JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 563–576
© 2010 The Authors
Journal compilation © 2010
Royal Pharmaceutical Society
of Great Britain
Received October 08, 2009
Accepted February 26, 2010
DOI 10.1211/jpp/62.05.0002
ISSN 0022-3573

Review

Proteases implicated in apoptosis: old and new

Kelly L. Moffitt, S. Lorraine Martin and Brian Walker

Biomolecular Sciences Group, School of Pharmacy, Queen's University of Belfast, Belfast BT9 7BL, Northern Ireland, UK

Abstract

Objectives The role of proteases in the regulation of apoptosis is becoming increasingly apparent. Whilst many of these proteases are already characterised, some have yet to be identified. Traditionally caspases held the traditional role as the prime mediators of apoptosis; however, attention is now turning towards the contribution made by serine proteases.

Key findings As unregulated apoptosis is implicated in various disease states, the emergence of this proteolytic family as apoptotic regulators offers novel and alterative opportunities for therapeutic targets.

Summary This review presents a brief introduction and overview of proteases in general with particular attention given to those involved in apoptotic processing.

Keywords apoptosis; diphenyl phosphonate; protease; proteolytic; serine

Introduction

Unregulated apoptosis, defined as programmed cell death, can be due to a disruption in the balance and control of both intra- and intercellular proteolytic activity, thus leading to various disease states. Many proteases involved in apoptotic processes have yet to be identified; however, several are already well characterised. Caspases traditionally held the predominant role as prime mediators of execution. Increasingly, research is implicating serine proteases within apoptotic processing, particularly in the generation of nuclear events such as condensation, fragmentation and DNA degradation observed in late stage apoptosis.[1–5] Serine proteases therefore are emerging as providing additional or alternative therapeutic targets and, as such, offer increased opportunities for research into their identification, cellular location and apoptotic contribution.

In conjunction with the fundamentals of proteolytic activity, accurate research is dependent upon availability of selective and specific target protease inhibitors. Some traditional inhibitors, once thought to be target-specific, are now deemed to target a broad spectrum of proteases, which can potentially lead to ambiguities within established research into apoptosis.

Proteases

Proteases, also termed peptidases, are defined as proteolytic enzymes, degrading proteins into smaller segments via cleavage of peptide bonds. Being critical for normal functioning of all cells and organisms, they are involved in a variety of physiological processes including post-translational modifications. Amino acids are the building blocks of protein synthesis and their recycling through protein degradation is energetically more efficient compared with being synthesized *de novo*. Therefore, proteases are pivotal biological catalysts, disassembling protein molecules, thus restoring the pool of free amino acids required for protein synthesis.

Their importance is clearly indicated, primarily by the estimation of 8000 papers relating to this field being published each year and secondly, by the fact that protease genes represent approximately two percent of the total genome.[6,7]

Ordinarily, proteolytic activity, an irreversible process, is well regulated and monitored within the cell, thus reducing the potential for autolysis and inappropriate proteolysis. This is important given that cells themselves are largely constructed of protein. This regulation occurs either at the molecular level with the majority of proteases being synthesized as

Correspondence: Kelly L. Moffitt, Biomolecular Sciences Group, School of Pharmacy, Queen's University of Belfast, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK.
E-mail: k.moffitt@qub.ac.uk

pro-enzymes that require activation, usually via proteolysis, or at the cellular level with proteases being stored within membrane bound protective organelles such as lysosomes. In addition, enzymatic activity can be regulated by inhibition by other proteins.

Proteases have restricted activity and positional specificity. Those restricted to acting near the ends of polypeptide chains are termed 'exopeptidases' while, in comparison, 'endopeptidases' target internal bonds. In relation to exopeptidases, those acting at a free amino-terminus result in the liberation of a single amino acid residue (aminopeptidases), a dipeptide (dipeptidyl-peptidases) or a tripeptide (tripeptidyl-peptidases). For those acting on the carboxy-terminus, a single amino acid residue is liberated by the carboxypeptidases, whilst a dipeptide by peptidyl-dipeptidases (Figure 1). The primary determinants for endopeptidase activity are the amino acids near the scissile peptide bond on either side. In addition, sequence specificity allows preferences for cleavage of peptide bonds at particular amino acids.

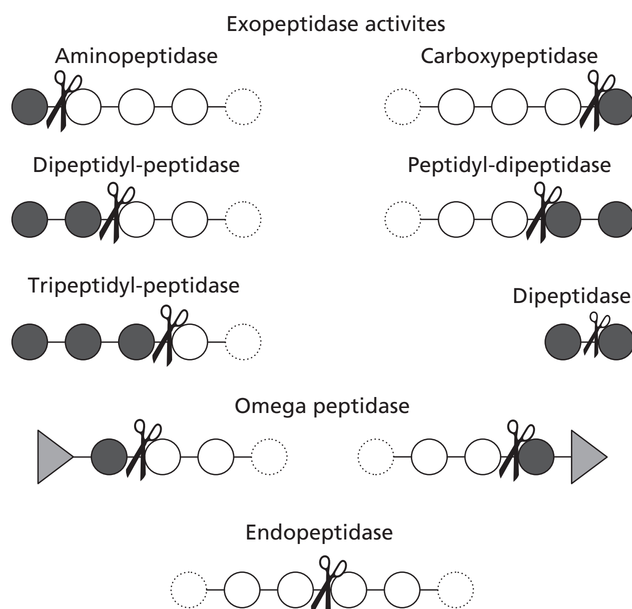

Figure 1 Various types of protease activity shown as exo- and endopeptidases. Adapted from Barrett[7]

Classification of proteases

Proteases are named and classified into family groups and subgroups according to their catalytic mechanisms. Six mechanistic classes have been recognised and documented within the *Merops* Database: cysteine, threonine, serine, metallo, aspartic and glutamic proteinases (Table 1) (http://merops.sanger.ac.uk). In addition, there is recognition that novel proteases may exist whose catalytic mechanism has yet to be identified. For each catalytic type of protease there are specific inhibitors.

Cysteine proteases

Cysteine proteases possess widespread biological roles including apoptosis, major histocompatibility complex (MHC) class II immune responses, prohormone processing and extracellular matrix remodelling.[8]

Whilst cysteine proteases can be grouped into two main superfamilies, the family of enzymes related to interleukin (IL) 1β converting enzyme (ICE) and the papain superfamily, the common catalytic mechanism involves a nucleophilic cysteine thiol in a catalytic triad.

The acid-acting papain-like family has a cysteine and histidine residue in its catalytically active site and is the main enzyme subgroup within cysteine protease classification. Papain-like enzymes within mammalian cells include lysosomal cathepsins B and L, whose role contributes to homeostatic turnover of cellular proteins. If these acid-preferring proteases encounter a biologically normal or alkaline environment, they tend to become irreversibly inactivated.[7] Cystatins, present both intra- and extracellularly, are natural inhibitors of this group of enzymes. Binding tightly and irreversibly, they protect cells, tissues and the circulation from harmful proteolytic activity.

Calpains, calcium-dependent cysteine proteases, belong to the papain superfamily. These function as cytosolic heterodimeric enzymes, consisting of 30 and 80 kDa subunits. Calpastatin, a specific endogenous inhibitor, interacts with the calcium-binding domains of the large and small subunits, thus providing an effective regulatory inhibitory effect within the cell. A regulatory role within cellular signalling has been suggested for these enzymes (reviewed by Chapman *et al*.[8]).

The ICE superfamily contains the caspases, important members of the cysteine protease family due to their

Table 1 The six catalytic types of proteases

Catalytic type	Primary catalytic group	Secondary catalytic group	Example
Serine	Hydroxyl group of serine	Imidazole nitrogen of histidine (sometimes ε-amino group of lysine)	Chymotrypsin
Threonine	Hydroxyl group of threonine	Amino-terminal α-amino group	Proteasome
Cysteine	Thiol group of cysteine	Imadazole nitrogen of histidine	Papain
Aspartic	Carboxyl groups of two aspartic acid residues		Pepsin
Metallo	Zinc atom (occasionally another metal)	Carboxyl group of glutamate	Aminopeptidase N
Glutamic	Glutamine (Gln-53) and glutamate (Glu-136) residues		Scytalidoglutamic peptidase

Adapted from Barrett.[7]

involvement in programmed cell death, apoptosis. Whilst there are several members of the caspase family, all show strict specificity for hydrolysis of aspartyl bonds. Caspases act either as initiator or executioner within the apoptotic processes. Executioner caspases are activated from their proenzymatic form by the actions of other caspases within a cascade reaction.

Threonine proteases

Threonine proteases were first described in 1995, being classed as N-terminal nucleophile hydrolases.[9] Enzymatic activity is provided by a catalytic triad consisting of threonine, lysine and glutamic acid, with the postulation that the hydroxyl group of Thr1 functions as the reactive nucleophile of peptide hydrolysis. The most described and well documented example of threonine proteases is the proteasome, responsible for the degradation of proteins within the eukaryotic cell. Protein hydrolysis results in the predominant production of oligopeptides of approximately nine amino acids, which are subject to further proteolysis within the cell.

The *Merops* database classifies four families within threonine proteases – T1, T2, T3 and T5, of which T1 contains two subfamilies T1A and T1B.

