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MINI-REVIEW

Microbial and fungal protease inhibitors—current and potential applications

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Abstract Proteolytic enzymes play essential metabolic and regulatory functions in many biological processes and also offer a wide range of biotechnological applications. Because of their essential roles, their proteolytic activity needs to be tightly regulated. Therefore, small molecules and proteins that inhibit proteases can be versatile tools in the fields of medicine, agriculture and biotechnology. In medicine, protease inhibitors can be used as diagnostic or therapeutic agents for viral, bacterial, fungal and parasitic diseases as well as for treating cancer and immunological, neurodegenerative and cardiovascular diseases. They can be involved in crop protection against plant pathogens and herbivorous pests as well as against abiotic stress such as drought. Furthermore, protease inhibitors are indispensable in protein purification procedures to prevent undesired proteolysis during heterologous expression or protein extraction. They are also valuable tools for simple and effective purification of proteases, using affinity chromatography. Because there are such a large number and diversity of proteases in prokaryotes, yeasts, filamentous fungi and mushrooms, we can expect them to be a rich source of protease inhibitors as well.

Keywords Fungi · Microorganisms · Protease · Protease inhibitor · Disease · Crop protection

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Introduction

Applications of protease inhibitors are intimately connected to the proteases they inhibit. So, as a preface to protease inhibitors, an overview of proteases with the modes of regulation of their proteolytic activity is provided. Then, known microbial and fungal protease inhibitors are reviewed, with the emphasis on protein (Tables 1 and 2) rather than small-molecule protease inhibitors (Table 3). Finally, their potential applications in the fields of medicine, crop protection and biotechnology are described, based on their target proteases. Microorganisms (prokaryotes, yeasts and filamentous fungi) and higher fungi or mushrooms have been selected for review since protease inhibitors of microbial origin have already proven useful in many different applications. Higher fungi have emerged as a valuable source of new protease inhibitors with unique characteristics only in the last decade and therefore offer great potential for future applications.

Proteases and protease inhibitors

Proteases, also called peptidases or proteolytic enzymes, constitute a large group of enzymes that catalyse the hydrolysis of peptide bonds. Cleavage of peptide bonds can be general, leading to complete degradation of protein substrates into their constituent amino acids, or it can be specific, leading to selective protein cleavage for post-translational modification and processing. Peptidases that cleave peptide bonds at the termini of polypeptide chains are called exopeptidases, while endopeptidases cleave peptide bonds within the polypeptide chain. Peptidases are classified according to their catalytic type into aspartic, cysteine, glutamic, serine and threonine peptidases, according to the main, functional amino acid

Table 1 Families of protein peptidase inhibitors of fungal and microbial origin (Rawlings and Barrett 2011)

Family[a]	Common name	Families of peptidases inhibited	Distribution[b]						
			Bacteria	Archaea	Fungi	Protozoa	Plants	Animals	Viruses
I1	Kazal	M10, S1A, S1D, S8A, S9A	××	×	–	××	×	××××	–
I2	Kunitz-BPTI	S1A, S7	××	–	–	×	×	××××	×
I4	Serpin	C1A, C14A, S1A, S7, S8A, S8B	××	××	×	××	××	××××	××
I9	YIB	S8A	×	–	××	–	–	–	–
I10	Marinostatin	S1A, S8A	××	–	–	–	–	–	–
I11	Ecotin	S1A	××	–	–	××	–	–	–
I16	SSI	M4, M7, S1A, S8A, S8B	××	–	–	–	–	–	–
I31	Thyropin	A1A, C1A, M10A	×	–	–	–	–	×××	–
I32	IAP	C14A	–	–	××	×	–	×××	××
I34	IA3	A1A	–	–	×	–	–	–	–
I36	SMI	M4	×	–	–	–	–	–	–
I38	Aprin	M10B	××	–	–	–	–	–	–
I39	α_2M	A1A, A2A, C1A, C2A, C11, M4, M10A, M10B, M12A, M12B, S1A, S1B, S8A	×××	××	–	–	×	×××	–
I42	Chagasin	C1A	××	××	–	××	–	–	–
I43	Oprin	M12B	××	–	–	–	–	××××	×
I48	Clitocypin	C1A, C13	–	–	×	–	–	–	–
I51	I^C	S1A, S10	×××	××	××	×	××	×××	×
I57	Staphostatin B	C47	×	–	–	–	–	–	–
I58	Staphostatin A	C47	×	–	–	–	–	–	–
I63		M43B, S1A	×	–	×	××	××	××××	×
I66	Cnispin	S1A	–	–	××	–	–	–	–
I69		C10	××	–	–	–	–	–	–
I75	CIII	M41	×	–	–	–	–	–	××
I78		S1A, S8A	××	–	×	–	–	–	–
I79	AVR2	C1A	–	–	×	–	–	–	–
I85	Macrocypin	C1A, C13, S1A	–	–	×	–	–	–	–
I87	HflKC	M41	××	–	–	–	×	–	–

[a] Underlined families include protease inhibitors exclusively of microbial and/or fungal origin

[b] × denotes the number of sequence homologues found in each group of organisms: × less than 10, ×× 11–200, ××× 201–1000 and ×××× more than 1000

residue at the active site. Metallopeptidases, on the other hand, are those whose catalytic activity depends on the presence of a divalent metal ion bound within the active site. In the MEROPS database (http://merops.sanger.ac.uk/), peptidases are classified further into families, according to their sequence similarity, and into clans, according to their structural similarity. There are 226 peptidase families assigned in the MEROPS database (Release 9.5, July 2011) and 57 clans, based on structural data (Barrett 2001; Rawlings et al. 2010). Peptidases are present in all living organisms, including viruses, bacteria, archaea, protists, fungi, plants and animals. Serine peptidases form the most abundant class, followed by metallo-, cysteine, aspartic and threonine peptidases. There has been an explosive growth of the number of peptidase families observed in eukaryotic organisms, there being 100 peptidases in bacterial genomes and half as many in archaeal genomes and from 400 to 700 peptidase genes in plant and mammal genomes. Furthermore, there is a striking difference between the compositions of eubacterial and eukaryotic degradomes, (i.e. the complete set of proteases present in an organism). Sixteen peptidase families constitute the core of the nearly ubiquitous peptidase families present in all living forms. Additional 34 peptidase families are widely distributed in eukaryotic organisms, while another ten are unique to higher metazoan organisms, performing mainly limited proteolysis in extracellular environments (Page and Di Cera 2008; Rawlings et al. 2010). In addition to the MEROPS database, information on proteases can be found in several other online databases, including the Degradome database (http://degradome.uniovi.es/) (Quesada et al. 2009) and the Proteolysis Map (PMAP) (http://www.proteolysis.org/) that comprises five

Table 2 Protein protease inhibitor families that include members of microbial and fungal origin

Protease inhibitor	MEROPS family	Proteases inhibited	Inhibitory mechanism and structural characteristics	Functional characteristics	References
α_2-macroglobulin					
α_2-macroglobulin	I39	Endoproteases of all catalytic classes	Trapping	Microbial members have a protective role against endogenous proteases; while in animals they have important roles in innate immunity	(Armstrong and Quigley 1999; Sottrup-Jensen 1989)
Serine protease inhibitors					
Ovomucoid (Kazal-type)	I1	Chymotrypsin (S1) and subtilisin (S8) families	Tight-binding, Laskowski	Described in Stramenopiles oomycetes (fungus-like microorganisms distantly related to fungi); e.g. involved in pathogenicity of *Phytophtora infestans*	(Tian et al. 2004; Rawlings and Barrett 2011)
Aprotinin	I2	Chymotrypsin family (S1)	Tight-binding, Laskowski	Broad inhibitory specificity	(Ascenzi et al. 2003; Rawlings and Barrett 2011)
Peptidase B inhibitor	I9	Subtilisin family (S8)	Bacterial inhibitors are propeptides of subtilisin-like proteases. Fungal inhibitors are separate polypeptides, e.g. *Pleurotus ostreatus* proteinase A inhibitor 1 POIA1 and yeast proteinase inhibitor 2 YIB2	They are potent but unstable inhibitors, gradually degraded by subtilisin	(Ascenzi et al. 2003; Dohmae et al. 1995; Kojima et al. 1999; Kojima et al. 2005; Kojima et al. 1997; Rawlings and Barrett 2011)
Marinostatin	I10	Proteases of family S8 (subtilisin) and certain proteases of family S1 (chymotrypsin)	Tight-binding, Laskowski. Structure stabilized by two internal ester bonds that are essential for their inhibitory activity	Exclusive to marine bacteria	(Kanaori et al. 2005; Rawlings and Barrett 2011)
Ecotin	I11	Chymotrypsin family (S1)	Tight-binding, Laskowski for primary binding site. Active as dimers, each monomer binds the protease at two binding sites	Ecotins from enterobacteria and parasites perform a protective role against host digestive proteases and target host proteases to facilitate colonization. Structure enables inhibition of multiple proteases with the chymotrypsin fold	(Eggers et al. 2001; Eschenlauer et al. 2009; Rawlings and Barrett 2011)
Streptomyces subtilisin inhibitor (SSI)	I16	Family S8 (subtilisin, kexin), family S1 (trypsin, plasmin) and the metalloprotease griselysin (family M4)	Tight-binding, Laskowski	Exclusive to bacterial Actinomycetales order. They probably control endogenous proteases involved in proteolytic activation of transglutaminase	(Kantyka et al. 2010; Taguchi et al. 1997; Tsuyuki et al. 1991; Rawlings and Barrett 2011)
Carboxypeptidase Y inhibitor	I51	Serine carboxypeptidase Y (family S10)	Tight-binding. In addition to a phospholipid binding site, there are two binding sites in I^C responsible for protease inhibition	First member (I^C) was isolated from *Saccharomyces cerevisiae*, and it belongs to the phosphatidylethanolamine-binding protein family. Binds anionic phospholipid membranes, anchoring the intracellular localization to vacuolar membranes	(Mima et al. 2006; Mima et al. 2005; Rawlings and Barrett 2011)
Cnispin, cospin and *Lentinus* peptidase inhibitor	I66	Strong inhibition of trypsin, weak inhibition of chymotrypsin (family S1)	Tight binding, Laskowski	Described only in basidiomycete mushrooms. Representatives from *Lentinus edodes*, cnispin from *Clitocybe nebularis* and cospin from *Coprinopsis cinerea* have been characterized. A defensive role against predatory insects has been shown, but a regulatory endogenous role is also possible	(Avanzo et al. 2009; Odani et al. 1999; Sabotič et al. 2012; Rawlings and Barrett 2011)

Table 2 (continued)

