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Review

# Proteinaceous cysteine protease inhibitors

**G. Dubin**

Faculty of Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Krakow (Poland),
Fax: +48 12 664-6902, e-mail: gdubin@mol.uj.edu.pl

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**Abstract.** Studies of proteinaceous cysteine protease inhibitors originated with the discovery of cystatins in the 1960s. Since that time, a rich and fascinating world of proteins that control and regulate a multitude of important physiological processes, ranging from the basics of protein turnover to development and brain function, has been uncovered. Failures in such important and complex systems inevitably lead to pathologies. Many threatening diseases such as cancer or neurological disorders, to mention only some, are attributed to deregulation of protease-inhibitor balance. Moreover, important aspects of infection pathology and host defense rely on proteolysis and protease inhibition. Recent advances in the field of protease inhibitors have drawn attention to the possible use of this collected knowledge to control related pathological processes. This review attempts to familiarize the reader with proteinaceous cysteine protease inhibitors by providing an overview of current knowledge. The work primarily highlights biological processes in which the inhibitors are involved and focuses on pathologies resulting from aberrant protease-inhibitor balance, pointing out emerging possibilities for their correction.

**Key words.** Protease; proteinase; peptidase; inhibitor.

## Introduction

The recent decade has witnessed tremendous development in the field of proteinaceous cysteine protease inhibitors. Though the prototype cystatins discovered in the 1960s remain the best-characterized group, several new large and a few smaller families are now recognized. Accordingly, the number of known physiological processes involving these proteins is rapidly increasing. In fact, it seems that wherever proteases are involved, proteinaceous regulators/protectants also appear on the stage. The role of such systems is best appreciated when the balance becomes upset. Consequently, deregulated proteolytic activity has been demonstrated in a number of diseases, such as cancer or neurodegenerative disorders, to mention a few. Achievements of genetics, especially targeting of specific genes and transgenesis, made it possible to prove or reject many previously developed concepts and to gain better insight into the true role of systems studied. In some cases it was possible to generate a comprehensive overview by merging separate reports into a consistent description of regulated pathways. However, our understanding of many underlying processes is still only superficial. The inhibitors are not only important in regulating endogenous protease activity. Many pathogens, including viruses, bacteria and parasites, use cysteine proteases to evade target organism defense mechanisms, which points to the general protective role of host inhibitors. In turn, the pathogens themselves use protease inhibitors to evade the host defense system. This brief overview illustrates a huge potential for the application of our increasing knowledge, and hence the academic and commercial interest in protease inhibitors and proteases.

Apart from opening opportunities, the increasing amount of data available also poses a problem of efficient mining of resources. Fortunately, efforts have been made by many researchers to develop a uniform classification of protease inhibitors. Recently, the starting point for queries and further contribution to classification of protease inhibitors has been developed. The MEROPS

database (merops.sanger.ac.uk), which previously listed only proteases, has launched a new service aimed at providing comprehensive information and uniform classification of protease inhibitors [1].

This review endeavors to introduce the reader to the fascinating world of cysteine protease inhibitors by giving a brief overview of the current knowledge, trends and emerging possibilities. Because of space limitations, no attempts were made to completely cover all topics or to discus all aspects of the physiological roles of protease inhibitors. In large families encompassing many homologous proteins, the focus is mostly on human inhibitors. A multitude of structural information available on cysteine proteases and their inhibitors is only briefly mentioned to reserve space for discussion of the physiological relevance of the systems under study (for a recent review on structural studies see [2]).

## Cystatins

The term 'cystatins' refers to a superfamily of cysteine protease inhibitors encompassing a large number of homologous proteins active against papain family proteases (MEROPS family C1; distinct representatives may also inhibit members of other families, see below). Chicken egg white cystatin, which was described in the late 1960s, is the prototype of the group. Today, representatives ranging from protozoa to mammals are known. Twelve functional cystatins from humans have already been described. They divide into three groups (types) based on distinct structural details, but also reflecting their distribution in the body and physiological roles. Type 1 cystatins are polypeptides with ~100 amino acid residues which possess neither disulphide bonds nor carbohydrate side chains (cystatins A and B). They are found mainly intracellularly, but can also appear in body fluids at significant concentrations. Type 2 encompasses cystatins C, D, E/M, F, G (CRES), S, SN and SA characterized by two conserved disulphide bridges, larger size (~120 residues), and a presence of a signal peptide for extracellular targeting. Cystatins belonging to this group are found in most body fluids. Finally, type 3 contains kininogens, high molecular mass proteins with three tandemly repeated type 2-like cystatin domains, only two of which, however, are able to inhibit cysteine proteases. Kininogens are intravascular proteins of blood plasma.

Elucidation of a tertiary structure of several representative members of the family defined a characteristic 'cystatin fold'. The mechanism of C1 peptidases inhibition was demonstrated by the X-ray structure of papain complex where a tripartite edge of a cystatin molecule occupies the active site cleft of a target enzyme preventing substrate entry [3].

In general, cystatins function as a protection against lysosomal peptidases released occasionally at normal cell death or phagocyte degranulation, or 'intentionally' by proliferating cancer cells or by invading organisms, such as parasites (reviewed in [4]).

### Cystatin A (Stefin A)

Cystatin A, the type 1, 98-amino acid residue protein is encoded on chromosome 21 in humans. The tertiary structure of the protein has been determined by nuclear magnetic resonance (NMR) spectroscopy [5]. The expression pattern of cystatin A is quite restricted, with especially high levels in skin and some blood cells. In skin the protein takes part in the epidermal barrier formation. The boundary between body and environment is constantly maintained by reproduction of inner living epidermal keratinocytes which undergo a process of terminal differentiation and then travel to the surface as interlocking layers of dead stratum corneum cells. Much of the barrier function is provided by a cornified cell envelope (CE), an extremely tough protein/lipid polymer structure formed just below cytoplasmic membrane, which subsequently resides on the exterior of the dead cornified cells. The protein part of CE is formed by cross-linking of mainly involucrin, loricrin and small proline-rich proteins by both disulphide bonds and $N^{\varepsilon}$-($\gamma$-glutamyl) lysine isopeptide bonds formed by transglutaminases [6]. Cystatin A has been identified as a minor (2–5%) cross-linked component of CE and suggested to contribute to bacteriostatic properties of skin [7–9]. Indeed, Takahashi et al. [10] were able to demonstrate that cystatin $\alpha$ (a rat counterpart of cystatin A) depleted rat skin is more sensitive to *Staphylococcus aureus* colony formation compared to normal skin.

### Cystatin B (Stefin B)

A gene encoding the 98-aa cystatin B maps to chromosome 21 in humans. The tertiary structure of this type 1 cystatin has been elucidated by the X-ray crystallography [3]. The protein, found mainly intracellularly, is widely distributed among different cell types and tissues in mammals. As such, it is regarded as a general cytosolic inhibitor, probably protecting the cell against leakage of lysosomal enzymes (cystatin B is a tight-binding reversible inhibitor of cathepsins B, H, L and S). Nevertheless, loss-of-function alterations in the gene encoding this ubiquitous protein lead to disorders only in a very selective population of cells, suggesting that genetic redundancy of cystatins may largely act to compensate for its function. The principal cytopathology in cystatin B-deficient mice appears to be an apoptotic loss of cerebellar granule cells [11], while less pronounced gliosis and apoptotic neuronal loss are observed in other parts of brain [12].