Serine proteases

Serine proteases utilise a highly reactive serine hydroxyl group to cleave other proteins and perform a broad variety of physiological processes. Whilst possessing a conserved catalytic site consisting of histidine, aspartic and serine residues, this order and arrangement varies within their family classification (Table 2).[4] Representing the largest family of proteases, Barrett and Rawlings[10] classified serine proteases into evolutionarily unrelated clans based on the order of catalytic residues, and not active site structure or chemistry.[11] Clans in turn are subdivided into family groups. Individual family members are classified according to substrate specificity with chymotrypsin, trypsin and elastase being proto-typical of three defined subgroups.[12] Chymotrypsin preferentially cleaves at the carboxyl side of aromatic side chains such as phenylalanine. Trypsin cleaves at basic residues such as lysine, while elastase prefers smaller uncharged residues such as glycine.[13]

Functional diversity of serine proteases, in particular chymotrypsin-like, is achieved by generating variations on a common fold.[14] This fold consists of two open-ended β-barrels with the active site and substrate-binding clefts lying in between. Variations in substrate recognition occur due to differences in the identities of residues which lie within the substrate-binding cleft.[15] Generally, proteolytic activity is optimal at neutral or slightly alkaline pH values.

Table 2 Examples of catalytic arrangements of residues within serine proteases

Family	Name	Catalytic triad arrangement
S1	Chymotrypsin	Histidine, aspartate, serine
S2	α-Lytic endopeptidase	Histidine, aspartate, serine
S3	Togavirus endopeptidase	Histidine, aspartate, serine
S8	Subtilisin	Aspartate, histidine, serine
S9	Prolyl oligopeptidase	Serine, histidine, aspartate
S10	Carboxypeptidases	Serine, aspartate, histidine

Functionally, serine proteases participate in post-translational processing of various polypeptides. The majority of chymotrypsin (S1) family members function as endopeptidases. Primarily, these family members make a significant contribution to homeostatic maintenance. Most well known for their role in the digestion of dietary proteins they are, in addition, involved in the regulation of key amplification cascades through proteolytic activation of inactive zymogen precursors as manifested during blood-clotting and inflammatory processes.[4] Specifically, trypsin plays the role of proteolytically activating precursors of chymotrypsin and elastase.[7] Examples of physiological functions and sites of expression of chymotrypsin-like serine proteases are shown in Table 3, to demonstrate their multifunctionality and ubiquitous expression. At present, results from the Human Genome Project have indicated that 118 chymotrypsin-like proteases exist.[11] Tightly controlled serine proteolysis can occur through transcriptional regulation, for example, transcription of chymase and prostate-specific antigen occurs in response to cytokines and steroid hormones, respectively.[11]

Post-translational activation or deactivation through proteo- and auto-lysis is paramount for intracellular regulation, specifically in relation to proteases involved in digestive and blood clotting processes.[11] Furthermore, natural occurring inhibitors such as serpin play an important role in maintaining enzymatic homeostasis.[16] Regulation also occurs through environmental pH; for example, chymase is activated upon release from acidic granules into the neutral extracellular space.

The subtilisin (S8) family represent the second largest serine peptidase family and function as intracellular proteases or transmembrane proteins. Whilst the majority of enzymes are endopeptidases, the S8 family exhibit exopeptidase activity, through tripeptidyl-peptidase II (TPPII). TPPII aids intracellular protein recycling and degradation of neuropeptides and is generally localised to the liver and brain. A feature of several subtilisins is their multidomain structure, featuring a propeptide upstream and auxiliary modules downstream of the protease domain. The majority of subtilisins possess broad specificity profiles and are relatively easily engineered with new specificities unlike those of the chymotrypsin-like family.[11]

Metalloproteases

Metalloproteases are of enormous biological and medical importance, being involved in activities ranging from cell proliferation, differentiation and remodelling of the extracellular matrix to vascularisation and cell migration (reviewed by Chang and Werb[17]). Two closely related metalloprotease families exist: matrix metalloproteases (MMPs) and metalloprotease-disintegrins (ADAMs). The MMP family, consisting of over 20 enzymes, characteristically degrade the extracellular matrix (ECM) whilst ADAMs are secreted glycoproteins or transmembrane proteins containing disintegrins and metalloprotease domains, indicative of cell adhesion and proteolytic activity.[17]

Table 3 Physiologic functions and sites of expression of chymotrypsin-like serine proteases

Physiologic function	Proteases(s)	Site of expression (adult)
Blood coagulation	Factor VII	Liver
	Factor IX	Liver
	Factor X	Liver
	Factor XI	Liver
	Factor XII	Liver
	Plasma kallikrein	Liver
	Protein C	Liver
	Thrombin	Liver
Cell-mediated immunity	Azurocidin	Neutrophils
	Cathepsin G	Neutrophils
	Chymase	Mast cells
	Granzymes	T lymphocytes
	Myeloblastin	Neutrophils
	Neutrophil elastase	Neutrophils
	Tryptases	Mast cells
Complement	Factor B	Liver
	Factor C1r	Liver
	Factor C1s	Liver
	Factor C2	Liver
	Factor D	Adipocytes, macrophage lineages
	Factor I	Liver
	Haptoglobin	Liver
	Mannose-binding protein-associated serine proteases	Liver
Digestion	Chymotrypsin	Pancreas
	Elastases	Pancreas
	Enterokinase	Proximal small intestine
	Trypsins	Pancreas
Fertilisation	Acrosin	Sperm
	Prostate-specific antigen	Prostate
Fibrinolysis	Plasmin	Liver
	Tissue plasminogen activator	Endothelium
	Urokinase	Kidney
Growth stimulation and development	Hepatocyte growth factor	Liver
	Hepatocyte growth factor activator	Liver
	Hepsin	Liver
	Neuropsin	Brain
Kallikrein	Tissue kallikreins	Pancreas, kidney
Tissue remodelling	Apolipoprotein A	Liver
	Stratum corneum chymotrypic enzyme	Skin
Unknown function	Neurotrypsin	Brain
	Osteoblast serine protease	Bone
	Pancreatic endopeptidase E	Pancreas
	Prostasin	Prostate
	Protease M	Breast
	Transmembrane protease 2	Small intestine

From Di Cera and Krem.[11]

Catalytic activity is dependent upon a metal atom, with zinc being the main metal atom of importance; however activity is often retained when zinc is replaced by cobalt or manganese, in addition, metals other than zinc occur naturally within the active sites of some metalloproteases.[7]

Generally, metalloproteases function as exopeptidases, in particular aminopeptidases and carboxypeptidases, removing amino-terminal or carboxy-terminal amino acids of polypeptide chains. MMPs however, function as calcium-containing endopeptidases, regulated at the transcriptional level or by endogenous inhibitors known as the tissue inhibitors of metalloproteases (TIMPs).[18,19] Most MMPs are expressed as zymogens, whose activation is dependent upon regulated proteolytic activity.

Aspartic proteases

The activity of aspartic proteases is reliant upon the interacting carboxyl groups of a pair of aspartic acid residues. At present, the *Merops* database classifies 14 families and 16 subfamilies belonging to aspartic peptidases, with those of human origin being found in family A1, subclass A1A (pepsin A). Functioning only as endopeptidases, they are optimal within acidic pH environments. Aspartic proteases of biological and clinical importance include, amongst others, pepsin, renin and retropepsin.[7] Pepsin is the major secretory peptidase located within the stomach, where proteolysis of food proteins commences. Hydrochloric acid released from the gastric mucosa promotes an acidic environment wherein pepsin must remain stable and active. Renin, another secretory enzyme, is an exceptional aspartic protease, preferring to function at near neutral pH within blood plasma. Playing an important role in the regulation of mammalian blood pressure, it provides regulated proteolysis, releasing angiotensin-1 from its precursor angiotensinogen (reviewed by Barrett[7]). Retropepsin, the main protease of the human immunodeficiency virus (HIV), functions to separate, via proteolysis, individual viral proteins from newly synthesised polyproteins.[20] Consequently, this functional characteristic has led to retropepsin becoming a primary target for inhibitors as the basis of drug therapies in the treatment of acquired immunodeficiency syndrome (AIDS).

Glutamic acid proteases

The glutamic protease family, previously classified in family A4 of the aspartic endopeptidases, was reclassified in 2004 as the sixth catalytic type in the *Merops* database. One family, G1, is listed, termed 'Eqolisins' due to the importance of glutamate (E) and glutamine (Q) residues in the active sites.[21] To date, glutamic protease distribution is limited to filamentous fungi where they function as endopeptidases.[22]

Proteases involved in apoptosis

Proteolytic cleavage of intracellular proteins is a core feature of apoptosis, a term defined as programmed cell death. In addition, the mutation, absence or impaired regulation of proteases leads to diseased states.

Several proteolytic systems have been implicated in apoptosis and its associated processes. These are variable,

depending upon both the origin of apoptotic stimuli and the cell type involved.[23] Due to the diversity and quantity of proteases involved in apoptosis, experimental tools in the identification of unknown proteases within model systems have centred on the utilisation of protease inhibitors. Numerous inhibitors have been pharmacologically developed for therapeutic and clinical use.

Whilst many proteases involved in apoptotic processes have yet to be identified, there are several which are well established and characterised. The cysteine protease family members the caspases have traditionally been considered to play the predominant role as the prime mediators of the execution of the apoptotic programme. However, evidence has accumulated that noncaspases, including the cysteine proteases calpain and cathepsin B, and the proteasome (a threonine protease) also have roles in mediating and promoting cell death.[24] In addition, there is emerging evidence to suggest that serine proteases may function independently within the apoptotic signalling pathways, or interact with other mediators such as the caspases or Bcl-2 family proteins.[25,26]

Cysteine proteases

Cysteine proteases implicated in apoptotic processing and regulation primarily belong to those classified within the main papain family, C1, which incorporates several cathepsins including L, S, H and B, the calpain family, C2, and the caspase family, C14.

Calpains

Calpains, a family of nonlysosomal, cytoplasmic neutral cysteine proteases, can be ubiquitous or tissue specific. Ubiquitous ‘conventional calpains’ involved in promoting apoptosis are μ-calpain and m-calpain. Optimal proteolytic activity is dependent upon Ca^{2+} binding, with μ-calpain requiring micromolar Ca^{2+} concentrations whilst m-calpain requires millimolar concentrations.[24] Calpains have the ability to associate with membrane phospholipids in response to cellular stimuli. Following binding of Ca^{2+}, calpains undergo autolysis, which has the effect of increasing their activity in addition to lowering the requirement for Ca^{2+}.