Protease inhibitor	MEROPS family	Proteases inhibited	Inhibitory mechanism and structural characteristics	Functional characteristics	References
Aspergillus elastase inhibitor	I78	Human elastase-2 and endogenous *Aspergillus* elastases (family S1)	Unknown	Homologues have been found in a few other ascomycete species and in proteobacteria, but none was characterized biochemically	(Okumura et al. 2008; Okumura et al. 2006; Rawlings and Barrett 2011)
Serpin	I4	Chymotrypsin (S1) and subtilisin (S8) families. Some members inhibit also cysteine proteases of papain (C1) and caspase (C14) families	Trapping. Suicide inhibitors in which a rapid conformational change traps the cognate protease in a covalent complex	The physiological role of microbial serpins has been proposed to be to protect the cellulose-degrading apparatus (cellulosome) against proteolytic degradation. The only fungal serpin (celpin), was characterized from the anaerobic fungus *Piromyces* sp. E2	(Kantyka et al. 2010; Law et al. 2006; Roberts et al. 2004; Steenbakkers et al. 2008)
Cysteine protease inhibitors					
Thyropin	I31	Papain-like proteases (family C1), and equistatin inhibitor unit 2 inhibits an aspartic protease cathepsin D (family A1)	Tight-binding	Present in animals and in one bacterial pathogen (*Coxiella burnetii*) that has presumably acquired the gene by lateral transfer	(Kantyka et al. 2010; Rawlings and Barrett 2011)
Survivin	I32	Caspases-aspartate-specific cysteine proteases (family C14)	Tight-binding, several mechanisms	Survivin plays a dual role as a mitotic regulator of cell division and as an inhibitor of caspase activation in the process of apoptosis. Fungal homologues have been identified in ascomycete and a few basidiomycete genomes. The fission yeast homologue is a conserved chromosomal passenger protein	(Huang et al. 2005; Luthringer et al. 2010; Rawlings 2010)
Chagasin	I42	Protozoan and mammalian papain-like cysteine proteases (family C1)	Tight-binding	Parasitic chagasins are involved in regulating endogenous cysteine proteases essential for their life cycle. In bacteria and archaea, chagasins serve as endogenous regulators, and in some pathogenic species, they also serve a protective role against host proteases	(Kantyka et al. 2010; Santos et al. 2006)
Clitocypin (I48) and macrocypin (I85); together named mycocypins	I48, I85	Papain-like cysteine proteases (family C1) and legumain (family C13) and serine protease trypsin (family S1)	Tight-binding. Mycocypins are small and exceptionally stable proteins. They have a β-trefoil fold formed by the core six-stranded β-barrel that supports 11 loops which provide a versatile surface for the inhibition of several types of proteases	Unique to basidiomycetes. They probably have an endogenous regulatory role or a role in defence against pathogen infection and/or predation by pests. A defensive role for mycocypins is further supported by their high genetic variability and conformational stability as well as a broad inhibitory profile	(Brzin et al. 2000; Kidrič et al. 2002; Renko et al. 2010; Sabotič et al. 2007a; Sabotič et al. 2006; Sabotič et al. 2009b)
Staphostatins	I57, I58	Staphopains (family C47)	Tight-binding. Staphostatin A (I58) inhibits only staphopain A, and staphostatin B (I57) specifically inhibits staphopain B	Unique to bacteria. The staphostatin is always co-expressed with staphopain from one operon	(Dubin et al. 2007; Kantyka et al. 2010)
Streptopain inhibitor	I69	Streptopain (family C10)	Homologous to the streptopain propeptide and is secreted by *Streptococcus pyogenes* along with the target protease	Restricted to a few species of bacteria. Expression of streptopain and its inhibitor is probably co-regulated	(Kagawa et al. 2005)

Table 2 (continued)

Protease inhibitor	MEROPS family	Proteases inhibited	Inhibitory mechanism and structural characteristics	Functional characteristics	References
AVR2 protein	I79	Papain-like proteases (family C1)	Unknown	Avr2 is a virulence factor isolated from a phytopathogenic fungus *Passalora fulva* (formerly classified as *Cladosporium fulvum*) that causes leaf mould of tomato. Avr2 inhibits a set of defence-related plant cysteine proteases and is secreted upon infection and colonization	(Shabab et al. 2008; van Esse et al. 2008; Rawlings and Barrett 2011)
Metalloprotease inhibitors					
Streptomyces metallopeptidase inhibitor (SMI)	I36	Family M4 metalloproteases	Tight-binding, Laskowski	One metalloprotease inhibitor identified, SMPI (*Streptomyces* metalloprotease inhibitor), isolated from *Streptomyces nigrescens*	(Kantyka et al. 2010; Rawlings and Barrett 2011)
Aprin	I38	Bacterial metallopeptidases of subfamily M10B metzincins	Tight-binding	Unique to bacteria and limited to species from Enterobacteriaceae and Pseudomonadaceae. Aprins probably have a protective role as they are encoded with the protease in one operon	(Kantyka et al. 2010; Rawlings and Barrett 2011)
Oprin	I43	Family M12 metallopeptidases	Tight-binding	Animal oprins found in serum provide natural resistance to the effects of snake venom peptidases	(Rawlings and Barrett 2011)
Proeosinophil major basic protein	I63	Pappalysin-1 (family M43)	Tight-binding	None of the microbial homologues has been characterized (only six found in bacteria, none in fungi)	(Rawlings and Barrett 2011)
Bacteriophage lambda CIII protein	I75	Metalloendopeptidase FtsH (family M41)	Tight-binding	CIII enhances the lysogenic response by inhibiting the host metallopeptidase. Homologues have been found in *Escherichia coli* and *Salmonella enterica* and presumably represent retained phage genes	(Kobiler et al. 2007; Rawlings and Barrett 2011)
HflC and HflK	I87	Metalloendopeptidase FtsH (family M41)	Tight-binding	HflC and HflK are cytoplasmic membrane proteins in a hetero-multimeric complex (HflKC) which interacts with and regulates substrate selection for FtsH	(Akiyama 2009; Rawlings and Barrett 2011)
Aspartic protease inhibitors					
Saccharopepsin inhibitor (IA3)	I34	Yeast proteinase A or saccharopepsin (family A1) from *Saccharomyces cerevisiae*	Tight-binding	The high specificity is the result of the structural stabilization of the IA3 inhibitor in complex with saccharopepsin since the unstructured inhibitor in solution forms an alpha helix upon interaction with the enzyme active site	(Green et al. 2004; Phylip et al. 2001)

Table 3 Small-molecule inhibitors of fungal and bacterial origin grouped into four categories according to specificity of inhibition (modified from Rawlings (2010)

Inhibitor name	Source organism	Peptidases inhibited
Compounds inhibiting peptidases of different catalytic types		
Acivicin	*Streptomyces sviceus*	C26, T3
Amastatin	*Streptomyces* sp. 1.24819	C15, M1, M17, M28E, M42
Antipain	*Streptomyces michigaensis*, *S. yokosukaensis*	C1A, C2A, C3B, C11, C14B, C25, C39, S1A, S8A, S9A, S10, S28, S49, S53, T1A
Bacitracin A	*Bacillus licheniformis*	C1A, M3, M16, S8A
Bestatin	*Streptomyces olivoreticuli*	M1, M8, M17, M19, M20A, M28E, S33
Chymostatin	*Streptomyces hygroscopicus*	C1A, S1A, S8A, S10, S19, S29, S49, S53, T1A
E64	*Aspergillus japonicus*	C1A, C2A, C10, C11, C25, C28, C47, C57, C86, S1A
Leupeptin	*Streptomyces exfoliatus*	C1A, C2A, C11, C14B, C25, C84, S1A, S1D, S8B, S9A, S49, S53, T1A
Puromycin	*Streptomyces alboniger*	M1, S28
Tyropeptin A	*Kitasatospora* sp. MK993-dF2	S53, T1A
Compounds inhibiting peptidases of the same catalytic type from more than one family		
Actinonin	*Streptomyces* sp.	M1, M12A
Ebelactone A	*Streptomyces aburaviensis*	S9C, S10
Elastatinal	*Streptomyces griseoruber*	S1A, S49
Matlystatin A	*Actinomadura atramentaria*	M1, M10A
Pepstatin	*Streptomyces* spp.	A1A, A2A, A5, A11A, A22A
Phosphoramidon	*Streptomyces tanashiensis*	M2, M4, M11, M12B, M13, M16A, M27, M36
Probestin	*Streptomyces azureus*	M1, M49
Talopeptin	*Streptomyces* spp.	M4, M13
Compounds inhibiting more than one peptidase in the same family		
Elasnin	*Streptomyces noboritoensis*	S1A
Leuhistin	*Bacillus laterosporus*	M1
Matlystatin B	*Actinomadura atramentaria*	M10A
Piperastatin A	*Streptomyces lavendofoliae*	S10
TMC-95	*Apiospora montagne*	T1A
Compounds specific for a single peptidase		
Arylomycin A2	*Streptomyces* sp. Tu 6075	Signal peptidase I S26.001
Belactosin A	*Streptomyces* sp.	Chymotrypsin-like activity of the proteasome T01.012
Epoxomicin	Actinomycete strain No. Q996-17	Proteasome catalytic subunit 3 T01.012
Fumagillin	*Aspergillus* sp.	Methionyl aminopeptidase 2 M24.002
Poststatin	*Streptomyces viridochromogenes*	Prolyl oligopeptidase S09.001

different databases (CutDB, PathwayDB, ProteaseDB, SubstrateDB and ProfileDB) (Igarashi et al. 2009).

The occurrence of proteases in all living organisms indicates their critical role in essential metabolic and regulatory functions in many biological processes. Proteases are important in the production of nutrients for growth and proliferation. Extracellular proteases catalyse the hydrolysis of proteins into smaller peptides and amino acids for subsequent absorption into cells, constituting a very important step in nitrogen metabolism. Proteases perform critical regulatory functions in numerous physiological processes since they regulate the fate, localization and activity of many proteins, modulate protein–protein interactions and contribute to the generation, transduction and amplification of molecular signals. Proteases are involved in a wide span of cellular and metabolic processes, including regulation of gene expression, DNA replication, transport of proteins, cell growth and differentiation, cell cycle, heat shock response, SOS response to DNA damage, misfolded protein response, oxidative stress response and programmed cell death (Lopez-Otin and Bond 2008; Rao et al. 1998). Furthermore, in plants, proteases are important in the build-up and breakdown of seed storage proteins during seed germination, protein remobilization upon organ senescence and in many developmental processes such as embryogenesis, chloroplast biogenesis, photomorphogenesis, hormone signalling, flower development, pollen–pistil interaction and local and systemic

defence responses against pathogens and herbivores (Simoes and Faro 2004; Salas et al. 2008; Schaller 2004; van der Hoorn 2008). Moreover, in animals, proteases are involved in tissue morphogenesis and remodelling, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, haemostasis, blood coagulation, inflammation, immunity, autophagy and senescence (Lopez-Otin and Bond 2008).