Decreased levels of cystatin B activity in humans lead to a type of progressive myoclonus epilepsy (PME), the Unverricht-Lundborg disease (EPM1; OMIM 254800), while null phenotypes were not reported. EPM1, the autosomal, recessive neurodegenerative disorder, is caused in most cases by dodecamer repeat expansion in the promoter region or rarely by point mutations in the coding region (OMIM 601145) [13]. Unlike other PMEs, EPM1 is not associated with inclusion bodies or mitochondrial defects. The most consistent pathological findings in the disease are a marked loss of Purkinje cells in the cerebellum, neuronal loss in medial thalamus and spinal cord, and proliferation of Bergmann glia. EPM1 onset occurs at 6–14 years of age and shows a phenotype consisting of myoclonic seizures and progressive neurologic decline. A comparable phenotype develops in cystatin B-deficient mice. The role of cystatin B in the maintenance of normal neuron structure and survival is well documented. However, neuronal cell death seems to be independent of epileptic seizure events in the mouse model since the latter, unlike the former, depend on genetic background [12]. It remains unclear how the loss of cystatin B activity causes decreased neuron survival (increased apoptosis) and how it may be linked to hyperexcitable neurons in EPM1.

**Cystatin C**

Cystatin C is the most thoroughly studied mammalian cystatin, being the most abundant extracellular inhibitor of cysteine proteases. It was originally described in 1982 by Grubb and Löfberg [14]. The gene encoding the protein is located in cystatin multigene locus on chromosome 20. Mature human cystatin C is composed of 120 amino acid residues, but is synthesized as a preprotein with a 26-residue signal peptide. The protein is produced by a wide variety of tissues and has the most widespread body distribution in the group, being found in all body fluids. Concerning significant concentrations and good inhibitory properties the protein can be viewed as a major extracellular cysteine protease inhibitor of mammals. Cystatin C has been described accordingly to its ubiquitous distribution as an 'emergency' inhibitor able to scavenge any cysteine peptidases released to body fluids [15]. Nevertheless, cystatin C-deficient mice show no gross pathological abnormalities, and the phenotype manifests itself only when challenged with tumor cells, where decreased metastasis is observed. Unfortunately, different challenge conditions were not reported [16].

A point mutation in the cystatin C gene is responsible for hereditary cystatin C amyloid angiopathy (HCCAA; OMIM 105150). This dominantly inherited Icelandic type of amyloidosis results in paralysis and development of dementia due to multiple strokes and, generally, death from cerebral hemorrhage before 40 years of age. The L68Q point mutation [17, 18] results in abnormal protein aggregation responsible for the pathology – deposition of material with characteristics of amyloid in vessels (mainly mutated cystatin C aggregates). Since the aggregation increases significantly with temperature, it was suggested that medical intervention to abort febrile periods in carriers of the disease might reduce the formation of L68Q cystatin C aggregates [19]. The effects of such treatment were, however, not reported. The inhibitory activity of the mutant form remains unaffected, though plasma levels, and therefore total inhibitory pool, are decreased. Nevertheless, it seems unlikely that increased proteolysis associates with this primarily conformational disease. Further concerning the cystatin C-related abnormalities, Shi and colleagues [20] demonstrated decreased inhibitor levels in arteriosclerosis and abdominal aortic aneurysm, inflammatory diseases that involve extensive extracellular matrix degradation and vascular wall remodeling. The observed decrease in cystatin C seems, however, an effect of the inflammatory process rather than a causative factor, nevertheless contributing to the vascular injury. The authors demonstrated that proinflammatory interferon $\gamma$ (IFN-$\gamma$)stimulates elastolytic activity production by arterial smooth muscle cells, while anti-inflammatory tumour growth factor $\beta_1$ (TGF-$\beta_1$), having a stimulatory effect on cystatin C production, is decreased in affected subjects, correlating with decreased inhibitor production [20]. Interesting reports on antiviral activity of cystatin C exist, though the mechanisms involved have not been elucidated [21, 22].

**Other human cystatins**

All type 2 cystatin genes are grouped in a cystatin multigene locus on chromosome 20 (with the exception of cystatin E/M located on chromosome 11).

Cystatin D is found in saliva and in small amounts in tears [23]. In the light of current data, cystatins D, S, SA and SN can be seen as specialized glandular inhibitors. All these proteins most probably play a protective role against endogenous and/or pathogen-derived proteases. Interestingly, cystatins C and D were shown to inhibit coronavirus replication in vitro, demonstrating their possible role in protection against respiratory infections (mostly common colds) [22, 24]. Such a role seems especially attractive for further investigation, keeping in mind the threat of coronavirus infection caused severe acute respiratory syndrome (SARS).

Salivary cystatins S, SA and SN share high sequence identity (90%) and were all originally isolated from saliva (after which they received their names). Later the proteins were also shown in tears, urine and seminal plasma. The gene encoding cystatin S contains regions that resemble those of other hormone-responsive eucaryotic genes. A complex neural, hormonal and developmental regulation

of salivary cystatin genes was consistently demonstrated [25]. To mention several examples of the protective role of those proteins against exogenous proteases, the level of salivary cystatins is elevated during experimental *Trypanosoma cruzi* infections in rat [26]. They also inhibit cysteine protease activity and growth of *Porphyromonas gingivalis* [27]. The best demonstration of the protective role of salivary cystatins, however, comes from an experiment in which papain is administered to the oral cavity of rats, resulting in a dramatic increase in cystatin S levels [28].

Cystatin E/M expression is largely limited to epithelial cells, and as with cystatin A, it can be a substrate for transglutaminases [29]. It is downregulated in mammary tumour cells compared to surrounding ones [30]. The protective role of the protein against neoplasia was studied in greater detail by Shridhar and colleagues [31]. In contrast, upregulation in metastatic compared to primary cells in oropharyngeal squamous cell carcinomas was reported and proposed to promote metastasis by rescuing tumour cells from tumour necrosis factor (TNF)-induced apoptosis [32]. Further connecting cystatin E/M function with the epithelium, a locus responsible for spontaneous harlequin ichthyosis in mice was identified as nonsense mutation of the cystatin E/M gene. The defect in the homozygous state is characterized by anomalous epidermal cornification, abnormal hair follicles and juvenile lethality due to dehydration. The observed abnormality was proposed as a model of human harlequin ichthyosis, a recessive genetic disorder characterized by similar symptoms (OMIM 242500) [33]. Later, however, the cystatin E/M gene was excluded as a major candidate for the disease in humans [34]. Nevertheless, interesting findings arise from investigations in model mice. The observed ichthyosis phenotype is associated with excessive cross-linking of epidermal components by transglutaminase 3 (TGase3). The activation of the latter was linked to the increased activity of legumain, which in turn processes cathepsins, enzymes that directly activate TGase3. Surprisingly, the role of cystatin E/M is not to regulate cathepsins but legumain, an enzyme belonging to the CD clan, standing at the top of the cascade (for more information on cystatin inhibition of clan CD enzymes see below) [35]. It cannot be excluded that cystatin E/M deficiency plays a role in human cornification disorders other than harlequin ichthyosis.