Calpain substrates include fodrin, actin, filamin, p53, Bax and procaspase-3 and -9. Due to the cytoskeletal nature of some target substrates it may be suggested that calpains have a role in destruction of cellular architecture during apoptosis. Furthermore, a recent study by Yousefi *et al.*[27] demonstrated calpain-mediated autophagy-related gene (Atg)5 cleavage. Atg5, a gene product necessary for autophagosome formation, enhances susceptibility towards apoptotic stimuli. Activation or overexpression of calpains is observed in diseases marked by excessive cell death. For example, considerable activation of calpains is seen in brain tissue from patients with Alzheimer’s disease.[28]

Inhibitors of calpains

Calpastatin, a 110 kDa endogenous protein inhibitor, is commonly expressed and is highly specific for calpains, functioning by targeting the Ca^{2+} binding domains of both the large and small calpain subunits. During apoptosis, calpastatin is regulated by other proteases including caspases. Development of calpain inhibitors has proved difficult due to specificity; however, compounds which interact with the Ca^{2+} binding sites in calpain enzymes have proved more successful.

Cathepsins

Cathepsins are endosomal/lysosomal proteases, the majority being cysteine proteases (cathepsins B, C, F, H, K, L, O, S, T, V, W and X). Others are serine (cathepsins A and G) or aspartate proteases (cathepsins D and E). Synthesised as inactive zymogens, they undergo proteolytic activation within optimal pH conditions. Traditionally seen as contributors to tumour invasion and increased metastasis through extracellular degradation, they are increasingly becoming implicated in apoptotic pathways, particularly in the pathway triggered by cytokines and antiblastic drugs (i.e. those utilised in cancer chemotherapy).[23,29–33] The lysosomal apoptotic pathway is being established alongside the traditional extrinsic and intrinsic forms. Predominant cathepsins involved in apoptosis are B and D, with L playing a supportive role. Whilst recognising that cathepsin D is an aspartate protease, it has been included in this section of cysteine proteases for clarity and continuity of theme.

Cathepsins, tumour progression and metastasis

The most abundant of the cathepsins is ‘B’, whose housekeeping duties include digestion of cell proteins, nucleic acids, complex carbohydrates and lipids through exo- and endopeptidase activity.[34] Expression is found in many cancer cell lines including cervical, breast, prostate and glioblastoma. Increased serum and associated tumour levels of cathepsin B correlates with increased disease state in patients with cervical carcinoma.[35] Cathepsin B’s contribution to tumour progression is founded in its ability to degrade the interstitial matrix and basement membranes, allowing local invasion and metastasis of cancer cells.

Similarly, cathepsin D’s ability to degrade the extracellular matrix implicates it in the promotion of malignant invasion. Overexpression and secretion of cathepsin D by oestrogen correlates with high metastatic potential in breast cancer (reviewed by Garcia *et al.*[36]).

Cathepsin S, a protease particularly stable at extracellular pH, is observed to have increased activity and expression levels in astrocytomas. These levels directly relate to prognostic outcome and as such may be considered as a useful prognostic marker and additionally become a target for anti-invasive therapy.[37,38]

Cathepsins during apoptosis

Although demonstrated as promoting tumour cell invasion and metastasis, cathepsins contribute towards cell death. Chemotherapy-induced cell death has been associated with noncaspase dependent release of cathepsin D from lysosomes.[39] Furthermore, gene expression and cellular content of cathepsin D are upregulated in pyramidal neurons of Alzheimer’s disease brains.[40] Downregulation inhibits apoptosis induced by tumour necrosis factor-α (TNF-α), TRAIL (TNF-related apoptosis-inducing ligand) and three chemotherapy drugs in addition to impairing cytochrome *c* release from mitochondria.[39] Cathepsin B has been

demonstrated to be involved in apoptosis of hepatocytes, neurons and immune cells.[41–43]

As cathepsins are lysosomal, and apoptotic signal transduction pathways are cytoplasmic, mitochondrial and nuclear, translocation of cathepsins B and D to alternative subcellular locations occurs in response to apoptotic stimuli.[44–46] Translocation may be tightly regulated or due to lysosomal membrane disruption. Additionally, lysosomal membranes can be weakened during normal ageing processes as observed in neuronal cells (reviewed by Yamashima[34]). Release of cathepsin B can be mediated by active caspase-2 or -8.[32] Cytosolic cathepsins B and D promote mitochondrial release of cytochrome *c* potentially through cleavage of Bid.[32,47,48] Cathepsins, like caspases, may form a reaction cascade, with cathepsin D being activated upstream of both cathepsin B and mitochondrial events. Cathepsin D directly cleaves and activates cathepsin B.[49–50] Two p53 binding sites in the promoter region of cathepsin D intimate a role for p53 in the cathepsin D upregulation.[30]

Relationship between cathepsins and caspases

Cathepsin B readily cleaves caspase-1 and procaspase-11 (although these caspases do not contribute to apoptotic processes), with weak cleavage activity shown towards caspase-2, -6, -7 and -14.[46] Cathepsins B, H, K, L, S and X fail to directly cleave executioner caspases pro-caspase-3 and -7, suggesting cathepsins promote caspase activation by stimulating preceding mitochondrial events including the cleavage of Bid. This process in turn affects the precise balance between pro- and anti-apoptotic Bcl-2 family proteins.[51,39] Utilisation of cathepsin B and D inhibitors blocks the caspase-3 cascade, thereby preventing or delaying apoptosis.[23] The serine protease cathepsin G directly activates caspase-7.[52]

Conversely, Zhu and Uckun[53] reported that incubation with cathepsin inhibitor Z-Phe-Gly-NHO-Bz induced rapid apoptosis in various human leukaemia and lymphoma cell lines which was caspase- and p53- and MAP kinase independent.

Clearly interpretation of research into the dual role of cathepsins within apoptosis and tumour progression can be perplexing. Cathepsins are well documented as promoting tumour progression and metastasis and their expression is clearly upregulated in many tumour cell lines. However, additionally they are recognised as contributing to the apoptotic programme and, as such, their inhibition leads to either enhanced or diminished cell death. A suggested interpretation is that upon apoptotic stimulation cathepsins play a pro-apoptotic role. However, within tumour cells where the regulatory apoptotic mechanism is dysfunctional, cathepsin expression is upregulated and extracellular proteolysis of matrix proteins occurs.

Inhibitors of cathepsins: stefins and cystatins

Cystatins are tight and reversible binding inhibitors of the papain-like cysteine proteases including cathepsins B, H, L and S.[54] Sharing extensive sequence homology, this superfamily is subdivided into three families; the stefins, the cystatins and the kininogens. Cathepsin D inactivates human kininogen, stefins and cystatin C indicating a biological role in regulation of cysteine protease activity (reviewed by Turk and Bode[54]).

Stefins, with a molecular weight of approximately 11 kDa, are potent reversible and competitive inhibitors for cysteine proteases. They exist both intra- and extracellularly. Stefin A (cystatin A) may play a role in the prevention of apoptosis as it contributes to the prevention of lysosomal instability.[55] Stefin B (cystatin B) is distributed uniformly amongst different tissues. It binds tightly to cathepsins H, L and S and less tightly to cathepsin B.[56]

Cystatins, molecular weight 13 kDa, are endogenous, natural inhibitors of cathepsins L and B, and reside intra- and extracellularly. The most investigated member of this family is the ubiquitously expressed cystatin C, the most potent inhibitor against cathepsins B, H, L and S.[57] Cystatin F binds tightly to cathepsins F, K, L and V and less tightly to cathepsins S and H. It has no inhibitory effect on cathepsins B, C and X.[58]

Kininogens are single chain proteins which are strong inhibitors of papain and cathepsin L but weaker inhibitors of cathepsin H and B.[54] These inhibitors form technically reversible, but very tight complexes with cysteine proteases and block the active site.[59]

Caspases

Caspases are aspartic acid-specific proteases which take the form of inactive cytosolic zymogens, where they are converted into active enzymes by the effects of apoptotic inducers.[3] There are three subfamilies belonging to the caspases: an ICE subfamily, comprising caspases -1, -4 and -5; a CED-3/CPP32 subfamily, comprising caspases -3, -6, -7, -8, -9 and -10; and an ICH-1/Nedd2 subfamily, caspase-2.[60] Caspases are the most prominent group of proteases involved in the regulation, execution and progression of apoptosis and are activated by proteolysis autocatalytically or by other caspase family members.[61] Apoptotic caspases can be defined as 'initiator' caspases that trigger the apoptotic cascade; or 'executioner' caspases that disassemble the cell structure.

Inhibitors of caspases

There are several families of apoptotic inhibitors which exert their effect on various intracellular locations and pathways through interaction with the caspases. Caspase inhibitors bind to the active site of caspases forming either a reversible or irreversible linkage. These include ubiquitous baculoviral proteins and mitochondrial proteins.

The cowpox virus cytokine response modifier A (CrmA), a member of the serpin family, is a potent inhibitor of some active initiator caspases and those involved in inflammation.[62] Additionally XIAP, cIAP1 and cIAP2, members of the inhibitors of apoptotic proteins (IAP) family, are potent caspase inhibitors.[63,64]

The baculoviral cell survival factor p35 directly inhibits activation of most of the caspase family enzymes.[60,65–68]

Synthetic inhibitors are based on a peptide recognition sequence attached to a functional group. Functional groups available reported to target caspases include: aldehydes (CHO), chloromethylketones (CMK), fluoromethylketones (FMK), or fluoroacyloxymethylketones (FAOM).