Proteases of microbial and fungal origin offer a wide range of biotechnological applications. Alkaline proteases have been used in the detergent industry for over 50 years. Proteases with elastolytic and keratinolytic activities have been used in the leather industry for de-hairing and baiting of skins and hides. The food industry uses proteases in cheese making, baking, preparation of various protein hydrolysates, meat tenderization and manufacturing protein-rich diets. In the pharmaceutical industry, proteases have found uses as therapeutic agents as well as additives in preparations of slow-release dosage forms. Bioprocessing of used X-ray films for silver recovery involves the use of alkaline proteases. Proteases allow potential applications for the management of wastes from various food processing industries and from household activities. In addition to industrial and medical applications, proteases are used in basic research; for example, proteases with very selective peptide bond cleavage are used in protein sequencing, unselective proteinase K is used in nucleic acid isolation, and trypsin is widely used in maintaining animal cell cultures (Kumar and Takagi 1999; Rao et al. 1998).

There is also the downside to proteases as some are important virulence factors of many pathogenic bacteria, parasites and viruses. These proteases are involved in acquiring nutrients for growth and proliferation through host tissue degradation and evasion of host immune defences. In addition to colonizing and facilitating dissemination functions, they are also involved in evading the host immune system by interrupting the cascade pathways, disrupting the cytokine network, excising cell surface receptors and inactivating host protease inhibitors (Maeda 1996; Travis and Potempa 2000; Supuran et al. 2002).

Because proteases play essential roles in life and death processes in all living organisms and because peptide bond hydrolysis is irreversible, anomalies in proteolytic activities lead to numerous pathological conditions, including cancer, neurodegenerative disorders and inflammatory and cardiovascular diseases, as well as bacterial, viral and parasitic diseases (Lopez-Otin and Bond 2008; Turk 2006). Activity of proteases is regulated on several levels, including regulation of gene expression at transcriptional and post-transcriptional levels, synthesis as inactive zymogens, blockade by endogenous inhibitory proteins, spatial and temporal compartmentalization, post-translational modification (glycosylation, phosphorylation, co-factor binding), proteolysis and degradation (Lopez-Otin and Bond 2008).

Protein protease inhibitors constitute a very important mechanism for regulating proteolytic activity. They can be classified approximately according to the class of proteases they inhibit (for example, serine or cysteine protease inhibitors). However, those composed of multiple inhibitor units and pan-inhibitors (such as α_2-macroglobulin of family I39) that target proteases of different catalytic classes prevent unambiguous classification. A more detailed classification is included in the MEROPS database (http://merops.sanger.ac.uk/), which follows a hierarchy similar to that for proteases. Protease inhibitors are grouped into families based on sequence homology and into clans based on protein tertiary structure. In release 9.5 (4 July 2011) of the MEROPS database, there are 71 families of protease inhibitors, and those with available three-dimensional structural data have been assigned to 39 different clans (Rawlings 2010). Of the 71 families, 27 include members of microbial and fungal origin (Tables 1 and 2). Of these, seven families include members of exclusively bacterial origin (I10, I16, I36, I38, I57, I58, I69), and five families include members of exclusively fungal origin (I34, I48, I66, I79, I85). In addition to protein protease inhibitors, the MEROPS database includes a list of small-molecule inhibitors that are well known and widely used. Many of them have been synthesized in the laboratory; however, those that occur naturally (Table 3) have been isolated from bacteria and fungi (Rawlings 2010).

There are two general mechanisms of protease inhibition, namely, irreversible "trapping" reactions and reversible tight-binding reactions. Trapping reactions work only on endopeptidases and are the result of a conformational change of the inhibitor triggered by cleavage of an internal peptide bond by the host protease (Fig. 1a). Only three families utilize a trapping mechanism: I4 (serpins), I39 (α_2-macroglobulin) and I50 (viral caspase inhibitors). Reversible tight-binding inhibition is widespread, the best known being the "standard canonical" or "Laskowski mechanism", in which the inhibitor has a peptide bond that is cleaved by the peptidase active site in a substrate-like manner. The inhibitor is only slowly released due to the conformational stability of the stabilized loop that can mimic a substrate. This mechanism has been conclusively demonstrated only for inhibitors of serine proteases. Other reversible tight-binding protease inhibitors physically block the protease active site by high-affinity binding to sites on either side of the active site (Fig. 1b, c). Binding of an inhibitor to the active site can also be irreversible, when an electrophilic reactive group of the inhibitor forms a covalent bond with an amino acid residue in the enzyme active site. There are also some inhibitors that block the exosites, to which substrate

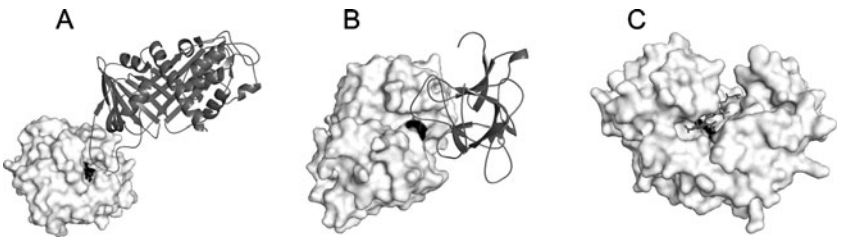

Fig. 1 Examples of protease inhibitors utilizing irreversible "trapping" reaction (**a**) and reversible tight-binding reactions (**b** and **c**). Proteases are shown in *light grey*, their active site residues in *black* and inhibitors in *dark grey*. **a** Serine protease trypsin in complex with serpin (family I39) (PDB ID 1K9O). The protease cleaves the reactive centre loop of serpin, which triggers a conformational change in the inhibitor and trapping of the protease in an inactive covalent complex. **b** Cysteine protease cathepsin V in complex with clitocypin (family I48) (PDB ID 3H6S). The inhibitor binds to the protease active site cleft and obstructs access of substrate. **c** Aspartic protease plasmepsin IV in complex with the small-molecule inhibitor pepstatin A (PDB ID 1LS5). The inhibitor binds in the active site of the protease

binding occurs in addition to the active site in some proteases (Christeller 2005; Rawlings 2010; Rawlings et al. 2004).

Microbial and fungal protease inhibitors

The families of protein protease inhibitors that include members of microbial and fungal origin are described in Table 2, and information of their distribution in taxonomic groups is given in Table 1. Protease inhibitors are described in groups according to the catalytic class of protease they inhibit and following the MEROPS inhibitor classification (Rawlings 2010; Rawlings and Barrett 2011). Since prokaryote-derived protease inhibitors have been reviewed recently (Kantyka et al. 2010), more information on protease inhibitors of fungal origin, including yeasts, filamentous fungi and mushrooms, is provided here.

Among the small-molecule protease inhibitors isolated from bacteria and fungi (Table 3), there are several that show broad inhibitory specificity and inhibit proteases of different catalytic classes. Several inhibit both serine and cysteine proteases (antipain, chymostatin, leupeptin), serine and metalloproteases (bestatin, puromycin), metallo- and cysteine proteases (amastatin) or metallo-, cysteine and serine proteases (bacitracin A). Of the small-molecule cysteine protease inhibitors, the best known is E-64 (1-[L-*N*-(*trans*-epoxysuccinyl) leucyl] amino-4-guanidinobutane), an irreversible inhibitor originally isolated from *Aspergillus japonicus* (Hanada et al. 1978) and routinely used as a class-specific cysteine protease inhibitor. A number of E-64 analogues have been synthesized in order to improve selectivity for a particular cysteine protease. Several inhibitors are specific for metalloproteases and inhibit more than one protease family (e.g. phosphoramidon). The only natural small-molecule inhibitor of aspartic proteases is pepstatin, originally isolated from various species of actinomycetes (Umezawa et al. 1970), which inhibits several families of aspartic proteases. It is a hexa-peptide containing the unusual amino acid statine (Rawlings 2010).

Applications of protease inhibitors in medicine

Proteases play an important part in almost every biological process; therefore, unregulated activity often leads to disease. In this review, only excessive proteolysis will be addressed as it is the one that can be reversed by protease inhibitors. Excessive proteolysis plays an important role in cancer and in cardiovascular, inflammatory, neurodegenerative, bacterial, viral and parasitic diseases. Due to the obvious relevance of protease inhibitors, they have been studied extensively with the intent to develop therapeutic drugs (Drag and Salvesen 2010; Turk 2006; Haq et al. 2010). Proteases that have a potential as therapeutic targets are reviewed, according to their catalytic type, for each group of disease-causing organisms and for other human diseases. Information on the availability of protease inhibitors for each protease described is provided, with the emphasis on those for which specific inhibitors have not yet been identified.

Viral diseases

Proteolytic processing of virus polyprotein into structural and non-structural proteins is an essential part of the viral replication cycle, making the proteases an important antiviral drug target. Several viral proteases have been studied as therapeutic targets. Although proteases of any virus could be potential antiviral targets, viruses that cause chronic diseases (e.g. HIV, herpes virus) and those that could cause large-scale epidemics (e.g. SARS coronavirus, dengue virus) have received most attention.

Several protease inhibitors acting against the human immunodeficiency virus 1 (HIV-1) protease, a homodimeric aspartic protease, have been used in treating HIV-1 infection.

These are all low molecular weight peptidomimetic inhibitors whose design has been based on the structures of the compounds binding to the protease active site. In therapy, they are usually used in combination with inhibitors of reverse transcriptase (Abbenante and Fairlie 2005; Anderson et al. 2009). In addition to a number of designed synthetic inhibitors, a potent peptidic inhibitor of HIV-1 protease of bacterial origin (ATBI) has been found in an extremophilic *Bacillus* sp. (Dash and Rao 2001; Vathipadiekal et al. 2010).

