proteasome and blocks the proteolytic destruction of viral proteins, thereby preventing presentation of viral antigens on the cell surface. Inhibition of *Tat* binding to the proteasome or of subsequent *Tat*-associated events will therefore allow the proteasome to degrade essential viral proteins and to present peptides derived from these on MHC class-I molecules for cellular destruction by cytotoxic lymphocytes.

Inhibition of the Protease-Inhibitory Function of the Human Immunodeficiency Virus *TAT* Protein

The HIV protein *Tat* binds to subunits of the 20S and 19S components of the proteasome. This binding inhibits the proteasomal protease activity. By so doing, *Tat* helps to maintain the amounts of viral proteins necessary for transcription and replication of the virus and decreases the presentation of virally-derived peptides to the immune system. Previous assay systems for identification of compounds affecting *Tat* function are based on the binding of *Tat* to the TAR of viral RNA, and do not contain the proteasome or any of its components. Thus they cannot detect binding to the proteasome.

In a fourth aspect, the invention consists of an assay method which allows the identification of compounds which interfere with the inhibition of proteasomal function by the HIV *Tat* protein. The assay contains proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), *Tat* protein (or a partial sequence of *Tat* which contains those regions of the protein that interact with proteasomal subunits) and protein or peptide substrates as appropriate for detection of proteolytic activity by measurement of fluorescence, absorption, luminescence or radioactivity.

Protease activity is decreased in the presence of *Tat* or peptides binding to the *Tat* binding sites to the 20S or 19S components of the proteasomal system. Compounds which bind to *Tat* or proteasomal subunits mediating the inhibition of protease activity such that protease activity in the assay system is increased are identified as *Tat* inhibitors and will block replication of HIV in infected cells. This novel assay will be of use in the identification of compounds with utility in the treatment of AIDS in infected patients.

The invention also provides a kit which comprises the components mentioned above necessary for carrying out an assay for identifying *Tat* inhibitors. Also the invention provides a method for identifying one or more *Tat* inhibitor(s) by use of the assay or kit. *Tat* inhibitor compounds, inhibitory amino acid sequences, and medicinal compositions derived therefrom (whether peptidic or peptidomimetic) useful in the treatment of AIDS in HIV infected patients and obtained or obtainable by the use of the method, assay or kit are also considered to form part of the present invention. Where legally permissible the invention also provides for a method of treatment of AIDS in an HIV infected patient by administration of a *Tat* inhibitor obtained according to the invention.

Example

Assay for the identification, and quantification of the effect, of compounds which interfere with the inhibition of proteasomal protease activity by HIV-*Tat*

Preparation of Plates

Standard multiwell (96 or 384 wells per plate) plates are used. To each well are added 50µl of buffer (30mM-TrisHCl, 10mM-KCl, 5mM-$MgCl_2$, 0.5mM-dithiothreitol, pH 7.8), containing compounds to be tested or equivalent vehicle. At this stage the plates are ready for incubation with assay solutions.

Proteasome solution

20S proteasomes are purified by standard methods and stored at 4° in TBK600 (20mM-TrisHCl, 600mM-KCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4, to which is added 5mM-NaN_3 as preservative). This solution is diluted with TBK0 (20mM-TrisHCl, 5mM-$MgCl_2$, 3mM-dithiothreitol, pH 7.4) so that the final concentration is that of TBK160.

Tat solution

Tat is obtained by purification from extracts of *Escherichia coli* containing the *Tat* gene and appropriate expression vector. It is stored in PsP buffer (50mM-Na phosphate, 100mM-NaCl, 1mM-EDTA, 1mM-dithiothreitol, pH 7.0).

Preincubation

Proteasome and *Tat* solutions are mixed and incubated at 37° for 20min, such that 50µl of the resulting mixture contain 1pmol of proteasomal protein and 2pmol of *Tat*.