Threonine proteases

Proteasome, the first described threonine protease, is to date the only identified protease within this family implicated in apoptosis. Its proteolytic housekeeping duties are primarily responsible for the degradation of proteins in eukaryotic cells. The 26S protease complex is central to the ubiquitin-dependent pathway with the proteolytic core of the complex represented by the 20S proteasome.[69–70]

Proteasome

The 26S proteasome is a large (2 MDa) ATP-dependent proteolytic complex found in the cytosol and nucleus of eukaryotic cells. It is composed of the 20S proteasome and a pair of symmetrically disposed 19S regulatory particles (Figure 2).[71] Targeted substrate proteins are labelled for destruction by the covalent attachment of a small protein named ubiquitin. Ubiquitinated proteins are bound to the outer structures of the 26S proteasome and in the presence of energy supplied through the hydrolysis of ATP they unfold leading to conformational change, thus permitting passage through a narrow pore formed by subunits, to undergo proteolysis. A de-ubiquitinating enzyme salvages the ubiquitin molecule for reuse.

The 20S proteasome is described as being a unique high-molecular-mass (700 kDa) threonine protease complex of 22–31 kDa subunits possessing several distinct catalytic activities, including chymotrypsin-like, trypsin-like and peptidylglutamyl peptide hydrolysing actions.[1] The overall proteolytic contribution made by these catalytic groups varies between cell types.[72] The fundamental structure of the proteasome is described as a tube formed by the stacking of four rings on top of one another.[73] Each ring is constructed of seven α- or β-subunits. The outer rings, composed of α-subunits, control access of protein molecules to the proteolytic inner chamber with the inner rings, composed of β-subunits, carrying the catalytic sites.

Functions of proteasome

The ubiquitin–proteasome system degrades and processes proteins for disposal by ATP/ubiquitin-dependent or ubiquitin-independent proteolytic mechanisms.[74–76] Intracellular degradation is essential for homeostasis, allowing removal of misfolded and short-term regulatory proteins. Additionally, the proteasome is believed to be responsible for the turnover of defective proteins during ageing.[77,78] With proteolytic activity confined to the inner cavity, substrates include cell growth and differentiation regulatory proteins alongside transcription factors such as p53.[79]

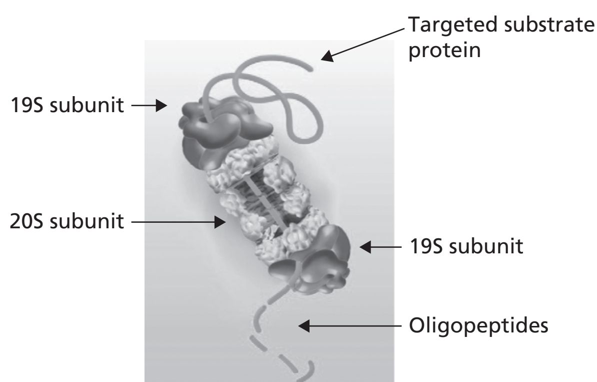

Figure 2 Schematic diagram of the proteasome. Image credit: U.S. Department of Energy Genomics:GTL Program, http://doegenomestolife.org

It has been suggested that proteasomes are essential as positive regulators of cell proliferation and may have a role in protecting the cell against apoptosis.[80] The relationship between the proteasome and apoptosis has been highlighted in several cell lines.[1,81,82] In particular, apoptotic death has been induced in the monocytic cell line U937 following the addition of lactacystin, a microbial metabolite which covalently binds to the amino-terminus of the proteasome catalytic β-subunit, thus selectively inhibiting its proteolytic activities.[77,81,83]

Inhibitors of proteasome

The involvement of the proteasome in various pathophysiological processes makes it a potential target for synthetic inhibitors. In particular, proteasome inhibitors are potent anti-angiogenic agents *in vivo* which can contribute to their overall effectiveness as potential anticancer agents.[84]

PS-341 (Velcade, bortezomib), an example of the boronic acid peptide inhibitors, is a potent and specific proteasomal inhibitor. It was the first proteasomal inhibitor to enter clinical trials and has been approved for the treatment of relapsed and refractory multiple myeloma.[85] PS-341 acts through both the extrinsic and intrinsic apoptotic signalling pathways.

In 1998, Lynas *et al.*[86] reported that peptidyl α-keto aldehydes, previously shown to be excellent inhibitors of serine and cysteine proteases, had a profound inhibitory effect on the proteasome.[87] The cell permeable Bz-LLL-COCHO (a peptidyl-α-keto-aldehyde) targets all proteasomal subunits, a therapeutically advantageous property as most proteasomal inhibitors mainly target only one catalytic subunit, with most focusing on the chymotrypsin-like activities.

The natural product lactacystin specifically inhibits proteolytic activities of the proteasome.[83] Incubation of cells with this inhibitor reduced proteasome activity and increased apoptosis as measured by TUNEL and caspase-3 activity.[79,88]

Proteasome inhibition by benzyloxycarbonyl-Leu-Leu-leucinal (Z-LLLal) induces apoptosis through accumulation of the tumour suppressor transcription factor p53, which is normally degraded by the proteasome.[1] Use of Z-LLLal, a reversible ubiquitin–proteasome inhibitor, also markedly accelerates cleavage of Bcl-2 to a 22 kDa fragment, thus promoting cell death through the apoptotic pathway via a caspase-3 like protease.[79] This inhibitor, a leupeptin analogue, decreases degradation of ubiquitin-conjugated proteins by the 26S protease complex without affecting its ATPase or isopeptidase activities.[79]

Accumulation of various proteins following proteasome inhibition contributes to release of apoptotic factors from mitochondria and subsequent activation of caspases. However, the induction of apoptosis following incubation with

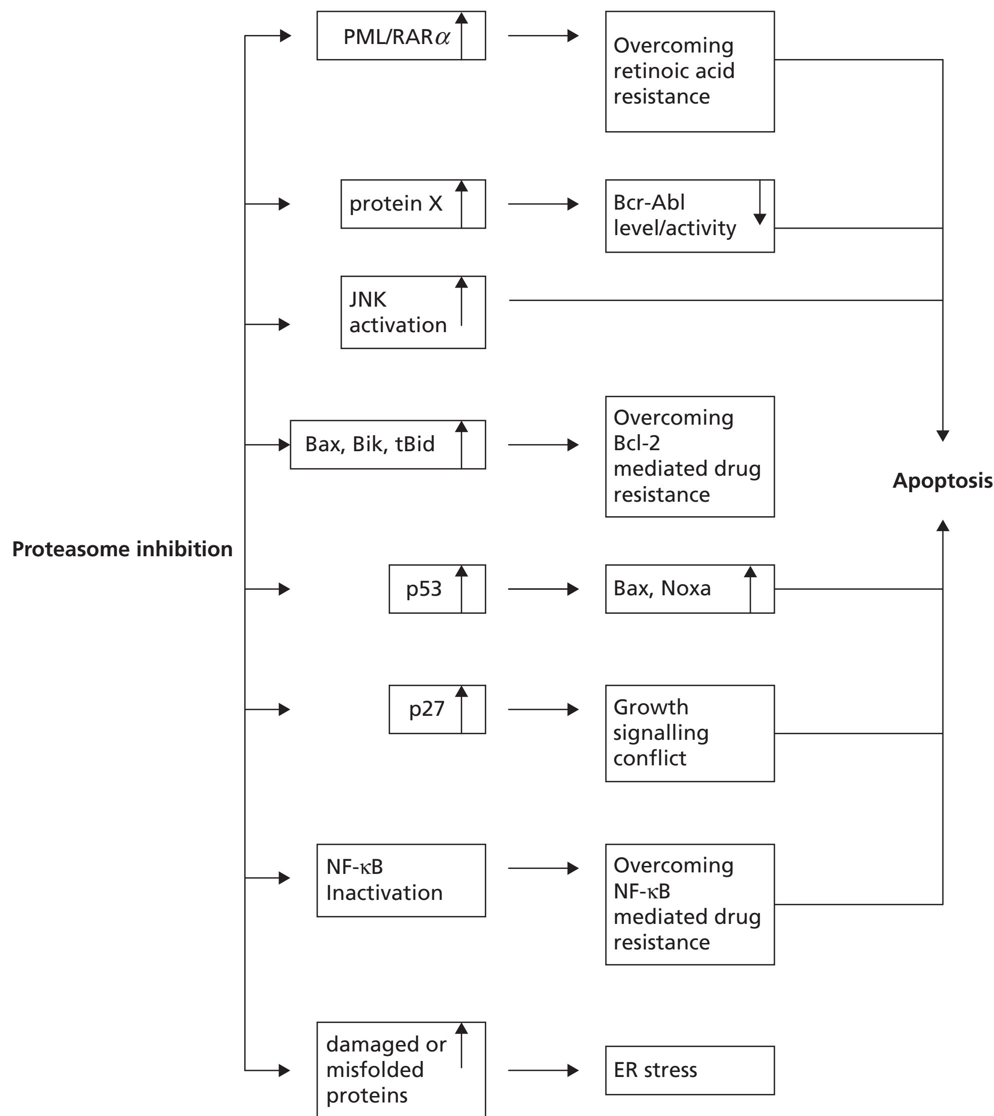

Figure 3 Mechanisms of induction of apoptosis by proteasome inhibitors. Adapted from Almond and Cohen[84]

proteasome inhibitors varies between cell types and is the outcome of various signalling targets (Figure 3).