Other targeted viral proteases belong to the serine catalytic type. Human cytomegalovirus (HCMV) is one of eight human herpes viruses and is widespread in populations worldwide, with infection rates of 80–100%. It causes asymptomatic infections in healthy individuals but high morbidity and mortality in immunocompromised individuals. A few inhibitors of the cytomegalovirus protease have been described from bacterial (*Streptomyces*) and fungal (*Cytonaema*) origins (Stoeva and Efferth 2008; Anderson et al. 2009). An additional target in antiviral therapy against cytomegalovirus is the proteasome, where the aim is to hinder the recruitment of host proteins by the virus for its replication. Several synthetic and a few natural proteasome inhibitors (e.g. lactacystin from *Streptomyces lactacystinaeus*) are known and have been reported to obstruct replication of several viruses, including influenza virus, herpes simplex virus type 1, paramyxovirus and rhabdoviruses, as well as cytomegalovirus (Kaspari and Bogner 2009). The serine proteases NS3 and NS2 of flaviviruses are targets for antiviral drug development against hepatitis C virus and dengue virus, with the former blood-transmitted virus causing various liver diseases (including cirrhosis and liver cancer) and the latter being a mosquito-transmitted disease causing dengue hemorrhagic fever. Several structure-based designed low molecular weight inhibitors are in different phases of clinical evaluation (Anderson et al. 2009; Lange et al. 2010; Tomlinson et al. 2009).

The coronavirus associated with severe acute respiratory syndrome, SARS, encodes a chymotrypsin-like cysteine protease M^{Pro} that is similar to picornavirus 3C protease. Since the 2003 SARS global outbreak, several strategies of structure-based design of low molecular weight protease inhibitors have been applied in the search for antiviral drugs against SARS (Anderson et al. 2009; Sirois et al. 2007). The first to be considered were the protease inhibitors targeting the picornavirus 3C protease. The picornaviruses, which encode a 3C protease, are important human and animal pathogens such as poliovirus, hepatitis A virus, coxsackievirus, human rhinovirus and foot-and-mouth disease virus. Inhibitors targeting the 3C protease of human rhinoviruses that cause common cold, as well as 3C proteases of other picornaviruses and coronaviruses, have been developed based on structural data, but none has yet successfully passed all the phases of clinical evaluation (Neubauer et al. 2009; Wang and Liang 2010).

Due to the rapid development of resistance in viruses, the search for novel strategies for developing inhibitors targeting different sites on proteases is encouraged, including the search for novel lead compounds from natural sources and structure-based drug development.

Bacterial diseases

Bacterial pathogens employ an array of virulence factors that enable their colonization, evasion of host defences and dissemination. Proteases are important virulence factors of many pathogenic bacteria, which play roles in acquiring nutrients by direct degradation of host tissue components. The even more important aim of disrupting host immune response and signalling cascades has been reviewed by Potempa and Pike (2009). Most currently available antibiotics target bacterial cell wall synthesis or protein synthesis. In the light of rapidly spreading antibiotic resistance, bacterial proteases are promising targets for the design of novel antibiotics. Metalloproteases are most abundantly represented in primary and opportunistic pathogens, although all catalytic classes are found. These proteases are often associated with mobile genetic elements (plasmids, pathogenicity islands, integrated phages), and their expression is not constitutive but regulated through environmental or cellular signals (Travis and Potempa 2000; Wladyka and Pustelny 2008).

Omptins (outer membrane proteins T) are aspartic proteases (family A26) found in several Gram-negative bacteria, including the pathogenic species *Escherichia coli* (OmpT), *Yersinia pestis* (Pla), *Shigella flexneri*, *Shigella dysenteriae* (SopA), *Salmonella enterica* (PgtE), *Legionella pneumophila* (Lpa) and plant pathogens *Agrobacterium tumefaciens* and *Erwinia pyrifoliae* (PlaA). Omptins are bacterial virulence factors and, in addition to their proteolytic activity, possess adhesive and invasive activities. They modulate the coagulation system since they act as plasminogen activators (OmpT, Pla, PgtE, Lpa), inactivate tissue factor pathway inhibitor (OmpT, Pla, PgtE), degrade thrombin-activatable fibrinolysis inhibitor (Pla, PgtE), degrade the complement system proteins (Pla, PgtE) and antimicrobial peptides (OmpT, Pla, PgtE), and process autotransporters (OmpT, SopA). Lipopolysaccharide (LPS) is required for their enzymatic activity. Omptins have a unique catalytic mechanism that combines the elements of both serine and aspartic proteases, and partial inhibition by serine protease inhibitors has been reported (Hritonenko and Stathopoulos 2007; Valls Seron et al. 2010; Yun et al. 2009). Other than a weak substrate-based peptide inhibitor with a D-Arg, shown to inhibit OmpT (Dekker et al. 2001), and a colicin immunity protein shown to protect colicin E2 from degradation by OmpT in *Escherichia coli* (Duche et al. 2009), no specific inhibitors have been reported.

No specific inhibitor has so far been found for the exfoliative toxin A, a glutamate-specific serine protease (family S1) produced by *Staphylococcus aureus*, which is the causative agent in staphylococcal scalded skin syndrome. The target of the toxin is a transmembrane glycoprotein desmoglein-1 of the cadherin superfamily, which plays an important role in keratinocyte cell–cell adhesion (Ladhani 2003).

Immunoglobulin A1 proteases (IgA1 proteases) are serine proteases (family S6) produced by several pathogenic bacteria, including species causing bacterial meningitis, *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*. The IgA1 proteases enable colonization of human mucosal surfaces by cleaving the secretory IgA antibodies, thus disrupting the specific immunity response. Except for an early report on substrate analogue inhibitors (Burton et al. 1988) and small synthetic peptide inhibitors (Bachovchin et al. 1990) of *Neisseria gonorrhoaeae* IgA1 protease, there has been no further development of IgA1 protease inhibitors (Mistry and Stockley 2006).

Other immunomodulating serine proteases are C5a peptidases from streptococci (family S8)—those of group A (*Streptococcus pyogenes* ScpA) and group B (*Streptococcus agalaciae* ScpB) streptococci have been described in more detail. *Streptococcus pyogenes* is the causative agent of pharyngitis and also causes rheumatic fever and skin infections, which can develop severe complications, including toxic shock syndrome. C5a peptidases are important for streptococcal pathogenesis as they specifically cleave complement C5a and therefore prevent the recruitment of phagocytic cells to the infection site (Cheng et al. 2002; Collin and Olsen 2003). Antibodies raised against C5a peptidase were used to inhibit C5a peptidase in vivo (Park and Cleary 2005), but no peptidase inhibitors have been described.

Serine proteases are important pathogenesis factors in bacteria involved in dental diseases. *Treponema denticola* is a spirochete implicated in the progression of periodontal diseases. A serine protease, trepolisin (also called dentilisin), of family S8 is an important pathogenesis factor, a mediator of cytopathic effects by degrading host proteins, including extracellular matrix components and host protease inhibitors (Sela 2001). The broad-range inhibitor of serine proteases (families S1 and S8), chymostatin, inhibits trepolisin; however, no specific inhibitors have been described. Another important oral cavity pathogen involved in periodontal disease, *Porphyromonas gingivalis*, in addition to a few cysteine proteases (discussed further in the following), produces a serine protease, a prolyl tripeptidyl peptidase PtpA (family S9), which is involved in degrading host connective tissue, providing nutrients for bacterial growth (Banbula et al. 1999). A substrate-based specific inhibitor of PtpA has been developed (Xu et al. 2008).

Bacterial type I signal peptidases SPase (family S26) are serine proteases widespread among Gram-negative and Gram-positive bacteria and are membrane proteases required for processing newly synthesized secreted proteins. In addition to their essential role in bacterial viability, they are important antimicrobial drug targets as they are involved in the secretion of many virulence factors (Paetzel et al. 2000). Synthetic penem inhibitors have been developed for inhibiting both Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*, *Staphylococcus epidermidis*) SPases. The various penem derivatives display different degrees of activity against these pathogenic bacteria SPases (Allsop et al. 1995; Harris et al. 2009). Recently, substrate-based peptide aldehydes have been shown to be promising inhibitors of *Escherichia coli* and *Staphylococcus aureus* SPases (Buzder-Lantos et al. 2009). However, the in vitro inhibitory activity and in vivo antimicrobial activities of these inhibitors did not correlate well, so further optimization of and search for novel SPase I inhibitors are expected.

No specific inhibitors have been described for the streptococcal pyrogenic exotoxin B (SpeB, streptopain), a cysteine protease (family C10) produced by all strains of *Streptococcus pyogenes*. It is a multifunctional protease and an important pathogenesis factor with several immunomodulating activities, including release of proinflammatory molecules, degradation of extracellular matrix, cleavage of IgG in the hinge region and degradation of other immunoglobulins. In addition to class-specific cysteine protease inhibitors, a peptide derivative was shown to inhibit SpeB, as well as α_2-macroglobulin and an S-nitrosylated form of α_1-protease inhibitor (Collin and Olsen 2003).

IdeS (family C66) is another cysteine protease from *Streptococcus pyogenes* that specifically cleaves IgG, its only known substrate (Vincents et al. 2004). In addition to specific, inhibitory IgG antibodies (Akesson et al. 2006), synthetic reversible inhibitors were designed, with aldehyde compounds being the most promising; however, no specificity data are yet available for these inhibitors (Berggren et al. 2009).

Staphylococcus aureus causes a range of diseases, from mild skin infections to life-threatening disorders, including septicaemia, endocarditis, toxic shock syndrome and pneumonia (Lowy 1998). It expresses several extracellular proteases with proposed roles in pathogenicity, including a serine protease V8 (SspA), cysteine proteases staphopains A and B (ScpA, SspB) and a metalloprotease aureolysin (Aur) (Shaw et al. 2004). Staphopains A and B (family C47) are papain-like cysteine proteases that are co-expressed with their respective specific inhibitors staphostatins A and B (Dubin 2003). Their role in pathogenicity has not been determined. Staphopain B (SspB) is activated by the glutamyl peptidase SspA (V8 protease, family S1), which is expressed from the same operon (Shaw et al. 2004). V8

protease modulates the surface protein profile and so influences the binding of fibronectin by *Staphylococcus aureus* (McGavin et al. 1997).

Sortases are cysteine proteases (family C60) of Gram-positive bacteria that catalyse the covalent attachment of proteins to the cell wall peptidoglycan. They have been shown to contribute to the virulence of several important pathogens, including *Staphylococcus aureus*, *Bacillus anthracis*, *Listeria monocytogenes* and *Streptococcus pneumoniae*. Therefore, they have been considered to be important targets for the development of novel antiinfective agents (Suree et al. 2007; Clancy et al. 2010). *Staphylococcus aureus* sortase (SrtA) has been at the focus of sortase inhibitor development due to the emergence of antibiotic-resistant strains and the need for novel antimicrobial strategies. Several types of SrtA inhibitors have been investigated, including non-specific sulfhydryl modifiers, peptide analogues of the sorting signal, compounds from plants and marine organisms, and synthetic small-molecule inhibitors. Several inhibitors of SrtA have been described with varying strength and specificity; however, good in vitro inhibitory activity has not yet led to an effective in vivo sortase inhibitor (Maresso and Schneewind 2008; Suree et al. 2007; Clancy et al. 2010).