Cystatin F is expressed mainly in haematopoietic and blood cells of the immune system [36, 37]. The expression is readily regulated during differentiation. Unlike other type 2 cystatins, high levels are found intracellularly. The protein is also secreted and found in blood plasma, though in small amounts. The intracellular compartment where cystatin F is localized is subject to discussion. The inhibitor seems to be at least in part directed to granules, possibly lysosomes. Taken together, the expression pattern, inhibitory profile and intracellular localization predispose cystatin F as possible regulator of antigen processing and/or presentation [38–40]. Levels of messenger RNA encoding the protein are elevated in some metastatic cancer cell lines. Antisense strategy demonstrated that switching off the expression decreases the metastatic potential of these tumour cells. Since the abnormally activated proteases are generally attributed to metastatic potential, the above findings associating cystatin F overexpression with metastasis together with some reports suggesting a similar action of cystatin E/M and decreased metastasis observed in cystatin C-deficient mice (see above) seem contrary to the common concept and await deeper investigation of the underlying mechanisms [41].

**Kininogens**

A single gene encoding human kininogen is located on chromosome 3. Due to alternative splicing, two forms differing by the length of C-terminal region are present in plasma. The proteins are designated H- and L-kininogen, respectively, due to their higher and lower molecular weights. Kininogens, like most intravascular proteins, are expressed in liver. Each molecule carries a triple repeat of cystatin type 2-like motif in the N-terminus, two of which are active against cysteine proteases (domains 2 and 3). Domain 2, moreover, is able to inhibit calpain. Due to high levels of the protein in plasma, kininogens constitute a major cysteine protease inhibitory activity in the circulation. Smaller amounts are found in other body fluids, and, accordingly, in secretions and the extracellular space in tissues single-domain cystatins constitute the predominant inhibitory activity against cysteine proteases [42].

Most research concerning kininogens concentrates on the kallikrein-kinin system, where bradykinin, a small peptide released from kininogens, plays a central role. In the classical view, the system is activated on negative surfaces (such as glass) simultaneously with the intrinsic pathway of the blood coagulation cascade by the action of factor XII. In short, activated factor XII (XIIa) releases kallikrein, which in turn cleaves kininogen to release a signaling molecule – bradykinin. Recently, physiologically relevant triggering has been described, discussion of which, however, remains beyond the scope of this review (for more information see [43]). Regardless of the pathway utilized, bradykinin is released. The molecule mediates, among others, smooth muscle contraction, vascular permeability increase (inflammatory exudation) and pain production (for detailed information see [44]). Kininogens are, moreover, active in a number of processes involved in the regulation of vascular biology, such as vascularization, endothelial cell apoptosis, interference with thrombin-mediated platelet activation and others which are understood less clearly.

Though kininogens, as presented here, play multiple biological roles, their deficiencies do not produce pronounced phenotypic effects. Several asymptomatic cases of H-kininogen deficiencies in humans have been described, the first known being the Fitzgerald trait [45]. Even more pronounced is the fact that a complete deficiency of both H and L kininogens, as seen in the Williams trait, is also asymptomatic [46]. A Brown Norway-Katholiek rat strain carries a congenital kininogen deficiency and is widely used as a model of such an abnormality, though the production of a third form of kininogen (T kininogen), not found in humans, is normal in that strain. As in human counterparts, the Katholiek strain does not show any physiological difference from parental, normal kininogen-producing strain. The most pronounced manifestations of the described deficiencies include abnormalities in surface-activated coagulation and fibrinolysis (however, not associated with bleeding or thrombosis), and in kinin formation. No pathologic processes related to abnormal protease activity associated with the lack of kininogen inhibitory activity have been described.

**Inhibition of legumain and calpain**

In general, cystatins are characterized as inhibitors of C1 family proteases; however, separate cases of cystatin fold adaptation to the inhibition of cysteine proteases belonging to other families have been reported.

Legumain, a C13 family cysteine protease, is potently inhibited by cystatins C, E/M and, with a higher $K_i$, by cystatin F. Interestingly, it has been demonstrated that the binding site for legumain is distinct from that of papain. A ternary complex may be formed allowing the cystatin to inhibit both proteases simultaneously [47]. Moreover, the main physiological function of cystatin E/M seems to be legumain and not C1 family protease inhibition (see above).

The calcium-dependent cysteine proteases – calpains (family C2) – are inhibited by the second cystatin domain of kininogens. Due to exclusively intracellular localization of calpains, the ability of intravascular kininogens to inhibit those enzymes is thought to serve as a protection against their activity at accidental release in pathological states.

**Chagasin family**

Protozoan parasites transmitted by insect bites, such as *Trypanosoma cruzi* (a causative agent of Chagas' heart disease in Latin America), *Trypanosoma brucei* (responsible for sleeping sickness in sub-Saharan Africa), *Leishmania* sp. (leishmaniasis) and *Plasmodium* sp. (malaria) produce large quantities of papain-like (family C1) lysosomal proteases. In plants and mammals, such proteases – cathepsins – are regulated by endogenous inhibitors of the cystatin family. A search for similar regulators of parasite cysteine proteases resulted in the discovery of a novel inhibitor family distinct from cystatins and other known groups. Chagasin was the first described member of the group after which the family was named. Chagasin is a *T. cruzi* inhibitor of the endogenous cysteine protease cruzipain [48]. Subsequently, homologues were identified in the genomes of all mentioned parasites as well as in bacteria [49]. While genomes of all but one of those organisms encode cruzipain-like proteins, the function of *Pseudomonas aeruginosa* chagasin homologue remains puzzling since the bacterium does not seem to produce any family C1 proteases. Some of the identified proteins were recombinantly expressed and demonstrated to inhibit papain-like cysteine proteases [50]. In *T. cruzi* and *Leishmania mexicana* the production of inhibitor correlates inversely with the expression of endogenous proteases, while other species were not tested. Chagasins are predicted to possess an all-$\beta$ immunoglobulin-like fold and, though not homologous to cystatins or thyropins, were proposed to have convergent trends in the mode of interaction with cysteine proteases. No experimental evidence is available, however.

Chagas' disease with 16–18 million cases worldwide and 21,000 deaths annually, sleeping sickness affecting annually about half a million and causing 66,000 deaths, leishmaniasis averaging 12 million cases and 57,000 annual deaths and finally malaria with 273 million cases annually causing over 1 million deaths per year (World Health Organization, www.who.int) cause serious public health problems in many countries, especially since prevention is difficult. Moreover, only a few effective drugs are available, which often cause considerable side effects. Furthermore, rapidly advancing drug resistance calls for development of new therapeutics. Cysteine proteases have been suggested as suitable targets since the inhibition of these enzymes has proven effective against protozoan parasites in model infections ([51] and references therein). The compounds tested, however, require further optimization to decrease toxicity and improve bioavailability and specificity, which may be, at least partially, facilitated by structural studies on the chagasin family inhibitors.