Serine proteases

Implication of serine proteases in apoptosis was suggested as early as 1987, where serine protease inhibitors prevented apoptosis induced in SK-MEL-109 melanoma cells.[89] By 1994, the relationship between serine proteases and apoptosis was further established when it was demonstrated that introduction of chymotrypsin and trypsin into tumour cells led to cell death that was indicative of apoptosis.[90] Abate and Schroder[2] found that lipopolysaccharide caused a concentration-dependent toxicity in a macrophage cell line, which was completely inhibited by the serine protease inhibitors *N*-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK). Suggestions have been made that serine proteases may be required for the formation of the apoptosome within the intrinsic apoptotic pathway.[91] In 2003, a novel trypsin-like serine protease isolated from macrophages was identified by Chen *et al.*[4] and designated EOS. This discovery led to the suggestion that other serine proteases may be involved in macrophage apoptosis. More recently, it has been suggested that serine proteases are required for the generation of nuclear events such as condensation, fragmentation and DNA degradation observed in late stage apoptosis.[92]

Other serine proteases with apoptotic functionality have already been well documented, and include Omi/HtrA2, granzyme B, thrombin and AP24. It is noted that serine proteases have been implicated as functioning both up- and downstream of caspase-3.

Although several apoptotic serine proteases have been identified and described, research continues to highlight many unidentified serine proteases, in particular chymotrypsin-like with an apoptotic role.[26,93] However, identification of these proteases remains elusive.

Granzyme B, granzyme A, thrombin and AP24

Granzyme B is a member of a family of serine proteases expressed exclusively by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Upon receptor-mediated fusion of a CTL or NK cell with an infected target cell, granzymes are extruded in a perforin-dependent manner into the target cell and induce apoptosis.[94] Granzyme subfamilies have trypsin-like, chymotrypsin-like and elastase-like specificities and are expressed as zymogens within cytolytic granules. The major role for granzymes is the destruction of virus-infected or other potentially harmful cells. Granzyme B possesses the strongest apoptotic activity of the family. It triggers increased mitochondrial membrane permeability, cytochrome *c* release and downstream caspase activation by cleaving Bid.[95] Moreover, granzyme B cleaves at aspartate residues and activates the caspase cascade through direct interaction with several procaspases.[96] In addition, it has the ability to cleave some caspase substrates e.g. poly (ADP-ribose) polymerase (PARP) and Bid, as does the less effective granzyme A. This characteristic may be due to granzyme B possessing structurally similar active sites to the cysteine proteases of the caspase family.[96]

Granzyme A, the only granzyme to be found as a dimer, is the most abundant protease in CTL cell granules. It allows CTL cells to kill target cells under conditions where granzyme B activity is inhibited. Granzyme A has trypsin-like activity and cleaves both the nucleolar phosphoprotein nuclelolin and pro-IL-1β *in vitro*.[97,98] It is implicated in chromosomal degradation, a process which occurs during apoptosis, in addition to cleaving downstream caspase substrates.[94,99] In contrast to granzyme B, DNA fragmentation induced by granzyme A is caspase-independent (for review see Johnson[24]).

At high concentrations thrombin, a trypsin-like protease, impairs tumour cell growth by cell cycle arrest and caspase-dependent apoptosis.[12] This observation is not observed in normal cells, indicating an important role for thrombin as a carcinogenic therapeutic tool.

AP24, a chymotrypsin-like serine protease, is activated by a variety of apoptotic stimuli and is implicated in initiating internucleosomal DNA fragmentation. This occurs indirectly through the inactivation of leucocyte elastase inhibitor (LEI) via translational modification. Inhibition of AP24 is observed by overexpression of Bcl-2 in HL-60 cells.[100]

Omi/HtrA2

The mature serine protease Omi (also known as HtrA2), is identified as being a mitochondrial direct BIR-3-binding protein and caspase activator.[101] BIR motifs bind directly to caspases, thus inhibiting their activity.[67] Omi belongs to the heat shock response serine proteases (HtrAs) whose role is essential for cell survival at high temperatures.[102] In addition to being an apoptotic factor, it acts as a molecular chaperone for other mitochondrial proteins.[103]

Ordinarily, Omi is sequestered within the intermembrane space of the mitochondria, preventing proteolytic damage to healthy cells. Cytosolic translocation occurs upon loss of mitochondrial integrity following apoptotic stimulation. Over expression of Omi/HtrA2 within the cytoplasm of mammalian cells induces apoptosis, indicating a functional role for the protease within the mitochondrial apoptotic pathway.[101] Omi induces apoptosis in human cells in a caspase-independent manner through proteolytic activity and in a caspase-dependent manner through its ability to disrupt caspase–IAP interaction.[101] The IAP-binding motif of Omi begins at residue 134; this requires proteolytic processing at residue 133 to remove its N-terminal leader sequence.[101] This leader sequence also contains a typical mitochondrial targeting sequence within its first 60 residues, which upon Omi's localisation within the mitochondria is removed by mitochondrial processing peptidases.[101] Omi, like the mitochondrial proapoptotic protein Smac/DIABLO, disrupts interaction of caspase-9 with XIAP, thus permitting the promotion of procaspase-3 activation.[104,105] Furthermore, Omi participates with other apoptotic factors in the overall sensitivity of cells to apoptosis.[101]

Tripeptidyl-peptidase II

Tripeptidyl-peptidase II (TPPII), a subtilisin protease, was first discovered by Balow *et al.*[106] in 1983 whilst attempting to find a peptidase specific for phosphorylated sequences. Due to its characteristic sequential removal of tripeptides from a free N-terminus, the enzyme was originally termed tripeptidyl aminopeptidase, however it was renamed in 1986.[107] TPPII exists in both cytosolic and membrane-bound form exhibiting ubiquitous distribution in eukaryotes.[108] Structurally TPPII resembles the proteasome. Electron microscopic examination reveals a rod-shaped particle approximately 50 nm in length and 17 nm in diameter with a longitudinal segmentation pattern and an internal channel running end-to-end.[109,110] Subunits (138 kDa) form oligomeric complexes with a molecular weight of approximately 4 MDa. These complexes are essential for complete enzymatic activity, and proteolytic control is achieved through association and dissociation of the subunits.[111]

TPPII acts as both an exo- and an endopeptidase within an optimal environment of pH 7.5.[109] As an exopeptidase, TPPII demonstrates broad substrate specificity although preferential hydrolysis occurs after hydrophobic residues. In addition it does not cleave before or after proline residues; however, cleavage can occur during endopeptidase activity.

The relationship between TPPII and the proteasome is continually being debated, with TPPII considered as playing a complementary and/or substitutive role. The proteasome produces oligopeptides with a mean length of 3–22 residues, which are further degraded by TPPII into tripeptides. TPPII compensates to a degree for the loss of proteasome in proteasome-inhibitor-adapted cells.[109,112]

TPPII plays a role in pathological conditions such as muscle sepsis, cancer cachexia, apoptosis, antigen presentation and obesity.[108,112–119] It is critical for MHC class I peptide generation through antigen processing.[118] TPPII is implicated as participating in *Shigella*-induced macrophage pro-inflammatory apoptosis through the promotion of caspase-1 maturation.[116] Furthermore, studies have suggested that TPPII plays a role in TRAIL and TNF-α mediated apoptotic pathways.[120] If TPPII activity is blocked, then oligopeptides are not effectively degraded, potentially leading to peptide accumulation, which in turn will have an effect on cellular metabolic pathways. The membrane variant of TPPII

degrades and inactivates cholecystokinin' a neuropeptide family involved in food intake regulation, and, as such, may provide a role in pharmacological targeting of obesity and its related diseases.[108]

Inhibitors of serine proteases

Serpins

Studies demonstrating the effect of the naturally occurring serine proteinase inhibitors (serpins) in regulating apoptosis also provide indirect evidence for the role of serine proteases in this process. The serpin family, known for displaying broad-spectrum anti-serine proteinase activity, are reported to be involved in cell death regulation.[16] Whilst several members of the serpin family have been shown to inhibit apoptosis such as cytokine response modifier A (CrmA), serine protease inhibitor 1 (SPI-1), proteinase inhibitor 9 (PI-9), plasminogen activator inhibitor 2 (PAI-2), protease nexin 1 (PN-1), intriguingly, serine protease inhibitor 2 (SPI-2) has been implicated in the induction of neural apoptosis.[121]

Chloromethyl ketones

Chloromethyl ketones are often described as synthetic serine protease inhibitors, however they exhibit pronounced and extensive inhibitory activity towards the –SH group in cysteine proteases.[122–125] In addition, they are known to alkylate glutathione.[126,127] Because they exhibit this broad reactivity, some of the biological effects observed are attributable to their inhibition of other biological targets.[126] Much work into the role of chloromethyl ketones was carried out by Shaw *et al.*[128] and Coggins *et al.*,[129] recognising that whilst chloromethyl ketones, in particular TPCK and TLCK, demonstrated inhibitory effects, some degree of cross-reactivity should be expected.

N-α-tosyl-L-phenylalanine chloromethyl ketone and N-α-tosyl-L-lysine chloromethyl ketone

TPCK (Figure 4) is an irreversible inhibitor of chymotrypsin, of chymotrypsin-like serine proteases and of some cysteine proteases. Originally synthesised as an irreversible inhibitor, it was reported as reacting specifically and irreversibly with the histidine residue in the active centre of proteases with high affinity for chymotrypsin and chymotrypsin-like enzymes.[130] In addition to chymotrypsin proteases, a number of proteins are reported to be TPCK targets and include: protein kinases, aldehyde dehydrogenase, fibroblast interferon, transcription factor TFIIIC, EFTu, luciferase and a viral oncoprotein (reviewed by Gillibert *et al.*[131]). Reports that TPCK targets a serine protease leading to the inhibition of internucleosomal DNA fragmentation has since been disregarded.[132] These findings indicated that TPCK should not solely be classified an inhibitor of proteases.