The cysteine protease clostripain (family C11) is a secreted protease of the anaerobic bacterium *Clostridium histolyticum*, a causative agent of gas gangrene (clostridial myonecrosis). Clostripain selectively hydrolyses arginyl bonds and constitutes an important clostridial virulence factor (Jozwiak et al. 2005; Manabe et al. 2010). In addition to oxidizing agents, thiol-blocking agents and heavy-metal ions that all inhibit clostripain, good reversible inhibitors have been described, namely, aziridine peptide esters, which are thus promising lead compounds for the development of specific clostripain inhibitors (Schirmeister and Peric 2000; Barrett et al. 2004).

Gingipains are extracellular cysteine proteases (family C25) produced by the oral pathogenic bacterium *Porphyromonas gingivalis*, a major etiological bacterium of chronic periodontal disease. Gingipains comprise two arginine-specific cysteine proteases (RgpA and RgpB) and a lysine-specific cysteine protease (Kgp). They constitute the major virulence factor of this periodontopathogenic bacterium as they are involved in multiple facets of its virulence and survival, including the destruction of periodontal tissues, disruption of the host immune system by inactivation of host proteinase inhibitors and deregulation of several proteinase cascades, as well as acquisition of nutrients required for bacterial growth and survival in the periodontal pocket (Fitzpatrick et al. 2009; Travis and Potempa 2000). Due to their importance in pathogenesis, considerable efforts have been put into discovering or designing specific inhibitors of gingipains. Tetracyclines, inhibitors of prokaryotic protein synthesis, have been shown to have, in addition to their antibiotic activity, inhibitory activity against cysteine proteases through binding to the proteinase outside the substrate binding site (Imamura et al. 2001). Peptidyl chloromethanes have been used as specific Rgp and Kgp inhibitors for their characterization (Potempa et al. 1997), and compound A71561 was shown to attenuate *Porphyromonas gingivalis* virulence through specific Kgp inhibition (Curtis et al. 2002). Based on histatin cleavage specificity, small peptide analogues were designed, which specifically inhibit Rgp and Kgp (KYT-1 and KYT-36, respectively), which display attenuation of several virulence traits of *Porphyromonas gingivalis* (Kadowaki et al. 2004). Chlorhexidine, which has been used in oral healthcare preparations on account of its antimicrobial effects, also inhibits proteolytic activities, including those of gingipains. Moreover, chlorhexidine inhibitory activity against R-gingipains was enhanced by the addition of Zn(II), which has also been used in human oral health care (Cronan et al. 2006).

Metalloproteases are important virulence factors of many primary and opportunistic pathogenic bacteria and cause major infectious diseases such as cholera, salmonellosis, Legionnaires' disease, cystic fibrosis, botulism, tetanus and anthrax (Miyoshi and Shinoda 2000). They have either direct roles in host interaction or indirect roles in processing other important virulence factors. Therefore, much has been invested in the search for an effective protease inhibitor for use in treatment (Jacobsen et al. 2007), but none has yet been developed, which would be used in clinic. Metal chelators, including EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) and 1,10-phenanthroline, inhibit metalloproteases in general. The ubiquitous presence of metalloproteases prevents the use of broad-spectrum inhibitors, and the search for potent and specific inhibitors of individual metalloproteases that could find clinical applications is important.

The most studied bacterial metalloproteases are those of the thermolysin family (M4), including MpI protease of *Listeria monocytogenes*, coccolysin of *Enterococcus faecalis*, hemagglutinin/proteinase of *Vibrio cholerae* and *Helicobycter pylori*, pseudolysin of *Pseudomonas aeruginosa*, aureolysin of *Staphylococcus aureus*, *Legionella pneumophila* protease and λ-toxin of *Clostridium perfringens*. Inhibitors of thermolysin family proteases are of bacterial origin, including those isolated from *Streptomyces*, the small-molecule inhibitor phosphoramidon and protein inhibitor SMPI (*Streptomyces* metalloproteinase inhibitor) of family I36. Another family of inhibitors targeting bacterial thermolysins is family I8 of animal origin (Adekoya and Sylte 2009; Supuran et al. 2002; Rawlings and Barrett 2011).

Of the bacterial metalloproteases, the light chain domains that are zinc metalloproteases (family M27) of tetanus and botulinum neurotoxins (TeNT and BoNTs) from *Clostridium*

tetani and *Clostridium botulinum*, respectively, have also been studied extensively. Various β-aminothiols have been considered as selective BoNT and TeNT inhibitors and have been further developed into strong and selective pseudotripeptide inhibitors of BoNT/B (Blommaert et al. 2004; Supuran et al. 2002). *Clostridium histolyticum* collagenases and their homologues from *Vibrio* (family M9) are very effective in connective tissue degradation and hydrolyse triple helical regions of collagen under physiological conditions. They are targeted for both therapy and diagnosis of clostridial infections, and several types of compounds have been found to inhibit them. However, in addition to bacterial collagenases, they also inhibit vertebrate collagenases (Supuran et al. 2002; Barla et al. 2009).

No potent and selective inhibitor has yet been found for metalloproteases of family M10 from pathogenic bacteria, including serralysin from *Serratia*, *Pseudomonas* and *Erwinia*, aeruginolysin from *Pseudomonas aeruginosa*, mirabilysin from *Proteus mirabilis* and fragilysin from *Bacteroides fragilis*. They are, however, inhibited by protein inhibitors of bacterial origin belonging to family I38 and hydroxamate inhibitors, including batimastat (Supuran et al. 2002; Rawlings and Barrett 2011).

Another group of medicinally important bacterial proteases for which specific inhibitors have not yet been described comprises the metalloexopeptidases, which belong to several MEROPS families (M1, M2, M14, M15, M17, M18, M19, M20, M24, M28, M29, M32, M42, M54, M55 and M61). Of the metallocarboxypeptidases (belonging to families M14, M15, M20, M32) the zinc-containing D-Ala-D-Ala dipeptidase VanX (family M15) has been studied in view of its ability to mediate antibiotic resistance against vancomycin (Crowder 2006). Similarly, the family M19 of membrane dipeptidases includes members that degrade β-lactam antibiotics. Other carboxypeptidases, such as glutamate carboxypeptidases (family M20), have been studied with a view to clinical use in treating different types of cancer (Holz et al. 2003). Bacterial metalloaminopeptidases, which perform essential cellular functions in protein synthesis and maintenance, have been studied as targets for novel antibiotics. A few reviews on inhibitors designed to inhibit bacterial aminopeptidases of families M17 (e.g. leucyl aminopeptidases or LAPs), M1 (alanyl aminopeptidase) and M24 (methionine aminopeptidases) cover the natural and designed compounds that could serve as lead compounds for inhibitors aimed at this group of proteases (Mucha et al. 2010; Holz et al. 2003; Supuran et al. 2002; Rawlings and Barrett 2011). Bacterial metalloproteases that cleave immunoglobulin A (IgA proteinases, families M26 and M64) constitute important colonization factors for several pathogenic bacteria (e.g. *Streptococcus*, *Neisseria*, *Haemophilus*, *Clostridium*, *Prevotella*, *Capnocytophaga*, *Bacteroides*). No strong and specific inhibitor is available for these enzymes. The same is true also for another family of pathogenesis-related metalloproteases (family M23), including staphylolysin from *Pseudomonas aeruginosa* and lysostaphin from *Staphylococcus simulans*. In addition to their function in increasing virulence, staphylolysin and lysostaphin show bactericidal activity against *Staphylococcus aureus* and have been studied with a view to their use in countering drug-resistant staphylococci (e.g. methicillin- and vancomycin-resistant *Staphylococcus aureus*) (Barequet et al. 2009; Desbois and Coote 2011). The metalloprotease anthrax lethal factor LF (family M34) is a component of the anthrax toxin responsible for the major symptoms and death associated with *Bacillus anthracis* infection (Kim and Yoon 2006). An increased interest in anthrax vaccination and treatment methods has been provoked by the use of *Bacillus anthracis* spores as a bioweapon. Lethal factor (LF) inhibitors would provide two-fold protection, namely, in preventing early spore protection in macrophages and, later, inhibiting LF disruption of signalling pathways through inactivation of mitogen-activated protein (MAP) kinase kinases. The search for an LF inhibitor to use in combination with antibiotic treatment is aimed at finding a selective and potent LF inhibitor (Shoop et al. 2005; Turk 2008), which would be non-(cyto) toxic and have good biological stability and bioavailability (Johnson et al. 2009; Kim et al. 2011; Li et al. 2011).

Fungal diseases

The predominant fungal diseases afflict immunocompromised patients and are caused by opportunistic pathogens *Candida* sp. (e.g. *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*) and *Aspergillus* sp. (e.g. *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus calidoustus*), followed by other ascomycetes of genus *Fusarium*, basidiomycete genera *Malassezia*, *Cryptococcus* and *Trichosporon*, and zygomycete genera *Rhizopus* and *Mucor* (Boekhout et al. 2009). The importance of proteases in the pathogenicity of these opportunists is controversial; however aspartic, serine and metalloendopeptidases, as well as aminopeptidases, carboxypeptidases and dipeptidylpeptidases that are secreted by these species, have been proposed as the virulence factors that facilitate colonization and invasion by hydrolysis of host proteins or damage cells and molecules of the host defence system (Segal 2006; Yike 2011). The most studied are the secreted aspartic proteinases of *Candida albicans* (SAPs) (Naglik et al. 2003). Interestingly, the protease inhibitors targeted against the viral aspartic protease used in treating HIV infection also inhibited *Candida* SAPs and reduced occurrence of candidiasis in these patients. Secreted aspartic proteases are thus an important target for the development of new protease inhibitor based compounds for treating candidiasis (Braga-Silva and Santos 2011; Naglik et al. 2004; Dash et al. 2003).

Fungal proteases are also important fungal allergens, and most belong to the serine catalytic type. Addition of protease inhibitor during *Aspergillus fumigatus* and *Aspergillus niger* protease and antigen sensitization attenuated allergic inflammation and hyper-responsiveness in an animal model (Yike 2011). Secreted alkaline serine protease of *Aspergillus fumigatus* was shown to help evade the host immune response by degrading human complement proteins, and it is therefore a good target for drug development (Behnsen et al. 2010).

A network of proteolytic enzymes is very important for the survival of dermatophytes such as *Microsporum canis* and *Trichophyton rubrum*, the specialized pathogenic fungi that infect stratum corneum, nails or hair of healthy individuals. It includes the metalloendopeptidases, fungalysins (family M36), serine endopeptidases, subtilisins (family S8A) and several exopeptidases (dipeptidyl peptidases (family S9), aminopeptidases (family M28) and carboxypeptidases (family S10)) (Monod 2008).