**Clitocypin**

Clitocypin has been isolated in large quantities from fruit bodies of an edible mushroom *Clitocybe nebularis* by affinity chromatography on carboxymethylpapain Sepharose. It is the first proteinaceous inhibitor characterized from higher fungi. The protein forms a non-covalent ho-

modimer inhibiting C1 family proteases: papain, cathepsins L and B and bromelain. A 1:1 inhibitor dimer/enzyme binding ratio was observed, suggesting that either only half of the monomers are active or binding of one domain hinders the other. The amino acid sequence of clitocypin has been determined both by direct sequencing and by gene cloning. It shows no obvious similarity with any known cysteine protease inhibitors or other proteins beside a lectin-like protein from the mushroom *Pleurotus cornucopiae*. The homology is, however, low and no hemagglutinating activity has been demonstrated for clitocypin [52]. The inhibitor is quite resistant to heat denaturation, which corresponds with a high $\beta$-structure content proposed on the basis of circular dichroism (CD) and Fourier transform infrared (FTIR) spectoscopy measurements [53]. The physiological target for clitocypin is not known. Control of endogenous proteases seems unlikely given the high amounts of inhibitor. Rather, the proposed protection against pathogen infection or predation by insects [52] seems more probable, though speculative. Up to now no further homologues have been identified.

## Staphostatins

Specific inhibitors of staphylococcal cysteine proteases (staphostatins) constitute yet another, recently described, novel inhibitor family. Unlike chagasins and clitocypin, however, detailed structural characterization has been accomplished, and clues for the function are available. Three members of the family have been described – staphostatins A and B from *Staphylococcus aureus* and staphostatin A from *Staphylococcus epidermidis*. Though, staphopains, the target enzymes, belong to the CA clan, no other tested members were inhibited. The specificity is even more stringent since a single inhibitor affects only one of three characterized staphopains (only *S. aureus* staphostatin A inhibits both *S. aureus* and *S. epidermidis* staphopain A). The inhibited protease is encoded in one operon with the inhibitor, strongly suggesting that the latter molecules were designed by nature to specifically control the activity of coexpressed enzymes [54, 55].

Structures of both *S. aureus* staphostatins as well as of staphostatin B/staphopain B complex have been solved, demonstrating a fold and mechanism of action previously not observed among cysteine protease inhibitors. Staphostatins are $\beta$-barrels, structurally resembling lipocalins, while the mode of action brings to mind the 'standard mechanism' utilized by some serine protease inhibitors. The staphostatin polypeptide chain spans the active site of the protease in a substrate-like mode, but remains uncleaved due to a distinct disposition of P1 glycine. Such a mechanism was confirmed by site-directed mutagenesis since substitutions of P1 glycine convert the inhibitors into substrates. Unfortunately, the structures have not explained the mechanism of strict specificity determination. Since staphopains are considered important virulence factors, it was suggested that structural studies performed may facilitate the development of low molecular weight inhibitors possibly effective in the struggle against staphylococci [56–58].

Staphopains are secretory enzymes, and staphostatins were demonstrated to reside intracellularly. The role for the latter was therefore proposed as protection of cytoplasm against misdirected staphopains accidentally appearing therein. Indirect evidence for such a role comes from studies on staphopain expression in *Escherichia coli*. The enzyme is toxic and may be produced only while coexpressed with the inhibitor [59]. Recently, direct evidence was presented by construction of a staphostatin B knockout strain which showed a markedly decreased growth rate [personal communication]. The suggested protective role, however, does not explain the need for extraordinary specificity.

## Calpastatin

Calpastatin is a specific inhibitor of calcium-dependent cysteine proteases – calpains. It does not inhibit any other cysteine proteases tested to date. Likewise, activity of calpains, is regulated only by calpastatin. (Though kininogens are also able to inhibit calpain proteases, their extracellular localization excludes them as regulators of intracellular calpains, and they are thought to serve only as protectants against accidental calpain release.) Considering the exclusive interactions, the in vivo occurrences of the proteins may not be viewed otherwise as $Ca^{2+}$-dependent proteolytic system (CaPS). Description of such system requires equal attention to both partners. Calpains will, therefore, be briefly discussed here together with calpastatin, stressing the contexts in which actions of the latter were directly investigated.

Calpains are intracellular, non-lysosomal, $Ca^{2+}$-dependent cysteine proteases. Two major isoforms, μ- and m-calpain (characterized, respectively, by μ- and m-molar $Ca^{2+}$ concentration dependence for activity), are ubiquitously distributed in all kinds of mammalian cells, whereas at least 12 tissue/cell-specific isoforms are also present. Most studies performed up to date concentrate on the former two proteins, while little is known about the latter. Calpains consist of an 80-kDa catalytic subunit (domains DI through DIV) and a 28-kDa regulatory subunit (domains DV and DVI). The large subunits of different calpains are products of distinct genes. Encoded polypeptides share ~ 60% sequence identity. Small subunits are identical. DII is a papain-like protease domain. DIV and

DVI belong to a family of $Ca^{2+}$-binding proteins with five EF-hands [60]. Different autolysis products are frequently found and are indicative of the activation of the system. The process is specific and limited, and results in a decrease of calcium levels necessary for half-maximal activity.

Calpastatin shares no sequence homology to any known proteins. It is encoded by a single gene located on chromosome 5 in humans. The corresponding protein is found in a number of different isoforms resulting from the use of divergent promoters and alternative splicing mechanisms. The role for such diversity remains largely unknown. High molecular weight species contain an N-terminal L domain (involved in regulation of L-type $Ca^{2+}$ channels) [61], and four repetitive inhibitory domains, each having three conserved regions termed A, B and C. Regions A and C bind to the calmodulin-like domains DIV and DVI, respectively, potentiating the inhibitory activity of calpastatin, while B binds to, and directly inhibits, the protease DII [62]. A synthetic peptide corresponding to the B region is a self-sufficient inhibitor of calpain. Lower molecular weight isoforms partially lack N-terminal parts. Testis-specific calpastatin consists of a T, instead of an L, N-terminal domain responsible for membrane association, with a part of domain II and entire domains III and IV. Moreover, multiple limited proteolysis products are detected upon system activation (due to calpain action) and as a result of proteolysis by caspases. At least some of these fragments retain inhibitory activity.

Though the intracellular CaPS attracted much attention over the years due to its ubiquitous expression within all mammalian cells and considerable pleiotropic role, there are still many questions, including some basic uncertainties, while well-established mechanisms are few. This review does not pretend to comprehensively deal with all CaPS-related problems and will only give an outline of current knowledge and enigmas (for detailed information see [63]).

In general, CaPS is involved in limited proteolysis of cellular components. As such it has been implicated in a number of basic processes, including signal transduction, apoptosis, proliferation and differentiation, cytoskeleton remodeling and others. In order to allow calpain to act in a large excess of calpastatin normally occurring in most cells, the protease and the inhibitor seem to depend, at least partially, on subcellular sequestering/relocation mechanisms. In unstimulated cells the CaPS components have a widespread cytosolic distribution; however, in some cells calpastatin was found in an aggregated state in granule-like structures, not surrounded by membranes, close to the nucleus. Phosphorylation on serine residues was suggested to play a role in such sequestration. Conversely, dephosphorylation resulted in redistribution of soluble inhibitor species [64]. Reports exist that phosphorylation also affects the inhibitory activity of calpastatin towards some calpain species [65]. Calpain, on the other hand, in response to some signals concentrates near the membrane out of the reach of calpastatin, while phospholipid interaction is a further activating process. Moreover, calpastatin degradation is observed during activation of the proteolytic system and by the action of caspases. However, whether this inactivates the inhibitor or only releases single inhibitory domains remains undetermined. It must be remembered, however, that reports counter to those presented above also exist, and different regulatory mechanisms have been proposed. For now, we are far from understanding the in vivo regulation of CaPS.