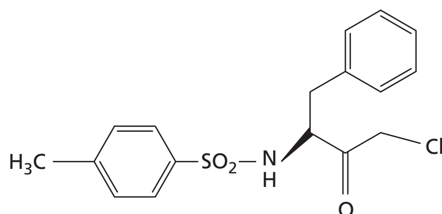

Figure 4 Structure of *N*-α-tosyl-L-phenylalanine chloromethyl ketone

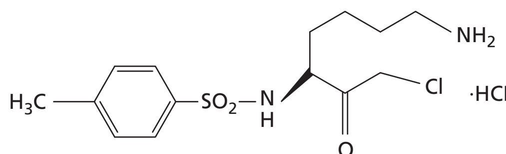

Figure 5 Structure of *N*-α-tosyl-L-lysine chloromethyl ketone

TLCK (Figure 5), like TPCK, is often described as a serine protease inhibitor and is generally referred to as an inhibitor specific for trypsin-like proteases. TLCK however, is also a cysteine protease inhibitor.[133] In particular, it targets cathepsin B so potentially any apoptotic effects reported following incubation with TLCK might be due to the inhibition of intracellular cysteine and serine proteases.[41]

Both TPCK and TLCK inhibit protein kinase C (PKC), an enzyme with a critical role in tumour promotion.[134] Whilst PKC catalyses the phosphorylation of serine and threonine residues in proteins it has, in addition, various roles.[135] The effects of TPCK and TLCK are cell-type specific and indeed have been suggested as being cytotoxic during long exposures, leading to cell necrosis.[25,91,136]

These inhibitors are involved in the prevention of phosphorylation of and subsequent degradation of IκB-α protein in response to phorbol 12-myristate 13-acetate (PMA), TNF-α and okadaic acid.[137–142] IκB-α remains bound to NF-κB, thus preventing its translocation into the nucleus thereby preventing apoptosis.

Ala-Ala-Phe-chloromethyl ketone

Ala-Ala-Phe-chloromethyl ketone (AAF-CMK; Figure 6) inhibits TPPII irreversibly, alongside demonstrating marginal inhibition of the chymotrypsin-like activity of the proteasome.[109] Some degree of nonspecificity is found, as AAF-CMK reversibly and potently inhibits TPPI.[143]

Diphenyl phosphonates

Diphenyl phosphonate groups are specific and potent irreversible inhibitors of serine proteases.[144] They are known to covalently react with the active site serine, affording a phosphonylated and irreversibly inhibited enzyme.[145,146] Further specificity is achieved by the addition of P1 position amino acid residues to the diphenyl phosphonate warhead, for example, phenylalanine will target chymotrypsin-like, leucine

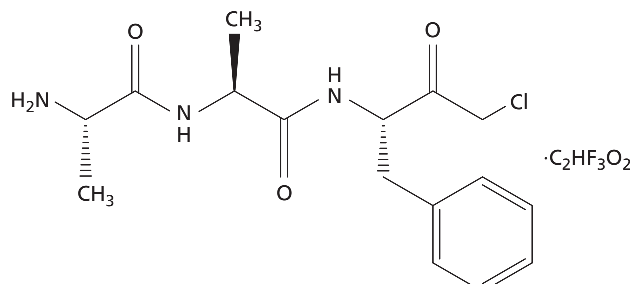

Figure 6 Structure of Ala-Ala-Phe-chloromethyl ketone

for trypsin-like and valine for elastase-like proteases. Diphenyl phosphonates are advantageous over other synthetic serine protease inhibitors as they show no activity against cysteine proteases, are nontoxic *in vivo* and are exceptionally stable under physiological conditions.[144,147,148] Visualisation of the targeted proteases can be obtained by the addition of an affinity label to the peptide. This can be biotinylated for identification with streptavidin or fluorogenic, which is detected with fluorescent microscopy or UV light.

Phenylmethylsulfonyl fluoride and Pefabloc

Phenylmethylsulfonyl fluoride (PMSF) is a nonspecific serine protease inhibitor, targeting proteases such as chymotrypsin, trypsin, thrombin and thiol proteases such as papain. PMSF is considered a highly toxic cholinesterase inhibitor, and as such, may be unsuitable for use within cell-model systems. Pefabloc (AEBSF) irreversibly inhibits a wide spectrum of serine proteases, thus is nonspecific in detection of serine subclasses. Less toxic than PMSF, it is more suitable for cell culture conditions.

Conclusions

Unregulated apoptosis, a process implicated in many disease states, is due in part to a disruption in the balance of intra- and intercellular proteolytic activities. Increasingly research is implicating serine proteases in apoptotic processing, with several already being identified – Omi/HtrA2, granzymes A and B and AP24. During the past few years much research focused on the 'unknown' serine proteases, with a plethora of papers published implicating serine proteases in apoptosis but falling short of their identification.[2,5,26,89,93] Identification is paramount as these serine apoptotic regulators might provide additional or alternative therapeutic targets.

This goal is dependent upon the availability of selective, potent, stable and specific target protease inhibitors. Chloromethyl ketones are described as synthetic serine protease inhibitors; however they exhibit broad- and cross-reactivity and are unstable in the presence of serum.[122–125,127–129] They are not ideal as cellular research tools as many of the biological effects observed are attributable to their inhibition of other biological targets.[126] In comparison, diphenyl phosphonates have many advantages as they show no activity against cysteine proteases, are nontoxic and are exceptionally stable under physiological conditions.[144,147]

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This review received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

References

1. Shinohara K *et al*. Apoptosis induction resulting from Proteasome inhibition. *J Biochem* 1996; 317: 385–388.
2. Abate A, Schroder H. Protease inhibitors protect macrophages from lipopolysaccharide-induced cytotoxicity: possible role for NF- kappa B. *Life Sci* 1998; 62: 1081–1088.
3. Grabarek J, Darzynkiewicz Z. In situ activation of caspases and serine proteases during apoptosis detected by affinity labeling their enzyme active centers with fluorochrome-tagged inhibitors. *Exp Hematol* 2002; 30: 982–989.
4. Chen C *et al*. A novel serine protease predominately expressed in macrophages. *J Biochem* 2003; 374: 97–107.
5. O'Connell AR *et al*. Characterization of a serine protease-mediated cell death program activated in human leukemia cells. *Exp Cell Res* 2006; 312: 27–39.
6. Sanchez EF *et al. Handbook of Proteolytic Enzymes*, 2nd edn. London: Academic Press, 2003.
7. Barrett AJ. Proteases. *Encyclopedia of Life Sciences*, www.els.net. Chichester: John Wiley & Sons Ltd, 2001.
8. Chapman HA *et al*. Emerging roles for cysteine proteases in human biology. *Annu Rev Physiol* 1997; 59: 63–88.
9. Seemuller E *et al*. Proteasome from *Thermoplasma acidophilum*: a threonine protease. *Science* 1995; 268: 579.
10. Barrett AJ, Rawlings ND. Families and clans of serine peptidases. *Arch Biochem Biophys* 1995; 318: 247–250.
11. Di Cera E, Krem MM. Serine proteases. *Encyclopedia of Life Sciences*, www.els.net. Chichester: John Wiley & Sons Ltd, 2005.
12. Stenson-Cox C *et al*. In the cut and thrust of apoptosis, serine proteases come of age. *Biochem Pharmacol* 2003; 66: 1469–1474.
13. Rawlings ND, Barrett AJ. Families of serine peptidases. *Methods Enzymol* 1994; 244: 19–61.
14. Lesk AM, Fordham WD. Conservation and variability in the structures of serine proteinases of the chymotrypsin family. *J Mol Biol* 1996; 258: 501–537.
15. Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases. *Protein Sci* 1995; 4: 337–360.
16. Bird PI. Serpins and regulation of cell death. *Results Probl Cell Differ* 1998; 24: 63–89.
17. Chang C, Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. *Trends Cell Biol* 2001; 11: S37–43.
18. Ray JM, Stetler-Stevenson WG. The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. *Eur Respir J* 1994; 7: 2062–2072.
19. Sternlicht MD, Werb Z. How metalloproteases regulate cell behaviour. *Annu Rev Cell Dev Biol* 2001; 17: 463–516.
20. Debouck C *et al*. Human immunodeficiency virus protease expressed in Escherichia coli exhibits autoprocessing and specific maturation of the gag precursor. *Proc Nat Acad Sci U S A* 1987; 84: 8903–8906.
21. Fujinaga M *et al*. The molecular structure and catalytic mechanism of a novel carboxyl peptidase from Scytalidium lignicolum. *Proc Nat Acad Sci U S A* 2004; 101: 3364–3369.
22. Sims AH *et al*. Glutamic protease distribution is limited to filamentous fungi. *FEMS Microbiol Lett* 2004; 239: 95–101.
23. Castino R *et al*. Lysosomal proteases as potential targets for the induction of apoptotic cell death in human neuroblastomas. *Int J Cancer* 2002; 97: 775–779.
24. Johnson DE. Noncaspase proteases in apoptosis. *Leukemia* 2000; 14: 1695–1703.
25. Gong B *et al*. Ionizing radiation-induced, Bax-mediated cell death is dependent on activation of cysteine and serine proteases. *Cell Growth Diff* 1999; 10: 491–502.
26. Egger L *et al*. Serine proteases mediate apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions. *Cell Death Diff* 2003; 10: 1188–1203.
27. Yousefi S *et al*. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol* 2006; 8: 1124–1132.