Although proteases are only one of the several groups of virulence factors of pathogenic fungi, they aid in the invasion of tissues and evasion of immune responses. Therefore, specific protease inhibitors aimed at them would constitute a valuable addition to the presently used antifungal drugs that target fungal cell wall and cell membrane integrity or DNA replication—and to which many pathogenic strains have acquired resistance (Marie and White 2009). The class-specific (broad-spectrum) aspartic protease inhibitor pepstatin has been shown to inhibit adhesion of *Candida albicans* and prevent invasion or mucosal tissue damage by inhibiting the SAPs (Naglik et al. 2004). Recently, it has been shown that ergosterol production transcriptional regulator (Sre1), which is activated by a metalloprotease Stp1, is essential for the survival of *Cryptococcus neoformans* in the presence of antifungal drugs that inhibit sterol biosynthesis. Therefore, regulators of the ergosterol pathway, including the metalloprotease Stp1, constitute promising targets for novel antifungal therapeutics to be used in combination with a sterol synthesis inhibitor for treating cryptococcosis in immunocompromised individuals (Bien et al. 2009).

Parasitic diseases

Protozoan parasitic diseases, including malaria, leishmaniasis and African and American trypanosomiasis, are some of the most important infectious diseases in the world, with high mortality and morbidity rates in developing countries. Reasons for their persistence, despite prolonged use of antiparasitic drugs, include their toxic side effects and the increasing emergence of drug resistance. Therefore, research over the past 15 years has been focused on identifying new targets for antiparasite treatment and on developing substances suitable for human therapy. Parasitic proteases constitute one of the very important druggable targets since they are key virulence factors due to their essential roles in cell metabolism and interaction with the host (Zucca and Savoia 2011; Renslo and McKerrow 2006; McKerrow et al. 2008).

Aspartic proteases plasmepsins (family A1), which are important for haemoglobin catabolism in parasites causing malaria (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*), have been targeted for the design of novel antimalarial drugs, i.e. selective protease inhibitors. Although plasmepsin inhibitors (including the broad-spectrum pepstatin) have been shown to have antimalarial effects, the main challenge still remains to design an inhibitor that would be active against different plasmepsins I, II and IV, and the histoaspartic protease HAP involved in haemoglobin degradation, but also inactive against the homologous human aspartic proteases (cathepsins D and E) (Zucca and Savoia 2011; Rosenthal 2003; Ersmark et al. 2006; Gil et al. 2011). In addition to plasmepsins, the cysteine proteases falcipains (family C1), metalloprotease falcilysin (family M16) and aminopeptidases have been targeted for development of new antimalarial protease inhibitors. A synergistic effect has been observed for their combined use (e.g. a cocktail of aspartic and cysteine protease inhibitor) (McKerrow et al. 2008; Rosenthal 2003; Zucca and Savoia 2011; Trenholme et al. 2010).

Cysteine proteases similar to falcipain (family C1) have been associated with the pathogenesis of trypanosomiases—cruzipain of *Trypanosoma cruzi* (Chagas disease) and brucipain or rhodesain of *Trypanosoma brucei* (sleeping sickness). Cruzipain (or cruzain) has been extensively studied and is considered to be a promising target for chemotherapy since it is critical for parasite viability in all stages of infection, especially for nutrition acquisition, tissue invasion and host immune response evasion (Duschak and Couto 2009). An irreversible inhibitor, K777, was effective in pre-clinical models of Chagas disease; however, a safer treatment would be achieved by utilizing a reversible and highly specific cruzipain inhibitor (Beaulieu et al. 2010; McKerrow et al. 2009; Brak et al. 2010). Cysteine proteases have been implicated in the pathogenesis of *Leishmania* species and are major virulence factors as they substantially modify the immune response. A surface metalloprotease gp63 or leishmanolysin (family M8) has been shown to be essential for establishing and maintaining the infection and would make a promising target for development of a selective protease inhibitor for antiparasitic chemotherapy (Olivier and Hassani 2010; Yao 2010). In addition to the computational design, development and optimization of a suitable protease inhibitor, based on the 3D structure of the target protease, the search for novel types of inhibitors from natural sources (Pereira et al. 2011), such as fungi and microbes, is important for identifying new lead compounds.

Serine proteases are also a neglected group of potentially targetable protozoan proteases involved in their pathogenicity, although several subtilisins (family S8) have been described from *Plasmodium falciparum* and *Toxoplasma gondii*, and two oligopeptidases (family S9) from *Trypanosoma cruzi* (McKerrow et al. 2008).

In addition to protozoan parasites, helminths, which include parasitic roundworms (nematodes) and flatworms (trematodes and cestodes), also cause important parasitic infections. Cysteine cathepsins (family C1), which are important for many aspects of the helminth–host relationship, are a potential target for developing antihelminthic drugs. Cysteine protease inhibitors have been shown to impair fecundity of the liver fluke (*Fasciola hepatica*) and blood fluke (*Schistosoma mansoni*) in animal models. However, the similarity of host cathepsins, together with their importance, calls for the design of a very specific and selective protease inhibitor. Alternatively, drug design could exploit bioavailability and pharmaco-kinetic and -dynamic properties to target parasitic proteases preferentially (Robinson et al. 2008). In addition to proteases, parasite-derived protease inhibitors play important roles in manipulating the host immune system and establishing a niche for the successful feeding and reproduction of helminth parasites. Therefore, protease inhibitors are also important targets for immunological control of helminth parasitic infections and make proteases that are insensitive to parasite-derived protease inhibitors valuable candidates for new types of antihelminthic therapy (Knox 2007; Stepek et al. 2006).

Cancer

The ability of tumour cells to invade extracellular barriers and to metastasize to distant sites is associated with the activity of proteases (Kos and Lah 1998). The major molecular mechanism, which involves the active role of various intra- and extracellular proteases, is the dissolution and remodelling of connective tissue and the basement membrane. It includes matrix metalloproteases (MMP), serine proteinases such as urokinase, tissue types of plasminogen activator (uPA, tPA) and plasmin, aspartic proteinase cathepsin D and cysteine proteinases cathepsins B, H, S and L (Schmitt et al. 1992). In addition to extracellular matrix remodelling, proteases regulate several other processes, leading to the progression of malignant disease, such as cell adhesion, migration, differentiation, proliferation and signalling of tumour cells. It is well accepted that tumour-associated proteolytic activity escapes the control of endogenous protease inhibitors and that treatment of patients with exogenous protease inhibitors may suppress the progression of the disease and improve the outcome of cancer patients. However, the treatment should specifically target the tumour-associated proteases that cause harmful actions and not those involved in the numerous physiological processes in cells and tissues. Several protease inhibitors failed in clinical trials due to lack of specificity, resulting in severe side effects and/or lack of clinical benefit in treated patients (Turk 2006; Coussens et al. 2002). New approaches to developing protease inhibitors applicable in therapeutic interventions include structure-based medicinal chemistry and development of molecular systems to deliver inhibitors to the site of action.

Among the small-molecule inhibitors of bacterial and fungal origin, peptidyl aldehydes such as leupeptin and antipain, hexapeptide pepstatin and epoxysuccinyl peptide E-64 and their analogues have been studied as anticancer agents. The thiol-protease specific inhibitor, E-64, originally isolated from *Aspergillus japonicus* (Hanada et al. 1978), has been studied extensively as a potential antitumour agent in cell culture and animal models. Derivatives of E-64, displaying selectivity between different cysteine proteases (Frlan and Gobec 2006), represent the next step towards their application in treating cancer and other diseases. They were designed on the basis of the X-ray crystal structures of individual cathepsins, and the most studied were cathepsin B specific inhibitors CA-074 and CA-030, cathepsin L specific inhibitors CLIK-148 and CLIK-195, and cathepsin X specific inhibitor AMS-36. Cathepsin S specific inhibitor CLIK-060 was designed on the basis of the structure of leupeptin and antipain (Katunuma 2011). Antitumour activity was exhibited particularly by CA-074, a specific inhibitor of the cysteine protease cathepsin B (Johansson et al. 2000), which appears to be crucial for tumour cell invasion (Lah et al. 2006). In animal models, it was shown that CA-074 reduces tumour growth, invasion and angiogenesis of many cancer types, including pancreatic cancer, melanoma and breast tumours, all tested on animal models. The cell permeability of epoxysuccinyl inhibitors was improved by esterification. The esters are less active than free acids; however, in cells, they are rapidly hydrolysed to their active form. Better cell permeability was demonstrated for ethyl ester E-64d and the methyl ester of CA-074, which are also highly soluble and effective for prolonged periods (Frlan and Gobec 2006).

Metalloproteases are an important group of proteases that have been considered extensively as targets for cancer therapy due to the many roles they play in carcinogenesis and tumour invasion, growth and dispersion. However, the diversity of endogenous metalloproteases and their numerous and versatile physiological roles have prevented the use of broad-spectrum metalloprotease inhibitors. Much effort is invested in determining which metalloproteases to target and in designing highly selective and potent protease inhibitors based on the structural characteristics of individual target metalloproteases (Coussens et al. 2002; Bialas and Kafarski 2009; Dorman et al. 2010; Overall and Kleifeld 2006).

Several proteases of the serine catalytic type have also been targeted for the design of specific protease inhibitors for use in cancer treatment, including the urokinase plasminogen activator and matriptase (Abbenante and Fairlie 2005; Bialas and Kafarski 2009; Ulisse et al. 2009). The broad-range microbial inhibitors of serine, cysteine and threonine proteases, leupeptin and antipain, were also shown to inhibit malignant transformation (Vaccari et al. 1999) or tumourigenesis (Hozumi et al. 1972). Threonine catalytic type proteolysis is present in proteasomes, and several chemical classes of natural and synthetic proteasome inhibitors have been considered as anticancer agents because of their preferential antiproliferative and proapoptotic activity on cancer cells (Kisselev and Goldberg 2001; Abbenante and Fairlie 2005; Bialas and Kafarski 2009; Cecarini et al. 2011; Chen et al. 2011).

The aspartic protease inhibitor pepstatin has also been frequently tested as an antitumour agent since cathepsin D was identified as an important tumour promoting factor (Greenbaum and Sutherland 1983; Benes et al. 2008).