The calpain-calpastatin proteolytic system participates in many processes crucial to normal function and survival of the organism which is best demonstrated by gene disruption in mice. The calpain small subunit homozygous knockout is embryonically lethal (since different calpains use the same small subunit, such a knockout expresses no calpain activity) [66, 67]. On the other hand, mice deficient only in μ-calpain develop normally. The only obvious manifestation of the latter deficiency is platelet dysfunction, which is surprisingly not associated with increased bleeding times. Such a mild phenotype suggests the possibility of deficiency compensation by different calpains [68].

Several functions of CaPS have been firmly established. However, though calpains were demonstrated to cleave a large number of proteins in vitro, their in vivo substrates and the pathways involved await thorough characterization. The role of calpain in cytoskeleton/membrane attachment remodeling is well-documented. Myoblast fusion is a widely exploited model. During muscle development myoblasts fuse to myotubes, which requires extensive remodeling. Throughout the process calpastatin levels decrease transiently at least partially due to degradation by caspase-1 [69]. Calpain antisense or microinjection with calpastatin prevents fusion [70]. Moreover, other studies demonstrate that CaPS plays an important part in membrane fusion in general (neural vesicle exocytosis, platelet and red cell aggregation, fertilization) [71]. Furthermore, CaPS involvement in cytoskeleton remodeling during migration and adhesion (focal adhesion disassembly) is demonstrated by fibroblasts derived from calpain small subunit knockout mice, which exhibit impaired migration, and by engineered calpastatin overexpression, reducing the ability of NIH-3T3 cells to spread [72, 73]. CaPS activation was implicated in certain apoptotic pathways since synthetic calpain inhibitors and injection or overexpression of calpastatin rescues some apoptotic cells [63, 74]. The decrease in calpastatin levels consistently enhances apoptotic cell death. Moreover, it was shown that calpastatin is cleaved by caspases, suggesting a possible intensifying mechanism [75]. CaPS

was demonstrated to play a role in long-term synaptic potentiation, a process underlying memory formation [76]. Involvement in proliferation and differentiation [77] as well as in cell cycle control/oncogenic transformation and a role in invasiveness were also reported [78, 79]. Studies on the influence of the system on signal transduction/gene expression are available [80].

Imbalance in the CaPS, mostly in the direction of excessive proteolysis, has been associated with a number of pathophysiological processes. Only some are indicated here since the topic itself would fill the pages of a large review article. The genetic diseases resulting from the loss of various calpain genes are not discussed at all. For more information the reader is directed to Huang and Wang [81] and Goll and colleagues [63]. In rheumatoid arthritis, increased calpain activity is observed in joint synovial fluid, which is associated with cartilage destruction [82]. Inactivating, anti-calpastatin antibodies were detected in affected subjects. A straightforward concept connects such inhibitor depletion with increased proteolysis. However, the preliminary reported high incidence of antibodies [83] could not be reproduced with different groups of patients [84]. Moreover, the lack of other evidence in favour of such an explanation raises a question of its generality. Similar autoantibodies were reported with low incidence in venous thrombosis and systemic sclerosis. Elevated calpain activity (associated with lowering of calpastatin levels) contributes to muscle and neuronal damage after ischemia and traumatic brain and spinal cord injuries. During such events, calpastatin degradation (at least partially owning to caspases) is observed [85]. Essential hypertension in human subjects was associated with decreased calpastatin levels in erythrocytes [86]. However, studies on rats have shown no correlation at least in this animal model [87, 88]. Anti-calpastatin antibodies found in women, targeting the acrosomal region of spermatozoa, were implicated in infertility [89]. Moreover, CaPS has importance in human neurodegenerative disease pathogenesis. The proteolytic system indirectly participates in the cleavage of amyloid precursor protein and has been implicated in the pathology of Alzheimer's disease [90]. Involvement in pathogenesis of Parkinson's disease was also suggested [91]. Increased activity of CaPS due to elevated $Ca^{2+}$ is well documented in the formation of eye cataract in a hereditary cataract ICR/f rat model. A similar role was suggested in humans [92]. The excessive activity of a CaPS was moreover associated with muscle wasting during disuse and disease. It was demonstrated that injection of synthetic calpain inhibitors prevents the decrease in muscle fibre diameter in *mdx* mice, a model of Duchenne muscular dystrophy [93]. Such studies may find application in preventing muscle mass loss in injury and disease (e.g. Duchenne muscular dystrophy) [94]. In keeping with the above information, $\beta$-adrenergic agonist-induced muscle hypertrophy is associated with decreased calpain activity due to elevated levels of calpastatin [95]. A similar effect of CaPS equilibrium displacement was demonstrated in chicken. Broilers, characterized by high fractional growth rate, exhibit lower calpain/higher calpastatin activities compared to layer chickens, characterized by the low value of the index [96]. Finally, overexpression of calpastatin transgene slows muscle wasting in a murine model of muscle disuse [94].

Taken together, such a ubiquitous proteolytic system with multiple functions, some beginning to be understood but many remaining to be elucidated, merits the efforts of thorough characterization. Already, the potential to address the treatment of some important disorders has emerged. The focus on calpastatin, rather than the typical calpain-oriented approach, might lead to interesting new findings. The introduction of specific low molecular weight inhibitors (e.g. [97]) and recent advances in molecular biology and genetics will surely facilitate further development in the coming years.

## Thyropins (thyroglobulin type-1 domain proteinase inhibitors)

Thyroglobulin type-1 domain (Thyr-1) is a cysteine-rich structural element found either as a single or repetitive module in a variety of functionally unrelated proteins such as a precursor of thyroid hormones, thyroglobulin, basement membrane protein, nidogen (entactin), testicular proteoglycan, testican, insulin-like growth factor binding proteins (IGFBPs), pancreatic carcinoma marker proteins (GA733), major histocompatibility complex class II (MHC II)-associated p41 invariant chain, equistatin, chum salmon egg cysteine protease inhibitor (ECI) and saxiphilin. The Thyr-1 domains of the four latter proteins were demonstrated to reversibly inhibit cysteine proteases of the papain family (family C1) and will be discussed here, whereas entactin and thyroglobulin reportedly do not inhibit, reserving an as yet undefined role for their Thyr-1 modules. IGFBPs are also briefly discussed, though their inhibitory function is less clearly understood. The other proteins were not tested for inhibition [98–102]. A general mechanism of Thyr-1 domain interactions with cysteine proteases was demonstrated by the crystal structure of p41-invariant chain fragment in complex with cathepsin L (see below) [103]. Similarly to cystatins, the Thyr-1 domain fold of some thyropins adapted to inhibition of proteases outside C1 family. In this case even a cross-class inhibition is observed. It was demonstrated that equistatin not only interacts with papain and other C1 family members but also inhibits an aspartic protease cathepsin D (family A1). However, no structural data are available in this case.

### MHC class II-associated p41-invariant chain fragment

A crucial function of MHC II is to present bound peptides, derived mainly from extracellular antigens, to $CD4^+$ T lymphocytes. The invariant chain (Ii) associates with MHC class II molecules in endoplasmic reticulum, its main function being to block the peptide binding site during early stages of intracellular transport. Upon arrival at endosomes/lysosomes, Ii is proteolytically degraded, thus allowing the MHC II to bind peptides derived from endocytosed extracellular antigens. Both antigen processing by means of limited proteolysis and Ii degradation are mediated by endosomal proteases.