Protease assay

To each well are added 50µl of proteasome/*Tat* mixed solution followed by 10µl of 0.11mM fluorescent substrate (Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin, SucLLVY-AMC). Plates are then incubated at 37° for a further 30min, before reaction is stopped by addition of 200µl of stop buffer (0.1M-chloracetic acid, 0.13M-Na acetate, 0.1M-acetic acid, pH 4.3). Fluorescence is measured in a Labsystems Fluoroscan Ascent FL fluorimeter with appropriate data handling system. Results are quantified with reference to standard solutions of aminomethylcoumarin.

Control assays

Control wells for proteasomal protease activity contain the same as above but without *Tat*. Controls for *Tat* protease activity contain the same as above but without proteasomal protein.

Principle of the assay

Fluorescent product is only released after cleavage of the substrate peptide. Plates incubated with buffer alone will show little or no hydrolysis and readings will be low. With proteasomes alone, cleavage of the substrate takes place and readings are high. When *Tat* is present, cleavage is inhibited and readings will therefore be lower than with proteasomal protein alone. The extent to which the effect of *Tat* is overcome is a measure of the efficacy of test compounds.

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Bacterial and viral diseases of plants and non-human animals are sources of major economic cost. Crops may fail to germinate or thrive, and storage during transportation to market or by the end-consumer is often limited by such infections. In horticulture, such diseases may, in addition, be the cause of blemishes and misshapen growth. The high standards now set for meat production demand that the animals involved are free of infection at the time of killing. Weight gain in young animals of agricultural importance may also be severely compromised by infectious disease. Increased resistance, particularly of a generic kind, can help to prevent these costly infections.

The proteasome is a ubiquitous organelle which exhibits an endonuclease activity with specificity for its RNA substrate [1]. This has now been characterised [2]— proteasomes destabilise sequences with AU-rich elements containing two or more AUUUA repeats in the UTR of the mRNA and may be identical with the RNase E-like activity reported by Wennborg *et al.* [3]. AU-rich elements are found in mRNAs of both RNA and DNA viruses and are zones which are sensitive to RNase attack. Such sequences are uncommon in eukaryotic mRNAs [2,3].

The endonuclease activity of the proteasome is associated with two α-type subunits, zeta and iota; of these, zeta has the greater activity [5]. The purified zeta subunit, which is soluble, retains the endonuclease activity and its selectivity for the AU-rich sequence. It degrades the RNA from Tobacco Mosaic Virus (TMV) but neither 5S ribosomal RNA nor globin mRNA [1]. A typical sequence of the proteasomal zeta subunit is shown by way of example in Table 2.

Table 2

PROTEASOMAL ZETA SUBUNIT

241 AA
MW 26469

MFLTRSEYDR GVNTFSPEGR LFQVEYDIEA IKLGSTAIGI QTSEGVCLAV EKRITSPLME PSSIEKIVEI DAHIGCAMSG LIADAKTLID KARVETQNHW FTYNETMTVE SVTQAVSNLA LQFGEEDADP GAMSRPFGVA LLFGGVDEKG PQLFHMDPSG TFVQCDARAI GSASEGAQSS LQELYHKSMT LKEAIKSSLI ILKQVMEEKL NATNIELATV QPGQNFHMFT KEELEEVIKD I

[SEQ ID No. 6]

Generation of Resistance to Bacterial or Viral Infection Damage

In a fifth aspect, the invention consists of the transfection of the gene for the proteasomal zeta subunit into the host genome (as, for example in plants, in [6]) and its expression under the control of a specific promoter which may be general (see [7]), or inducible by addition of exogenous compounds (see [8]). When this promoter is activated, zeta subunit is synthesised in the cytoplasm and destroys viral and bacterial RNA containing the recognition and cleavage site (AU-rich) sequences, hence generating resistance to the consequences of infection, preventing further spread of the causative organism.