28. Saito K *et al*. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proc Natl Acad Sci U S A* 1993; 90: 2628–2632.
29. Deiss LP *et al*. Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. *EMBO J* 1996; 15: 3861–3870.
30. Wu GS *et al*. Potential role for cathepsin D in p53-dependent tumor suppression and chemosensitivity. *Oncogene* 1998; 16: 2177–2183.
31. Shibata M *et al*. Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation. *Biochem Biophys Res Commun* 1998; 251: 199–203.
32. Guicciardi ME *et al*. Cathepsin B contributes to TNF-α-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome *c*. *J Clin Invest* 2000; 106: 1127–1137.
33. Zhu D-M, Uckun FM. Z-Phe-Gly-NHO-Bz, an inhibitor of cysteine cathepsins, induces apoptosis in human cancer cells. *Clin Cancer Res* 2000; 6: 2064–2069.
34. Yamashima T. Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog Neurobiol* 2000; 62: 273–295.
35. Makarewicz R *et al*. Cathepsin B in predicting the extent of the cervix carcinoma. *Neoplasma* 1995; 42: 21–24.
36. Garcia M *et al*. Biological and clinical significance of cathepsin D in breast cancer metastasis. *Stem Cells* 1996; 14: 642–650.
37. Flannery T *et al*. The clinical significance of cathepsin S expression in human astrocytomas. *Am J Pathol* 2003; 163: 175–182.
38. Flannery T *et al*. Cathepsin S expression: an independent prognostic factor in glioblastoma tumours-a pilot study. *Int J Cancer* 2006; 119: 854–860.
39. Emert-Sedlak L *et al*. Involvement of cathepsin D in chemotherapy-induced cytochrome *c* release, caspase activation, and cell death. *Mol Cancer Ther* 2005; 4: 733–742.
40. Cataldo AM *et al*. Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: evidence for early up-regulation of the endosomal-lysosomal system. *Neuron* 1995; 14: 671–680.
41. Roberts LR *et al*. Cathepsin B contributes to bile salt-induced apoptosis of rat hepatocytes. *Gastroenterology* 1997; 113: 1714–1726.
42. Felbor U *et al*. Neuronal loss and brain atrophy in mice lacking cathepsins B and L. *Proc Natl Acad Sci U S A* 2002; 99: 7883–7888.
43. Michallet MC *et al*. Cathepsin-dependent apoptosis triggered by supraoptimal activation of T lymphocytes: a possible mechanism of high dose tolerance. *J Immunol* 2004; 172: 5405–5414.
44. Li W *et al*. Uptake of oxidized LDL by macrophages results in partial lysosomal enzyme inactivation and relocation. *Arterioscler Thromb Vasc Biol* 1998; 18: 177–184.
45. Roberg K, Ollinger K. Oxidative stress causes relocation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomyocytes. *Am J Pathol* 1998; 152: 1151–1156.
46. Vancompernolle K *et al*. Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity. *FEBS Lett* 1998; 438: 150–158.
47. Johansson AC *et al*. Cathepsin D mediates cytochrome c release and caspase activation in human fibroblast apoptosis induced by staurosporine. *Cell Death Differ* 2003; 10: 1253–1259.
48. Blomgran R *et al*. Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. *J Leukocyte Biol* 2007; 81: 1213–1223.
49. Nishimura Y *et al*. Identification of latent procathepsins B and L in microsomal lumen: characterization of enzymatic activation and proteolytic processing in vitro. *Arch Biochem Biophys* 1988; 61: 64–71.
50. Rowan AD *et al*. Rat procathepsin B. Proteolytic processing to the mature form in vitro. *J Biol Chem* 1992; 267: 15993–15999.
51. Stoka V *et al*. Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route. *J Biol Chem* 2001; 276: 3149–3157.
52. Zhou Q, Salvesen GS. Activation of pro-caspase-7 by serine proteases includes a non-canonical specificity. *Biochem J* 1997; 324(Pt 2): 361–364.
53. Zhu DM, Uckun FM. Cathepsin inhibition induces apoptotic death in human leukemia and lymphoma cells. *Leuk Lymphoma* 2000; 39: 343–354.
54. Turk V, Bode W. The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett* 1991; 285: 213–219.
55. van Nierop K *et al*. Lysosomal destabilization contributes to apoptosis of germinal center B-lymphocytes. *J Histochem Cytochem* 2006; 54: 1425–1435.
56. Turk B *et al*. Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol Chem* 1997; 378: 141–150.
57. Lindahl P *et al*. Interaction of recombinant human cystatin C with the cysteine proteinases papain and actinidin. *Biochem J* 1992; 281(Pt 1): 49–55.
58. Langerholc T *et al*. Inhibitory properties of cystatin F and its localization in U937 promonocyte cells. *FEBS J* 2005; 272: 1535–1545.
59. Dickinson DP. Cysteine peptidases of mammals: their biological roles and potential effects in the oral cavity and other tissues in health and disease. *Crit Rev Oral Biol Med* 2002; 13: 238–275.
60. Cohen GM. Caspases: the executioners of apoptosis. *J Biochem* 1997; 326: 1–16.
61. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science* 1998; 281: 1312–1316.
62. Komiyama T *et al*. Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. *J Biol Chem* 1994; 269: 19331–19337.
63. Deveraux QL *et al*. X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 1997; 388: 300–304.
64. Roy N *et al*. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J* 1997; 16: 6914–6925.
65. Xue D, Horvitz HR. Inhibition of the Caenorhabditis elegans cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature* 1995; 377: 248–251.
66. Bump NJ *et al*. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p35. *Science* 1995; 269: 1885–1888.
67. Deveraux QL, Reed JC. IAP family proteins - suppressors of apoptosis. *Genes Dev* 1999; 13: 239–252.
68. Clem RJ, Miller LK. Control of programmed cell death by the baculovirus genes p35 and iap. *Mol Cell Biol* 1994; 14: 5212–5222.
69. Eytan E *et al*. ATP-dependent incorporation of 20S protease into the 26S complex that degrades proteins conjugated to Ubiquitin. *Proc Natl Acad Sci U S A* 1989; 86: 7751–7755.
70. Peters JM *et al*. Structural features of the 26 S proteasome complex. *J Mol Biol* 1993; 234: 932–937.
71. Peters JM *et al*. Distinct 19 S and 20 S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. *J Biol Chem* 1994; 269: 7709–7718.
72. Crawford LJ *et al*. Comparative selectivity and specificity of the proteasome inhibitors BzLLLCOCHO, PS-341, and MG-132. *Cancer Res* 2006; 66: 6379–6386.
73. Groll M *et al*. Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature* 1997; 386: 463–471.

74. Hochstrasser M. Ubiquitin-dependent protein degradation. *Ann Rev Genet* 1996; 30: 405–439.
75. Hershko A. Roles of ubiquitin-mediated proteolysis in cell cycle control. *Curr Opin Cell Biol* 1997; 9: 788–799.
76. Pickart CM. Targeting of substrates to the 26S proteasome. *FASEB J* 1997; 11: 1055–1066.
77. Coux O *et al*. Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem* 1996; 65: 801–847.
78. Baumeister W, Lupas A. The proteasome. *Curr Opin Struct Biol* 1997; 7: 273–278.
79. Zhang X-M *et al*. Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. *J Biochem* 1999; 340: 127–133.
80. Fujiwara T *et al*. Proteasomes are essential for yeast proliferation. cDNA cloning and gene disruption of two major subunits. *J Biol Chem* 1990; 265: 16604–16613.
81. Imajoh-Ohmi S *et al*. Lactacystin, a specific inhibitor of the Proteasome, induces apoptosis in human monoblast U937 Cells. *Biochem Biophys Res Commun* 1995; 217: 1070–1077.
82. Grimm LM *et al*. Proteasomes play an essential role in thymocyte apoptosis. *EMBO J* 1996; 15: 3835–3844.
83. Fenteany G *et al*. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* 1995; 268: 726–731.
84. Almond JB, Cohen GM. The proteasome: a novel target for cancer chemotherapy. *Leukemia* 2002; 16: 433–443.
85. Kane RC *et al*. Velcade: U.S. FDA approval for the treatment of multiple myeloma progressing on prior therapy. *Oncologist* 2003; 8: 508–513.
86. Lynas JF *et al*. Inhibitors of the chymotrypsin-like activity of proteasome based on di- and tri-peptidyl alpha-keto aldehydes (glyoxals). *Bioorg Med Chem Lett* 1998; 8: 373–378.
87. Walker B *et al*. Peptide glyoxals: a novel class of inhibitor for serine and cysteine proteinases. *Biochem J* 1993; 293(Pt 2): 321–323.
88. Andersson M *et al*. Caspase and proteasome activity during staurosporine-induced apoptosis in lens epithelial cells. *Invest. Ophthalmol Vis Sci* 2000; 41: 2623–2632.
89. Ruggiero V *et al*. Protection from tumor necrosis factor cytotoxicity by protease inhibitors. *Cell Immunol* 1987; 107: 317–325.
90. Williams MS, Henkart PA. Apoptotic cell death induced by intracellular proteolysis. *J Immunol* 1994; 153: 4247–4255.
91. Dong Z *et al*. Serine protease inhibitors suppress cytochrome c-mediated caspase-9 activation and apoptosis during hypoxia-reoxygenation. *Biochem J* 2000; 347(Pt 3): 669–677.
92. O'Connell AR *et al*. Caspase-dependant activation of chymotrypsin-like proteases mediates nuclear events during Jurkat T cell apoptosis. *Biochem Biophys Res Commun* 2006; 345: 608–616.
93. Murn J *et al*. Internucleosomal DNA cleavage in apoptotic WEHI 231 cells is mediated by a chymotrypsin-like protease. *Genes Cells* 2004; 9: 1103–1111.
94. Trapani, J.A. Granzymes. a family of lymphocyte granule serine proteases. *Genome Biol* 2001; 2: 3014.1–3014.7.
95. Alimonti JB *et al*. Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. *J Biol Chem* 2001; 276: 6974–6982.
96. Thornberry NA *et al*. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem* 1997; 272: 17907–17911.
97. Pasternack MS *et al*. Granzyme A binding to target cell proteins. Granzyme A binds to and cleaves nucleolin in vitro. *J Biol Chem* 1991; 266: 14703–14708.
98. Irmler M *et al*. Granzyme A is an interleukin 1 beta-converting enzyme. *J Exp Med* 1995; 181: 1917–1922.
99. Hayes MP *et al*. Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. *J Exp Med* 1989; 170: 933–946.
100. Wright SC *et al*. Activation of CPP32-like proteases is not sufficient to trigger apoptosis: inhibition of apoptosis by agents that suppress activation of AP24, but not CPP32-like activity. *J Exp Med* 1997; 186: 1107–1117.
101. Hegde R *et al*. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem* 2002; 277: 432–438.
102. Spiess C *et al*. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell* 1999; 97: 339–347.
103. Vaux DL, Silke J. HtrA2/Omi, a sheep in wolf's clothing. *Cell* 2003; 115: 251–253.
104. Suzuki Y *et al*. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 2001; 8: 613–621.
105. Verhagen AM *et al*. HtrA2 promotes cell death through Its serine protease activity and its ability to antagonize Inhibitor of Apoptosis Proteins. *J Biol Chem* 2002; 277: 445–454.
106. Balow RM *et al*. Tripeptidyl aminopeptidase in the extra-lysosomal fraction of rat liver. *J Biol Chem* 1983; 258: 11622–11628.
107. McDonald JK, Barrett AJ. *Mammalian Proteases: A Glossary and Bibliography*, *Vol. 2: Exopeptidases*. London, UK: Academic Press, 1986.
108. Rose C *et al*. Characterization and inhibition of a cholecystokinin-inactivating serine peptidase. *Nature* 1996; 380: 403–409.
109. Geier E *et al*. A giant protease with potential to substitute for some functions of the proteasome. *Science* 1999; 283: 978–981.
110. Macpherson E *et al*. Supramolecular structure of tripeptidyl peptidase II from human erythrocytes as studied by electron microscopy, and its correlation to enzyme activity. *Biochem J* 1987; 248: 259–263.
111. Tomkinson, B. Tripeptidyl-peptidase II. In: Barrett AJ *et al*., eds. *Handbook of Proteolytic Enzymes*, 2nd edn. London: Elsevier Academic Press, 2004: 1882–1885.
112. Gavioli R *et al*. c-myc overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells. *Nat Cell Biol* 2001; 3: 283–288.
113. Wray CJ *et al*. Tripeptidyl-peptidase II expression and activity are increased in skeletal muscle during sepsis. *Biochem Biophys Res Commun* 2002; 296: 41–47.
114. Hasselgren PO *et al*. Molecular regulation of muscle cachexia: it may be more than the proteasome. *Biochem Biophys Res Commun* 2002; 290: 1–10.
115. Chand A *et al*. Effect of cancer cachexia on the activity of tripeptidyl-peptidase II in skeletal muscle. *Cancer Lett* 2005; 218: 215–222.
116. Hilbi H *et al*. Tripeptidyl Peptidase II promotes maturation of caspase-1 in Shigella flexneri-induced macrophage apoptosis. *Infect Immun* 2000; 68: 5502–5508.
117. Hong X *et al*. Tumors acquire inhibitor of apoptosis protein (IAP)-mediated apoptosis resistance through altered specificity of cytosolic proteolysis. *J Exp Med* 2003; 197: 1731–1743.
118. Reits E *et al*. A major role for TPPII in trimming proteasomal degradation products for MHC Class I antigen presentation. *Immunity* 2004; 20: 495–506.
119. York IA *et al*. Tripeptidyl peptidase II is the major peptidase needed to trim long antigenic precursors, but is not required for most MHC class I antigen presentation. *J Immunol* 2006; 177: 1434–1443.