Two, alternatively spliced variants of Ii, namely p31 and p41, exist, which differ by an additional Thyr-1 domain in p41. A protein fragment corresponding to p41 Thyr-1 domain was demonstrated to efficiently inhibit cathepsin L, cruzipain and to a lesser extent cathepsin H as well as papain. Other cysteine proteases, such as cathepsins B, C and S, and proteases from other classes were not inhibited [98, 104]. The inhibitory properties towards antigen-processing proteases implicate possible modulation of antigen presentation by alternative splicing of Ii. Different lines of evidence in favor of the invariant chain exerting inhibitory and modulatory activity in vivo are delineated below.

Proportions of p41 in relation to p31 vary in different cells, suggesting a probable way to regulate antigen presentation. The former can modify the degradation pattern of the latter, demonstrating direct interference with proteolytic pathways. The half-life of p41 in endosomal compartments is longer, possibly allowing for presentation of epitopes processed further than those bound after p31 degradation. Although proteolytic processing of antigens is necessary for presentation in the context of MHC II, reports exist that excessive cleavage destroys the epitopes. Cathepsin L is capable of such destructive proteolysis; therefore its regulation by the p41-invariant chain fragment seems a tempting explanation of an epitope-shielding mechanism. Confirming such assumptions, it was shown that p41 is more effective in presenting a subset of antigens to T lymphocytes. However, the most straightforward evidence came from transfection studies. Cells expressing only p41 were more effective in presenting certain antigens to T cells than the ones expressing only p31. However, when a synthetic inhibitor of cysteine proteases was added, the two cell lines could not have been distinguished ([102] and references therein). Finally, it should be mentioned that the possibility of proteolytic activity modulation by Ii is not only restricted to the p41 fragment. Common, N-terminal regions of p31 and p41 share homology with cystatins, and accordingly p31 inhibits cathepsins L and H though with $K_i$ values several orders of magnitude higher than the p41 fragment [105].

Recently the structural determinants of cysteine protease inhibition by Thyr-1 have been revealed by crystallization of the p41 fragment in complex with cathepsin L. Although the fold of Thyr-1 differs from that of cystatins, the wedge shape and three-loop arrangement of the region of contact with the protease are reminiscent of the inhibitory edge of cystatins, demonstrating an example of convergent evolution. Some additional contacts are observed, however, which are responsible for determining more strict specificity towards target enzymes in contrast to rather non-selective cystatins [103].

### Equistatin

Equistatin is a 199-residue protein composed of three Thyr-1 domains. It was originally isolated as a papain-like cysteine protease inhibitor from the sea anemone *Actina equina* [100]. It is evident now, however, that only the first, N-terminal domain exerts inhibitory action towards this class of enzymes, whereas domain 2 inhibits cathepsin D but not the other aspartic proteases tested. Both types of enzymes may be inhibited simultaneously. The role of domain 3 remains unknown since it inhibits neither the cysteine nor aspartic proteases tested [106]. This example demonstrates how a Thyr-1 scaffold may be used to inhibit different classes of proteolytic enzymes and leads to speculation for a similar role of other Thyr-1 repeat-containing proteins. The physiological function of equistatin remains unresolved.

### Inhibitors from the eggs of chum salmon

Three different isoforms of a cysteine protease inhibitor were isolated from the eggs of chum salmon, *Oncorhynchus keta* (ECI). These small proteins, composed only of a single repeat of the Thyr-1 domain, inhibit papain and cathepsins B and L. Their physiological function remains unknown [99].

### Saxiphilin

Saxiphilin is a 91-kDa neurotoxin binding protein from the plasma of the North American bullfrog, *Rana catesbiana*. It binds saxitoxin, a tricyclic organic cation produced by various dinoflagellates and cyanobacteria. Saxitoxin intoxication, typically due to intake of contaminated shellfish, results in paralysis due to blockage of voltage-dependent sodium channels involved in the generation of action potentials. The compound is also widely used in studies on such sodium channels. Saxiphilin is homologous to transferrins, but carries an insertion of a tandem duplication of Thyr-1 and lacks $Fe^{3+}$ binding activity. Both Thyr-1 domains of saxiphilin have been demonstrated to inhibit papain, though only one is active against cathepsins B and L. There is no effect of papain

on binding of saxitoxin and vice versa. The physiological significance of the inhibitory function remains unknown. It was only speculated that it could play a role in regulation of saxiphilin degradation if it was internalized by cells in a manner similar to transferrin and later exposed to the lysosomal compartment [101].

**IGFBPs**

IGFBPs, proteins containing a single Thyr-1 domain, function as modulators of IGF (small peptide hormones stimulating proliferation and differentiation of diverse cell types) actions by binding and preventing them from interaction with specific receptors. The effect of IGFBPs is regulated, among other processes, by inactivation by proteolysis, in which different classes of proteases have been implicated. In turn the modulation of the latter process by IGFBPs themselves and in particular by their Thyr-1 domains was demonstrated. Some IGFBPs may inhibit the processing of other IGFBP species by different proteases. However, the detailed nature of IGFBP interactions with proteolytic enzymes remains largely unknown (for a current review see [107]).

**IAP family**

Inhibitors of apoptosis (IAPs) are a family of proteins distinguished by encompassing one or more characteristic, ~70-residue zinc binding BIR (baculovirus IAP repeat) domains. They were primarily characterized as inhibitors of apoptosis (proteins able to complement p35 mutation in baculovirus; see below) [108], though currently some BIR-containing proteins are known which do not seem to confer such a function. Apoptosis, programmed cell death, is one of the crucial events controlling the number of cells in multicellular eucaryotes. Deregulation of the process is observed in the pathogenesis of many illnesses, such as cancer, autoimmune and immunodeficiency diseases, neurodegenerative disorders and viral infections. Apoptosis is regulated by many signals which finally converge in a cascade of caspases. These cysteine proteases, with a substrate preference for aspartic acid residues, exist in the cells as inactive precursor proteins (zymogens) which become activated upon receiving proper signals and execute the apoptosis. Housing such destructive machinery implies a need of strict modulation/control by IAPs, which directly suppress caspase activity. The above surely presents too simplified picture of the control of apoptotic process and should serve only to illustrate the role of IAPs as caspase inhibitors. The detailed information may be found in a multitude of reviews concerning the process.

First reports on IAPs appeared only a decade ago when an apoptosis suppressor was found in baculovirus where it helps to maintain host cell survival long enough to permit virus replication [108]. Currently IAPs are known from organisms ranging from yeast to mammals. Eight human IAPs have been identified: XIAP (OMIM 300079), c-IAP1 and 2, NAIP, Survivin, ML-IAP, ILP-2 and Bruce. Overexpression of the first five has been shown to suppress apoptosis. In parallel, evidence has accumulated that different IAPs, in addition to caspase inhibition, exhibit unrelated activities such as regulation of cell cycle progression, protein degradation or modulation of receptor-mediated signal transduction. These findings, however, remain beyond the scope of this review, and the reader is directed to Deveraux and Reed [109] and Salvesen and Duckett [110] for a detailed introduction.

Unlike in previously described inhibitors, BIR domains may not be considered as independent inhibitory units. Also the mechanism of inhibition of different caspases by IAPs is dissimilar. Therefore, IAPs are grouped on the basis of the structural similarity of BIR domains and general function in apoptotic pathways rather than the inhibitory mechanism.