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Claims

1. An assay for identifying compounds which inhibit viral replication and pathogenesis by increasing proteasomal degradation of viral components or molecules induced by viral infection, comprising reacting a compound with proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), viral gene product, and protein or peptide substrates, measuring protease activity and identifying the compound as an inhibitor if the protease activity of the assay system is increased.
2. An assay for identifying compounds which inhibit viral replication and pathogenesis by increasing proteasomal degradation of viral components or molecules induced by viral infection, comprising reacting a compound with proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), viral ribo- or deoxyribo-nucleic acid or an oligonucleotide containing sequences recognised by proteasomal subunits or other host proteins binding to proteasomal subunits, measuring nuclease activity and identifying the compound as an inhibitor if the nuclease activity of the assay system is increased.
3. An assay according to claim 1 or 2 which in addition contains host proteins which bind virally-encoded protein or nucleic acid such that proteasomal proteolytic or nuclease activities are modulated.
4. An assay according to claim 1, 2 or 3 wherein activity is measured spectrophotometrically, fluorometrically, radiometrically or by chemiluminescence.
5. A kit which comprises proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), viral gene product, and protein or peptide substrates for carrying out an assay for identifying viral inhibitors.
6. A kit which comprises proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), viral ribo- or deoxyribo-nucleic acid or an oligonucleotide containing sequences recognised by proteasomal subunits or other host proteins binding to proteasomal subunits for carrying out an assay for identifying viral inhibitors.
7. Inhibitor compounds, inhibitory amino acid sequences, and medicinal compositions derived therefrom (whether peptidic or petidomimetic) useful in the treatment of viral disease in infected patients and obtained or obtainable by the use of the assay or kit according to any preceding claim.
8. A method of treatment of viral disease in an infected patient by administration of an inhibitor obtained according to the assay of claim 1, 2 or 3.
9. An assay for identifying compounds which change the concentrations of cytokines, lymphokines and other regulatory proteins by modulating the rate of proteasomal destruction of the specific messenger ribonucleic acids, comprising reacting a compound with proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), a synthetic oligonucleotide with a 3'-region containing

one or more AUUUA [SEQ ID No. 4] recognition sequences and with or without specific proteins which regulate nuclease activity through binding to one or more of the subunits of the 20S proteasome or of the 19S or 11S complexes, measuring nuclease activity and identifying any changes in the rate of cleavage of the oligonucleotide by the proteasomal endonuclease.

10. An assay according to claim 9 wherein the compound binds to the AUUUA sequence or sequences in the oligonucleotide, to one or more of the 20S proteasome or of the 19S or 11S complexes, or to the regulatory protein.
11. An assay according to claim 9 or 10 wherein nuclease activity is measured spectrophotometrically, fluorometrically, radiometrically or by chemiluminescence.
12. A kit which comprises proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), a synthetic oligonucleotide with a 3'-region containing one or more AUUUA recognition sequences and with or without specific proteins which regulate nuclease activity through binding to one or more of the subunits of the 20S proteasome or of the 19S or 11S complexes for carrying out an assay for identifying compounds which change the concentrations of cytokines, lymphokines and other regulatory proteins by modulating the rate of proteasomal destruction of the specific messenger ribonucleic acids.
13. Compounds, amino acid sequences and medicinal compositions derived therefrom (whether peptidic or peptidomimetic) useful in the treatment of inflammatory disease and obtained or obtainable by the use of the assay or kit according to any preceding claim.
14. A method of treatment of a patient having an inflammatory disease by administration of a compound obtained according to the assay of claim 9, 10 or 11.
15. An assay for identifying compounds which inhibit the function of the HIV *Tat* protein, comprising reacting a compound with a synthetic oligonucleotide having a TAR sequence upstream of one or more nuclease AUUUA recognition sequences, a proteasomal preparation having nuclease activity and comprising any or all of the 20S, 19S and 11S components and HIV *Tat* or a polypeptide containing the sequence of amino acids 48 to 57 of HIV *Tat* and/or sequences involved in the binding to the 20S, 19S or 11S proteasomal complexes, measuring nuclease activity and identifying the compound as an inhibitor if the nuclease activity of the assay system is increased.
16. An assay according to claim 15 wherein the compound binds to *Tat*, the added polypeptides, the TAR of the added oligonucleotide or proteasomal subunits mediating the inhibition of nuclease activity such that nuclease activity in the assay system is increased.
17. An assay according to claim 15 or 16 wherein nuclease activity is measured spectrophotometrically, fluorometrically, radiometrically or by chemiluminescence.
18. A kit which comprises a synthetic oligonucleotide with a TAR sequence upstream of two or more AUUUA sequences, a proteasomal preparation having nuclease activity and comprising any or all of the 20S, 19S and 11S components and HIV