Other human diseases

Irregular function of proteases is associated with a variety of other diseases, representing targets for therapeutic application of all catalytic types of protease inhibitors. Small-molecule inhibitors of bacterial and fungal origin, described already in the previous section, particularly epoxysuccinyl inhibitors, have been reported as potential protective agents in autoimmune, neurodegenerative, antiinflammatory and cardiovascular diseases; osteoporosis; muscular dystrophy; diabetes and others. CA-074 and CLIK-148 were demonstrated to cause a switch between Th1 and Th2 T cell response due to the different roles of cysteine cathepsins B and L in antigen processing and presentation (Katunuma 1997). Cathepsin S inhibitor CLIK-060 has been shown to suppress Sj gren syndrome, an autoimmune disease associated with the processing of α-foldin by cathepsin S (Saegusa et al. 2002). The cathepsin X inhibitor AMS-36 reduces the activation of integrin receptor LFA-1 (lymphocyte function-associated antigen 1), a molecule involved in T cell adhesion, proliferation and migration (Jevnikar et al. 2008). LFA-1 overexpression and activation by cathepsin X is typical of autoimmune diseases, particularly psoriasis. The same inhibitor significantly enhanced the proliferation of neuronal cells and neuritogenesis, preventing the processing of neurotrophic factor γ-enolase by cathepsin X (Obermajer et al. 2009). Epoxysuccinyl inhibitors have been tested in several animal models of neurodegenerative diseases. E-64 was shown to restore normal synaptic function in the APP/PS1 mouse model of Alzheimer's disease with overexpressed amyloid precursor protein (APP) and presenilin 1 (PS1) (Trinchese et al. 2008). E-64d and CA-074Me also reduced the accumulation of neurotoxic beta-amyloid peptides in brains, presumably inhibiting cathepsin B involved in processing the amyloid precursor protein (Hook et al. 2007). Aspartic proteases β-secretase (also known as memapsin-2 or BACE1 and belonging to MEROPS family A1) and γ-secretase (composed of two presenilins belonging to MEROPS family A22) are important therapeutic targets for treating Alzheimer's disease, and several compounds designed to reduce their activity are in clinical trials (De Strooper et al. 2010). Another important target of aspartic proteases is renin (family A1), which is part of the complex renin–angiotensin–aldosterone system that regulates blood pressure and electrolyte balance. Several inhibitors have been designed based on the renin structure and activity, and one of them, aliskiren, is the first non-peptide, orally administered, direct renin inhibitor available on the market for management of hypertension (Nguyen et al. 2008; Barrios and Escobar 2010). The very first protease inhibitor used in humans as a therapeutic agent, namely, an inhibitor of metalloprotease angiotensin-converting enzyme (ACE), was also hypertension related. It is an important regulator of the renin–angiotensin system, and ACE inhibitors are used for treating hypertension, but are also implicated in other cardiovascular and renal diseases (Abbenante and Fairlie 2005; Ondetti et al. 1977). The inhibitors of collagenolytic enzymes, such as cathepsins L and K, prevent bone remodelling and can be useful in treatment of osteoporosis. Several synthetic cathepsin K inhibitors are in different stages of clinical trials. In addition to osteoporosis, they are also considered for application in arthritis, atherosclerosis, obesity and cancer (Bromme and Lecaille 2009). Administration of cathepsin L inhibitor CLIK-148 significantly reduced invasion and metastasis formation in bones (Katunuma 2011). E64d, a methyl ester of E64, was tested for treating muscular dystrophy; however, its further development was stopped in phase III due to insufficient effectiveness and hepatic injury in rats (Fukushima et al. 1990). Antipain, leupeptin and pepstatin have also been tested for treatment of muscular dystrophy; however, persuasive benefit was not demonstrated in animal models. Specific inhibitors designed to target the serine aminopeptidase dipeptidyl peptidase IV are in clinical trials for management of type 2 diabetes (Abbenante and Fairlie 2005; Tahrani et al. 2011).

Applications of protease inhibitors in crop protection

Endogenous plant protease inhibitors constitute one of the plant defence strategies against pathogenic, parasitic and herbivorous organisms. They target the important proteolytic virulence factors of phytopathogenic bacteria, fungi, parasites and viruses, preventing their roles in nutrient acquisition and evasion of host defence. Furthermore, they target digestive proteases of herbivorous pests (e.g. insects, mites, slugs), preventing the utilization of food-derived organic nitrogen

for their growth and development (Ryan 1990; Haq et al. 2004). Since pests and pathogens depend on utilization of these proteases, there is a strong selection pressure operating to develop resistance to plant endogenous defensive protease inhibitors (Haq et al. 2004; Jongsma and Beekwilder 2008). Therefore, the search for novel protease inhibitors with potential protective function is very important for the development of environmentally friendly pest and pathogen management strategies. In addition to investigations aimed at augmenting crop endogenous resistance by conventional breeding, there are several protease inhibitors of plant origin that have been used in the preparation of genetically modified crop plants with superior ability for biotic stress resistance (Ferry and Gatehouse 2010).

The use of protease inhibitors for insect pest management has gained most attention. Insect pests cause major economic losses annually, of which lepidopteran (butterflies' and moths') larvae are considered the most destructive. Agricultural pests causing significant economic impact also belong to orders Coleoptera (beetles), Diptera (true flies), Hemiptera (e.g. aphids), Orthoptera (e.g. locust) and Thysanoptera (thrips). They cause either direct damage to crops by feeding or indirect damage by transmitting viral diseases or secondary microbial infections. The most notable for their destructive capacity are the migratory locust (*Locusta migratoria*), several beetles, including Colorado potato beetle (*Leptinotarsa decemlineata*), boll weevil (*Anthonomus grandis*), Japanese beetle (*Popillia japonica*), the western corn rootworm (*Diabrotica virgifera virgifera*) and many species of aphids belonging to all families of the superfamily *Aphidoidea*. Different catalytic types of proteases provide the predominant proteolytic activity in different groups of insect pests. While serine proteases are predominant in digestive proteolysis in most insect species (e.g. Lepidoptera, Diptera), cysteine proteases predominate in Hemiptera, Coleoptera and Thysanoptera. In addition, aspartic and metalloproteases complement protein digestion to different degrees in most insect orders (Terra and Ferreira 1994). Therefore, protease inhibitors targeting different groups of proteases have shown variable antinutritional effects when fed to different insect pests. The catalytic, class-specific, small-molecule protease inhibitors of microbial origin have often been used for proof-of-principle feeding experiments, but protein protease inhibitors were then employed to generate insect-resistant transgenic plants. These were predominantly of plant origin, with a few exceptions of animal-derived protease inhibitor genes (Haq et al. 2004; Dunaevsky et al. 2005; Malone et al. 2008). Other important proteins that have been used for constructing pest-resistant transgenic crops are insecticidal proteins derived from *Bacillus thuringiensis* (*Bt*), and genetically modified maize and cotton varieties that express *Bt* toxins have become an important component in agriculture worldwide and have reduced the use of pesticides and lowered production costs (Ferry and Gatehouse 2010; Kumar et al. 2008).

The involvement of endogenous protease inhibitors in natural plant resistance against herbivores is probably the basis of the adaptation of lepidopteran and coleopteran species to ingestion of protease inhibitors that has been observed for several species (Jongsma and Beekwilder 2008; Wu et al. 1997; Bonade-Bottino et al. 1999; Lara et al. 2000). They circumvent the antinutritional effect of the ingested protease inhibitors either by overexpression of native gut proteases or by production of insensitive proteases; some of which can degrade the ingested protease inhibitors (Ferry and Gatehouse 2010; Jongsma and Beekwilder 2008). Therefore, the pyramiding or stacking of different families of inhibitors to increase the spectrum of inhibitory activity has been shown to have synergistic effects, as well as combining protease inhibitor genes with genes of other insecticidal proteins, namely, lectins, *Bt* or other bacterial toxins and α-amylase inhibitors (Ferry and Gatehouse 2010; Schluter et al. 2010; Christou et al. 2006; Malone et al. 2008). Furthermore, the use of protease inhibitors of microbial and fungal origin could offer superior characteristics, such as stability, resistance to proteolytic degradation and diverse inhibitory patterns, for a more potent antinutritional or insecticidal effect. Only a few examples of utilization of microbial small-molecule inhibitors as antinutritional agents are available, e.g. aminopeptidase inhibitors of actinomycetes amastatin and bestatin against the red flour beetle (*Tribolium castaneum*) (Oppert et al. 2011), aspartic protease inhibitor pepstatin A from actinomycetes against the cowpea bruchid (*Callosobruchus maculatus*) (Amirhusin et al. 2007), the serine and cysteine protease inhibitor leupeptin from actinomycetes against western corn rootworm (*Diabrotica virgifera*) (Kim and Mullin 2003) and cysteine protease inhibitor E-64 from *Aspergillus japonicus* against Colorado potato beetle (*Leptinotarsa decemlineata*) (Bolter and LatoszekGreen 1997). The use of protein protease inhibitors is advantageous since transgenic plants expressing a pest resistance gene in a controlled manner represent a stable and cheap propagation source that would lower the amount of pesticides needed, making plant protection environment friendly. To our knowledge, the only examples of a protein protease inhibitor of microbial or fungal origin as an effective antinutritional agent are the cysteine protease inhibitors macrocypins (family I85) from the edible parasol mushroom (*Macrolepiota procera*), which have been shown to be detrimental to the growth and development of Colorado potato beetle larvae (Istinič et al. 2011). The high capacity for development of resistance to insecticidal proteins in major insect pests drives the search for effective protease inhibitors. Novel protein protease inhibitors aimed at serine proteases

would be useful for management of major agricultural pests such as beet armyworm (*Spodoptera exigua*), cotton bollworm (*Helicoverpa armigera* and *Helicoverpa zea*) and tobacco hornworm (*Manduca sexta*). A combination of serine and cysteine protease inhibitors would be useful against, e.g. boll weevil (*Anthonomus grandis*), cowpea weevil (*Callosobruchus maculatus*) and red flour beetle (*Tribolium castaneum*), and cysteine protease inhibitors would, for example, be useful against the Colorado potato beetle (*Leptinotarsa decemlineata*), western corn rootworm (*Diabrotica virgifera*), banana weevil (*Cosmopolites sordidus*) and pea aphid (*Acyrtosiphon pisum*). Microorganisms that are generally recognized as safe and edible mushrooms offer a valuable source of such novel protease inhibitors that would also be acceptable for use in crops for human consumption.

In addition to insect pests, other herbivores causing significant crop losses worldwide include mites and slugs. In both cases, cysteine proteases constitute the predominant digestive proteolytic activity. Plant cystatins have been shown to be detrimental to mite development and reproductive performance in feeding trials on one of the major mite pests on agricultural crops, the two-spotted spider mite *Tetranychus urticae* (Acari: tetranychidae) (Carrillo et al. 2011). Similarly, the growth of juvenile slugs *Deroceras reticulatum*, the important agricultural and horticultural pest, was significantly reduced when fed with leaf tissue overexpressing a plant cysteine protease inhibitor (Walker et al. 1999). Therefore, protease inhibitors of microbial and fungal origin have great potential for protecting plants from important mite pests and suppressing the growth rates of slug populations, in addition to their antinutritional characteristics for different insect pests.