120. Mllnarczuk I *et al*. AAF-cmk sensitizes tumor cells to trail-mediated apoptosis. *Leuk Res* 2004; 28: 53–61.
121. Thiemmara V *et al*. Serine protease inhibitor Spi2 mediated apoptosis of olfactory neurons. *Cell Death Differ* 2002; 9: 1343–1351.
122. Whitaker JR, Perez-Villasenor J. Chemical modification of papain. I. Reaction with the chloromethyl ketones of phenylalanine and lysine and with phenylmethylsulfonyl fluoride. *Arch Biochem Biophys* 1968; 124: 70–78.
123. Shaw E. Selective chemical modification of proteins. *Physiol Rev* 1970; 50: 244–296.
124. Eichinger A *et al*. Crystal structure of gingipain R: an Arg-specific bacterial cysteine proteinase with a caspase-like fold. *EMBO J* 1999; 18: 5453–5462.
125. Rozman-Pungerčar J *et al*. Inhibition of papain-like cysteine proteases and legumain by "caspase-specific" inhibitors: when reaction mechanism is more important than specificity. *Cell Death Differ* 2003; 10: 881–888.
126. Rossman T *et al*. Inhibition of macromolecular synthesis in *Escherichia coli* by protease inhibitors. Specific reversal by glutathione of the effects of chloromethyl ketones. *J Biol Chem* 1974; 249: 3412–3417.
127. Tsan M.F. Inhibition of neutrophil sulfhydryl groups by chloromethyl ketones. A mechanism for their inhibition of superoxide production. *Biochem Biophys Res Commun* 1983; 112: 671–677.
128. Shaw E *et al*. Evidence for an active-center histidine in trypsin through use of a specific reagent, 1-chloro-3-tosylamido-7-amino-2-heptanone, the chloromethyl ketone derived from Nα-tosyl-L-lysine. *Biochemistry* 1965; 4: 2219–2224.
129. Coggins JR *et al*. Affinity labelling of proteinases with tryptic specificity by peptides with C-terminal lysine chloromethyl ketone. *Biochem J* 1974; 137: 579–585.
130. Schoellmann G, Shaw E. Direct evidence for the presence of histidine in the active center of chymotrypsin. *Biochemistry* 1963; 2: 252–255.
131. Gillibert M *et al*. Another biological effect of tosylphenylalanylchloromethane (TPCK): it prevents p47phox phosphorylation and translocation upon neutrophil stimulation. *Biochem J* 2005; 386(Pt 3): 549–556.
132. King MA *et al*. Pro- and anti-apoptotic effects of an inhibitor of chymotrypsin-like serine proteases. *Cell Cycle* 2004; 3: 1566–1571.
133. Kaufmann SH *et al*. Specific proteolytic cleavage of poly (ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* 1993; 53: 3976–3985.
134. Solomon DH *et al*. N-alpha-Tosyl-L-lysine chloromethyl ketone and N-alpha-tosyl-L-phenylalanine chloromethyl ketone inhibit protein kinase C. *FEBS Lett* 1985; 190: 342–344.
135. Kikkawa U *et al*. Calcium-activated, phospholipid-dependent protein kinase from rat brain. Subcellular distribution, purification, and properties. *J Biol Chem* 1982; 257: 13341–13348.
136. Mlejnek P. Can application of serine protease inhibitors TPCK and TLCK provide evidence for possible involvement of serine protease Omi/HtrA2 in imatinib mesylate-induced cell death of BCR-ABL-positive human leukemia cells? *Leukemia* 2005; 19: 1085–1087.
137. Finco TS *et al*. Inducible phosphorylation of I kappa B alpha is not sufficient for its dissociation from NF-kappa B and is inhibited by protease inhibitors. *Proc Natl Acad Sci U S A* 1994; 91: 11884–11888.
138. Higuchi M *et al*. Protease inhibitors differentially regulate tumor necrosis factor-induced apoptosis, nuclear factor-kappa B activation, cytotoxicity, and differentiation. *Blood* 1995; 86: 2248–2256.
139. Grammer TC, Blenis J. The serine protease inhibitors, tosylphenylalanine chloromethyl ketone and tosyllysine chloromethyl ketone, potently inhibit pp70^{s6k} activation. *J Biol Chem* 1996; 271: 23650–23652.
140. Miyamoto S *et al*. Tumor necrosis factor alpha-induced phosphorylation of I kappa B alpha is a signal for its degradation but not dissociation from NF-kappa B. *Proc Natl Acad Sci U S A* 1994; 91: 12740–12744.
141. Lin Y *et al*. Activation of NF-kappa B requires proteolysis of the inhibitor I kappa B-alpha: signal-induced phosphorylation of I kappa B-alpha alone does not release active NF-kappa B. *Proc Natl Acad Sci U S A* 1995; 92: 552–556.
142. DiDonato JA *et al*. Phosphorylation of I kappa B alpha precedes but is not sufficient for its dissociation from NF-kappa B. *Mol Cell Biol* 1995; 15: 1302–1311.
143. Page AE *et al*. Purification and characterization of a tripeptidyl peptidase I from human osteoclastomas: evidence for its role in bone resorption. *Arch Biochem Biophys* 1993; 306: 354–359.
144. Oleksyszyn J, Powers JC. Irreversible inhibition of serine proteases by peptide derivatives of (alpha-aminoalkyl)phosphonate diphenyl esters. *Biochemistry* 1991; 30: 485–493.
145. Oleksyszyn J *et al*. Novel amidine-containing peptidyl phosphonates as irreversible inhibitors for blood coagulation and related serine proteases. *J Med Chem* 1994; 37: 226–231.
146. Belyaev A *et al*. Structure-activity relationship of diaryl phosphonate esters as potent irreversible dipeptidyl peptidase IV inhibitors. *J Med Chem* 1999; 42: 1041–1052.
147. Oleksyszyn J, Powers JC. Amino acid and peptide phosphonate derivatives as specific inhibitors of serine peptidases. *Methods Enzymol* 1994; 244: 423–441.
148. De Meester I *et al*. In vivo inhibition of dipeptidyl peptidase IV activity by Pro-Pro-diphenyl-phosphonate (Prodipine). *Biochem Pharmacol* 1997; 54: 173–179.