Tat or a polypeptide containing the sequence of amino acids 48 to 57 of HIV *Tat* and/or sequences involved in the binding to the 20S, 19S or 11S proteasomal complexes for carrying out an assay for identifying *Tat* inhibitors.

19. *Tat* inhibitor compounds, inhibitory amino acid sequences, and medicinal compositions derived therefrom (whether peptidic or petidomimetic) useful in the treatment of AIDS in HIV infected patients and obtained or obtainable by the use of the assay or kit according to any preceding claim.
20. A method of treatment of AIDS in an HIV infected patient by administration of a *Tat* inhibitor obtained according to the assay of claim 15, 16 or 17.
21. A synthetic oligonucleotide $TARAU_4$ (SEQ ID 1) for use in an assay or kit according to any of claims 15 to 18.
22. An assay for identifying compounds which inhibit the function of the HIV *Tat* protein, comprising reacting a compound with proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), *Tat* protein (or a partial sequence of *Tat* which contains those regions of the protein that interact with proteasomal subunits) and protein or peptide substrate(s), measuring protease activity and identifying the compound as an inhibitor if the protease activity of the assay system is increased.
23. An assay according to claim 22 wherein the compound binds to *Tat* or proteasomal subunits mediating the inhibition of protease activity such that protease activity in the assay system is increased.
24. An assay according to claim 22 or 23 wherein detection of proteolytic activity is by measurement of fluorescence, absorbance, luminescence or radioactivity.
25. A kit which comprises proteasomal protein (20S proteasomes separately with or without 19S and 11S complexes), *Tat* protein (or a partial sequence of *Tat* which contains those regions of the protein that interact with proteasomal subunits) and protein or peptide substrate(s) for carrying out an assay for identifying *Tat* inhibitors.
26. *Tat* inhibitor compounds, inhibitory amino acid sequences, and medicinal compositions derived therefrom (whether peptidic or petidomimetic) useful in the treatment of AIDS in HIV infected patients and obtained or obtainable by the use of the assay or kit according to any preceding claim.
27. A method of treatment of AIDS in an HIV infected patient by administration of a *Tat* inhibitor obtained according to the assay of claim 22, 23 or 24.
28. A method for generating resistance to the damaging effects on a host of bacterial or viral infection, comprising transfection of the gene for the proteasomal zeta subunit into the non-human host genome and its expression under the control of a specific promoter whereby when this promoter is activated, zeta subunit is synthesised in the cytoplasm and destroys viral or bacterial RNA containing the recognition and cleavage site (AUUUA) sequences.
29. A method according to claim 28 wherein the promoter is inducible by addition of at least one endogenous compound.
30. A method according to claim 28 or 29 wherein the host is a plant.

31. An isolated DNA segment encoding the proteasomal zeta subunit [SEQ ID No 6].
32. A vector containing the isolated DNA segment of claim 31 together with a specific promoter for control of the expression of the DNA.
33. A non-human host cell transformed with the vector of claim 32.
34. A plant carrying in its genome a transgene encoding the proteasomal zeta subunit.
35. An isolated DNA segment encoding a compound or a precursor of a compound according to any of claims 7, 13, 19 and 26.
36. A vector containing the isolated DNA segment of claim 35 together with a specific promoter for control of the expression of the DNA.