It has been demonstrated that XIAP, c-IAP1 and 2, NAIP and ML-IAP directly bind and inhibit caspases, but their inhibitory potency towards different caspases differs significantly, and some caspases are not affected by any of those proteins. The evidence on survivin interactions with caspses is far less convincing than those on former proteins [111–114]. The mechanism of inhibition was elucidated with the use of X-ray crystallography and turned out to differ completely for the inhibition of the effector caspases (3 and 7) as compared to inhibition of initiator caspase-9.

Inhibition of caspase-9 by BIR3 of XIAP is peculiar in that the inhibitor does not in any manner occlude the active site. Instead, it binds to the surface responsible for caspase homodimerization, thus preventing the process. As a result, the enzyme lacks a properly formed active site since caspases are active only as dimers where a loop of one monomer contributes to the formation of a catalytic site on the other [115].

A different mechanism, utilizing the general scheme of active site occlusion is used to inhibit caspases 3 and 7. A fragment of the polypeptide chain N-terminal to the BIR-2 domain (linker) is inserted directly into the active site, though in an orientation opposite to the substrate, thus escaping cleavage. In this arrangement BIR plays little or no role in the inhibition itself, but it is required to assure a productive conformation of a linker peptide. Indeed, the linker peptide alone is unable to bind caspases, though it is enough to fuse any protein to either the C- or N-terminal of the former to achieve inhibition [116–118].

Though completely divergent, those two mechanisms of caspase inhibition reserve one common role for the BIR domain in the promotion of apoptosis by Smac. The latter protein, released from mitochondria upon process induc-

tion, selectively binds the BIR domains utilizing an IAP binding motif (the first four N-terminal amino acids), thus abolishing IAP's affinity for caspases [119, 120]. Significantly, a similar motif constitutes a part of the caspase-9/XIAP interface, and in this case the effect of Smac seems to depend on competitive binding. The removal of effector caspase inhibition, on the other hand, relies on steric hindrance of the inhibitory linker peptide by BIR domain bound Smac.

It has to be stressed that the antiapoptotic effects of IAPs may be attributed only partially to their ability to inhibit caspases. Divergent antiapoptotic pathways mediated by IAPs exist, discussion of which, however, remains beyond of the scope of this review [121, 122].

Playing an important role in apoptosis regulation, it is no wonder that IAPs deficiency or deregulation is associated with multiple pathologies, especially with cancers. Unfortunately, our understanding of the mechanisms involved is still mostly superficial. It is thus impossible to say whether aberrant caspase activity or other IAP-associated mechanisms underlie the deleterious processes. Alterations of SMN, a gene adjacent to NAIP, are observed in nearly all patients with spinal muscular atrophy (OMIM 253300), the second most common autosomal recessive disease after cystic fibrosis. This lethal, neurodegenerative disorder is characterized by progressive loss of anterior horn cells (spinal cord motor neurons), leading to wasting of voluntary muscles. The accompanying loss of NAIP was reported to possibly affect the severity of the disease, although an unambiguous correlation has not been established [123, 124]. However, as already mentioned, the main context in which we view IAP-associated diseases is malignancies. It is consistent with the known ability of cancer cells to escape apoptosis. Survivin, a protein containing only a single BIR domain, is expressed in higher quantities in tumour cells compared wich normal ones. It has, however, not been determined whether this defines an antiapoptotic mechanism or merely reflects the high proliferation rate of such cells, since survivin involvement/upregulation in cell division has been demonstrated and high levels are also observed in fetal tissues [113]. As for other IAPs, aberrant expression of c-IAP2 was demonstrated in mucosa-associated lymphoid tissue (MALT) lymphoma [125]. ML-IAP is expressed at high levels in melanoma cells as compared with normal melanocytes [126], while c-IAP1 is potentially overexpressed in esophageal squamous cell carcinoma [127]. XIAP overexpression has been suggested to correlate with a poor prognosis in acute myelogenous leukemia [128], while levels of XIAP negative-regulatory protein XAF1 are reduced in many human tumour cells [129].

Besides NAIP, no reports of IAP deficiency-associated diseases exist. Clues concerning such a situation come from studies of knockout mice. XIAP- or NAIP1-deficient animals develop normally, suggesting functional redundancy and complementation by different IAPs. Indeed, c-IAP1 and -2 levels are elevated in the former. Only in pathological conditions of pharmacologically induced epileptic seizures is decreased neuron survival observed in NAIP1-deficient animals, which seems to agree with a modulator role of NAIP in spinal muscular atrophy [130, 131]. On the other hand, disruption of the survivin gene in mice results in embryonic lethality. The defect is, however, not associated with uncontrolled apoptosis, but rather with defects in mitosis complementing other reports of involvement of survivin in the process [132].

## Baculovirus p35

IAPs are not the only natural inhibitors of caspases. Before their discovery, studies on the baculovirus *Autographa californica* and its insect host *Spodoptera frugiperda* identified another protein, p35, which was able to counter hosts' antiviral defense (apoptosis) to allow virus multiplication [133]. The p35 protein efficiently prevents baculovirus-induced apoptosis and as such would present simply a nice example of IAP-independent apoptosis control found only in baculoviruses (since the homologues from other species have not been identified) were it not for another characteristic, a broad-spectrum caspase inhibition. The protein can function as a general suppressor of programmed cell death in diverse organisms ranging from worms to mammals when expressed ectopically, and as such serves widely as a tool in apoptosis studies. Moreover, targeted transfer of the p35-encoding gene was proposed as a possible remedy for diseases characterized by excessive apoptosis. The significance of baculovirus possessing a caspase inhibitor with such broad specificity is unknown, and the fact most probably only reflects conservation of apoptotic pathways in evolution.

The mechanism of inhibition was elucidated by solving the crystal structure of p35 in complex with caspase-8. P35 is a suicide inhibitor. The reactive site loop is cleaved at Asp87, upon which the inhibitor undergoes dramatic conformational changes, trapping the catalytic cycle at a thioester intermediate stage. The next step in the cycle, thioester hydrolysis, is prevented by eliminating solvent accessibility to the catalytic dyad by means of a repositioned amino terminus of p35 [134]. Such covalent suicide inhibition has long been known for serpins (see below). However, the latter trap the intermediate by distorting the active site, while p35 utilizes water exclusion without pronounced active site distortion.

Besides caspases, p35 was shown to inhibit another clan CD protease, *Porphyromonas gingivalis* gingipain-K (family C25). This case is the only known exception to the strict specificity of IAPs and p35 caspase inhibitors towards C14 family peptidases [135].

## CrmA

Yet another protein, CrmA (cytokine response modifier A), derived from cowpox virus, can potently inhibit caspases. Structurally, it belongs to a large family of serine protease inhibitors termed serpins that encompass proteins involved in a number of fundamental biological processes such as blood coagulation, fibrinolysis, complement activation, inflammation, angiogenesis, tumour suppression and more [136]. Though CrmA indeed inhibits a serine protease – granzyme B – it has developed a mechanism of cross-class inhibition of cysteine proteases, with caspases being its predominant target [137, 138]. CrmA serves as a representative for a group of homologous viral serpin-like caspase inhibitors utilized to evade the host defense. The viral serpins are not unique in cysteine protease inhibition. Human serpins, antichymotrypsin and squamous cell carcinoma antigen 1, are also capable of such cross-class inhibition [139]. The role of the process is, however, less clearly understood; therefore, only viral serpins will be discussed here.