Another advantage of the application of protease inhibitors in crop protection is that in addition to protection against herbivorous pests, they offer cumulative protection against nematodes and bacterial, fungal and viral pathogens (Haq et al. 2004). The use of protease inhibitors is one of many strategies to protect plants from parasitic nematodes. Their targets are intestinal proteases, mostly of the cysteine catalytic class. Therefore, heterologously expressed plant cysteine protease inhibitors—phytocystatins (family I25)—have been effective to different degrees against beet-cyst nematode (*Heterodera schachtii*), root-knot nematode (*Meloidogyne incognita*), potato cyst nematode (*Globodera pallida*) and burrowing nematode (*Radopholus similis*) (McCarter 2009; Haq et al. 2004). A plant-derived serine protease inhibitor offered satisfactory nematode resistance in transgenic wheat against the cereal cyst nematode (*Heterodera avenae*) (Vishnudasan et al. 2005; McCarter 2009). The antifungal effect of serine protease inhibitors has been determined with plant-derived protease inhibitors that target secreted proteases (mainly families S1 and S8) of phytopathogenic fungi involved in plant cell wall penetration by hyphae (Wong et al. 2010). In addition, aspartic and cysteine proteases that are also fairly widespread (Valueva and Mosolov 2004) represent potential targets for protective protease inhibitors. Similarly, phytopathogenic bacteria secrete proteases involved in pathogenesis, mainly aiding plant cell wall degradation. Together with pectinases, cellulases and hemicellulases, they contribute to massive degeneration of plant tissue, for example, in wilt (e.g. *Ralstonia solanacearum*) and soft-rot diseases (e.g. *Erwinia chrysanthemi*, *Erwinia carotovora*) (Kunkel and Chen 2006). Cysteine proteases belonging to families C48, C55, C58, C70 and C72, which have been implicated in the pathogenicity of many phytopathogens belonging to genera *Pseudomonas*, *Xantomonas*, *Ralstonia*, *Erwinia* and *Pantoea* as effectors of the type III secretion system, constitute potential targets for protective protease inhibitors (Kunkel and Chen 2006; Mosolov and Valueva 2006). The importance of polyprotein processing in replication of viruses, especially those of families Potyviridae and Comoviridae, indicates that protease inhibitors could mediate resistance to plant viruses by inhibiting target viral proteases. Indeed, a rice cystatin (oryzacystatin) expressed in tobacco plants conferred resistance to potato virus Y (PVY) and to tobacco etch virus (TEV) which correlated to increased inhibition of the target papain-like protease (Benchabane et al. 2010; Sudarshana et al. 2007).

In addition to their protective role against biotic stress, endogenous plant protease inhibitors have been implicated in the control of proteolytic systems in plants under abiotic stresses with a dehydration component such as drought, increased salt concentration and freezing (Vaseva et al. 2012). It has been shown that the changes in metabolism triggered by water deficit involve active involvement of regulated proteolysis that assists the protective proteins (dehydrins and chaperones) in the cellular response to the increased levels of denatured, aggregated or oxidatively damaged proteins that accumulate during dehydration stress (Brzin and Kidrič 1995; Benchabane et al. 2010; Bray 1993; Hoekstra et al. 2001; Feller 2004). Overexpression of endogenous protease inhibitors increased resistance to drought stress, as shown for overexpression of the cysteine protease inhibitors cystatins AtCYS1 and AtCYS2 in the model plant *Arabidopsis thaliana* (Zhang et al. 2008) and for overexpression of a serine protease inhibitor OCPI1 (*Oryza sativa* chymotrypsin inhibitor 1) in rice, with the latter showing improved drought resistance in terms of yield loss in the field (Huang et al. 2007). Although protease inhibitors have great potential in protecting crops under abiotic stress conditions, detailed knowledge of the specific roles of different proteases in response to abiotic stress is needed before exogenous protease inhibitors can be used to manipulate and improve drought resistance in plants (Vaseva et al. 2012).

Applications of protease inhibitors in biotechnology and research

Small-molecule protease inhibitors are routinely used as buffer additives when preparing protein extracts, in order to prevent proteolytic degradation during protein purification procedures. Broad-spectrum inhibitors that cover all the different catalytic classes are generally used, including pepstatin A for aspartic proteases, E-64 for cysteine proteases, chymostatin for serine proteases, antipain for cysteine and serine proteases, and leupeptin for cysteine, serine and threonine proteases; all of which were originally isolated from actinomycetes. For inhibition of serine proteases, synthetic protease inhibitors such as AEBSF (pefabloc; 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), the physiologically more stable derivative of PMSF (phenylmethanesulfonyl fluoride), are used. Chelating agents such as EDTA and 1,10-phenanthroline are generally used to inhibit metalloproteases and, for certain applications, inhibitors of actinomycete origin—phosphoramidon (M4 and M13 metallopeptidases inhibited) or bestatin (aminopeptidases inhibited).

Unwanted proteolytic degradation can cause severe reduction in yield of heterologously expressed proteins in various expression systems. Therefore, natural or engineered protease-deficient strains are often used as hosts for bacterial (e.g. *Escherichia coli* BL21), yeast (e.g. *Pichia pastoris* SMD 1163, 1165 or 1168) and filamentous fungal (e.g. *Aspergillus niger prt pep*) expression systems. However, these strains may not exhibit optimal bioprocessing characteristics due to lower fitness traits. Alternative strategies are therefore used for preventing proteolytic degradation of recombinant proteins, including modification of the recombinant protein sequence to remove protease cleavage sites without affecting protein function, expression with a stabilizing fusion partner, optimization of cultivation conditions (pH, temperature, medium composition, bioprocess strategy) and use of protease inhibitors. For secreted recombinant proteins, small-molecule inhibitors can be added to the culture medium to inhibit the predominant secreted proteolytic activity of the host organism that is often of the serine and aspartic catalytic type. Another strategy is co-expression of an appropriate protein protease inhibitor with the recombinant protein, which may, however, influence the yield or complicate the downstream purification procedures (Potvin et al. 2010; Sharma et al. 2009; Rozkov and Enfors 2004). In addition to protection against proteolytic degradation of recombinant protein during the expression process, protease inhibitors as fusion partners have other advantages. One example is the use of the bacterial periplasmic serine protease inhibitor ecotin (family I11) as a vehicle for secretion to the periplasmic space of *Escherichia coli* (Paal et al. 2009). Another example is the serine protease inhibitor from oyster mushroom, the *Pleurotus ostreatus* proteinase A inhibitor 1 (POIA1) (family I9) that serves as an intramolecular chaperone enabling proper refolding of the fused subtilisin protease from inclusion bodies expressed in a heterologous bacterial expression system (Kojima et al. 2005).

The strategy of using co-expression of protein protease inhibitors for reducing proteolytic degradation of heterologously expressed proteins has been successfully implemented in plant expression systems. Preparation of a protease-deficient host is, in this case, not applicable because of the essential roles of proteases in growth and development. Plant expression systems offer many advantages over bacterial and yeast expression systems (e.g. post-translational modification capability) and over animal cell lines (e.g. lower cost and contamination risks) for large-scale recombinant protein production, but have not yet been commercialized due to low levels of protein expression and of heterologous protein accumulation. The latter can be influenced by targeting the expression to specific organelles (e.g. endoplasmic reticulum) or tissues (e.g. seeds and tubers) or by co-expression with stabilizing fusion partners or protease inhibitors. Co-expression of protective protease inhibitors does not have any adverse effects on plant growth and development, as described previously for examples of protease inhibitor expressing, insect-resistant transgenic plants. Furthermore, the heterologously expressed protease inhibitors offer the added advantage of protection against proteolysis also *ex planta*, especially in the early steps of purification of crude protein extracts, thus minimizing the need for addition of protease inhibitors to the extraction buffers (Rivard et al. 2006; Desai et al. 2010; Doran 2006; Benchabane et al. 2008). So far, only protease inhibitors of plant and animal origin have been used as co-expression partners; use of microbial or fungal protease inhibitors could offer superior protective characteristics. However, the properties of the protein of interest and of the selected host plant species influence the selection of an appropriate protective protease inhibitor, and the choice still has to be made on a case-by-case basis.

Another important biotechnological application of protease inhibitors is in protein purification, where they can be used as ligands in affinity chromatography. Affinity chromatography is a simple one-step purification method of a molecule from a complex mixture based on specific and high affinity binding to a ligand immobilized on a solid support. Reversible protease inhibitors of microbial origin are excellent ligands for purification of their cognate proteases by affinity chromatography. Depending on the target protease, inhibitors with broad range or very specific inhibitory spectrum can be selected for a ligand. Attention must be paid to the strength of the inhibitor as purification is not possible when the binding is too weak (no binding) or too strong (ineffective elution) (GE 2007). The advantage of using microbial and fungal protease inhibitors is that many

of them display unique inhibitory profiles and resistance to proteolytic cleavage, as well as high thermal and broad pH range stability, with the latter being very convenient since harsh conditions may be used for immobilization to the matrix as well as for the several cycles of elution steps, usually involving extreme change in pH and/or ionic strength. The broad-range inhibitor of pepsin-like aspartic proteases, pepstatin A, has been used for purification of aspartic proteases from several different sources, including higher fungi (Sabotič et al. 2009a), plants (Payie et al. 2000) and insect recombinant enzyme expressed in a bacterial expression system (Volkov et al. 2004). Several different types of inhibitor have been used for purification of serine proteases, including the synthetic inhibitor benzamidine and plant- and animal-derived protease inhibitors of different families (Polanowski et al. 2003). Differences in the inhibitory spectra of immobilized protease inhibitors can be used in serial affinity chromatography, where, first, a broad-range protease inhibitor can be used to purify proteases from a crude protein extract, followed by the use of a highly specific inhibitor for isolation of a single protease.

Conclusions

The microorganisms of prokaryotic domains archaea and bacteria and of the kingdom of fungi, including higher fungi or mushrooms, constitute important sources of protease inhibitors. Microbial protease inhibitors are versatile in their structures and mechanisms of inhibition in ways that differ from those of other sources. They have therefore found countless applications in the fields of medicine, agriculture and biotechnology. The diversity of processes in which proteases are key players, together with their multiplicity, drives the search for further novel protease inhibitors that could find applications, directly or as leads in structure-based design. The number and diversity of proteases found in microorganisms (Rao et al. 1998) and higher fungi (Sabotič et al. 2007b) makes them a virtually inexhaustible source of novel protease inhibitors with unique features.

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