Due to a unique set of targeted enzymes, CrmA is not only an apoptosis inhibitor but also has anti-inflammatory functions interfering with caspase-mediated cytokine processing. The latter is best illustrated by experiments in which CrmA is exchanged with p35, resulting in fully viable virus (unlike in CrmA knockouts or inactive mutants which result in decreased yields), however, associated with an elevated immune response as demonstrated in the chicken chorioallantoic membranes model [140]. The importance of a homologous protein (Serp2) from myxoma virus, which causes uniformly lethal effects after intradermal injection into rabbits, was demonstrated by generation of highly attenuated deleted variants [141]. The double protection of the virus against host response by controlling both proinflammatory cytokine activation, which prevents inflammatory signal transmission from infected cells (caspase 1 = interleukin 1$\beta$ converting enzyme), and the extrinsic apoptotic pathway triggered by death receptor engagement (caspase 8) may be further supplemented by the ability to inhibit granzyme B. The last effect, however, seems to lack physiological relevance.

Utilization of the protective role of CrmA against Fas/Fas ligand-mediated apoptosis may prove advantageous for gene therapy. The pathway is utilized by cytotoxic T lymphocytes to eliminate adenovirus-infected cells, resulting in a rapid decrease in levels of transgene production. Coexpression of CrmA has been shown in several reports to significantly prolong the expression of transgene product [142]. The possibility of protection against xenograft rejection by CrmA transduction was also discussed [143].

## Propeptide-like inhibitors

Papain-like cysteine proteases are produced as inactive precursors. The lack of activity is due to potent inhibition by the N-terminal propeptides. Only at the target location does limited proteolysis of the propeptide occur, allowing the enzyme to exert its action. Fox and colleagues [144] were the first to show that synthetic proregions inhibit their cognate proteases. Later studies demonstrated that propeptides are competitive, slow-binding inhibitors and, unlike the broad spectrum cystatins, possess high selectivity for the enzymes from which they originate. The inhibition is pH dependent (acidification decreases the affinity) being in good agreement with the process of enzyme activation, since pH is one of the most common environmental parameters in triggering the process. Based on structural data the mode of inhibition was elucidated. The propeptide spans the protease active site in an orientation opposite to that of a substrate, thus escaping cleavage. Beside being inhibitors, the propeptides assist in proper folding and targeting, and stabilize the cognate enzymes. For a comprehensive review of the functions of CA1 protease propeptides, the reader is directed to Wiederanders [145].

In addition to protease-associated propeptides, homologous inhibitory proteins, expressed independent of the proteases as autonomous modules, have been described (reviewed in [146]). The first report comes from Denizot et al. [147] who identified cytotoxic T lymphocyte antigen-2 in mice as a protein homologous to the proregion of cathepsin L. The production of recombinant protein demonstrated the anticipated inhibitory activity towards certain cysteine proteases [148]. Similar autonomous proteins were described in the silk moth *Bombyx mori* [149]. Like true propeptides, propeptide-like proteins show selectivity towards a restricted set of CA1 proteases, but unlike the former, inhibitory properties are retained at low pH. Several new homologues were recently identified in genomic sequences of *Drosophila* and rat; however, the latter were not characterized on a protein level.

Selectivity of the propeptide-like inhibitors compared with rather nonselective cystatins has led to the suggestion that the proteins may prove suitable in targeting individual proteinases as insecticides for plant/pest control or as therapeutic agents for parasitic pathogens. It has been demonstrated that short peptides derived from propeptide regions are capable of inhibiting cognate enzymes and surprisingly retain selectivity. Structural studies of this class of proteins may facilitate the development of selective low molecular weight analogues. Already, based on the studies of propeptides, a promising leading structure for cruzipain inhibitor (for the importance of this enzyme see the note on chagasin family) and some selective inhibitors of cathepsins L and B were developed (reviewed in [145, 146]).

## Perspectives for the future

The field of proteinaceous cysteine protease inhibitors has witnessed a huge advancement in recent years, opening a great many new ways both for further academic studies as well as applicative developments. Compared to a single family known in the early 1990s, currently 10 are described, and the accompanying growth in a number of known inhibitors and processes involving these molecules is even higher. Most, if not all, aspects of such an important activity as proteolysis are accompanied by inhibitors exerting regulatory or protective functions. Advances in genomic studies have already enabled rough estimates of the number of inhibitors and proteases produced by many organisms; however much remains to be done to understand the detailed function of these proteins. Moreover, some inhibitors constituting yet unknown new families might have been easily overlooked in such comparative studies. It is probable that such families still await discovery, especially since known proteinaceous inhibitors of cysteine proteases outside the papain family are scarce. The future will certainly bring further development measured not only in a number of described inhibitors but more important in better understanding of regulated pathways.

The deregulation of protease/inhibitor systems has been demonstrated in many diseases, and for some it is quite well understood and documented as an underlying process. Evidence has accumulated that pathogens use proteases and inhibitors to invade host organisms and evade their defenses. (It should be remembered that 'pathogens' correspond not only to human and animal foes but also to a huge field of agricultural pests.) At the same time similar proteins constitute a part of host armory. The thorough understanding of involved processes opens possibilities for external intervention in pathological states. In most cases the direct involvement of proteinaceous inhibitors in such applications seems improbable. Nevertheless, the possible utilization of the molecules in directed gene transfer seems a very tempting idea. Moreover, developments in the field facilitate the growth in a number of possible targets and enable a structure-based approach to the design of suitable low molecular weight compounds, where such important issue as specificity may be easier to address.

Current concepts and developments in the field of therapeutic, low molecular weight, synthetic cysteine protease inhibitors encompass the struggle against viruses, bacteria and parasites as well as control of deregulated endogenous proteolytic enzymes. Inhibitors of viral proteases of different catalytic classes including a 3C cysteine protease of picornaviruses are being developed after the spectacular success of the HIV protease inhibitor. The possibility of employing protease inhibitors in the treatment of bacterial infections has been suggested. Along with proteases of other catalytic classes, suitable targets include cysteine proteases of *S. aureus* and *P. gingivalis*. The suitability of synthetic protease inhibitors hase been demonstrated in the struggle against parasites such as *Plasmodium*, *Trypanosoma*, *Leishmania* and *Schistosoma*. Moreover, therapeutic success achieved so far with synthetic inhibitors of angiotensin-converting enzyme extends the field of interest on host proteases. In this context cysteine protease activity control is considered mostly in association with tumour cell invasion, metastasis and apoptosis. In all therapeutical applications the issues of bioavailability, low toxicity and selectivity are important to address. While the inhibitors developed may not necessarily prove useful as drugs for the above reasons, they still have immense value as research tools in studying the biological function of targeted enzymes (for detailed review see [150]). As demonstrated in this review, proteinaceous inhibitors are often even more convenient specific tools to address the latter issue.

Agriculture is yet another major field of interest in protease inhibitors. Here again the proteinaceous inhibitors play a role. Engineering for pest resistance using protease inhibitor transgenesis has been widely discussed and proved a suitable and advantageous method [151].

The rapid growth of the field of proteinaceous cysteine protease inhibitors will most probably increase even more in the forthcoming years owing to the need to answer many crucial questions brought by recent studies, involvement in many general processes, commercial interest and a general trend in the life sciences.

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