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Preface

Proteinase Inhibitors and Activators Strategic Targets for Therapeutic Intervention

The tertiary structure of the first proteinase, human pancreatic trypsin, was elucidated more recently than the span of a single research lifetime. This was achieved as the result of a monumental effort, made more so by the requirement to use what was then the state of the art in computing, using index cards [1].

The new millenium starts with vastly superior tools available to the researcher, from discovery of new proteinases at the genomic level to interpretation of their function in the pathology of the disease state at the proteomic level. The availability of the isolated materials by molecular biological and biochemical techniques enables full and rapid chemical and biochemical characterization of the proteins and their substrates, to such an extent that recombinant Fixa can now be proscribed therapeutically in haemophilia B.

The value of the study of proteinases, both human and those of pathogens (e.g. plasmepsins in malarial propogation) in understanding the aetiology of disease (Table 1) and developing therapeutic approaches and an understanding of the challenges still to be faced in this area is best approached from the outstanding contributions of the researchers in this field. This conference [2] and the articles herewithin are representative of the forefront of the Proteinase activator and Inhibitor field.

PROTEINASES DISCOVERY AND MECHANISM

The current status of the field is that less than 40% have been identified of an estimated 700–1100 human proteases and ~ 150 proteinase inhibitors, of which so far 260 and 36 are annotated, respectively, as such; the total being estimated from the 9445 human mRNAs in GenBank to date [3]. Libraries of human cDNA from specific tissues can be screened and the gene products expressed without post-translational modification in procaryotic cell lines or in eucaryotic cell lines with, for example, glycosylation or gamma-carboxylation.

A novel matrix metalloproteinase, named Endometase, of 40% sequence homology to MMP-7 has been identified [4] from a human cancerous cell line from endometrial tissue. Characterization shows a K_{cat}/K_m value of 1850 $M^{-1}s^{-1}$ for the synthetic substrate Mca-PLGLDpa(Dnp)AR-NH2 and its inhibition by tissue inhibitors of metalloproteinases (TIMPs).

Proteinase products can be screened. Intracellular processing of amyloid precursor protein by the beta-site cleaving enzyme (BACE) deposits the β-amyloid variant Aβ1-42, which was shown by the SELDI technique [5] to be the major constituent of senile and neuritic plaques in the brain tissue of Alzheimer's disease patients. Intracellular cholesterol was shown to upregulate the deposition of Aβ1-42, suggesting a role in γ-secretase activity.

CATEGORIZATION OF PROTEINASES

Proteinases can be broadly categorized based on the chemical mechanism that they employ to catalyse the hydrolysis of amide bonds in their polypeptide substrates compared to a rate for uncatalysed amide hydrolysis in water of 10^{-9}–10^{-10} S^{-1} [6].

Serine, threonine and thiol proteases use activation of the catalytic residues, while metallo and aspartic proteinases use an activated water as the nucleophile. Analysis of the catalytic mechanism by crystallography is limited by growth of the crystals of the complexes with ligands and cofactors, especially where this involves a cell surface [7].

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PROTEINASE ACTIVATION: ACTIVATORS IN MECHANISM AND REGULATION

Serine Proteinases

Serine proteases play roles in haemostasis and thrombosis (the coagulation proteases, including FIXa, FXa and thrombin), regulated tissue remodelling involved in both physiological and pathological processes, including cancer invasion and metastasis, arthritis and various fibroproliferative vascular disorders (Table 1). The involvement of the enzymes can be either direct (e.g. urokinase and the enzyme that it generates, plasmin) or by activation of MMPs, (e.g. during inflammation, APC and complement proteases) and immune response (e.g. the mast cell proteinase tryptase). The coagulation proteases are now additionally considered to play a role in cancer, perhaps by activation of the cellular receptors, Par 1, 2 or 3. It is understood from classical proteinase biochemistry that the peptide on the 'primed' [8] side of the scissile bond is the leaving group during the catalytic process. Consequently, it has been proposed that the proteinase has low affinity for substrate sequence on the 'primed' side [9]. It has been the classical approach to develop inhibitors that only exploit the 'unprimed' sites, for example for thrombin (see Figure 1). However, some natural thrombin inhibitors are known to derive much of their binding affinity by interaction with the primed regions (e.g. nazuamide, hirudin).

It is, therefore, postulated that inhibitors that bind to the 'primed' sites of the target enzyme can be developed that have higher affinity for these regions that the natural sequence of the substrate.

The adenoviral serine proteinases highlight a further exquisite mechanism of proteinase regulation, where the proteinase is synthesized in an inactive form. The enzyme molecules enter the immature virus particles in a complex with viral DNA that increases its catalytic rate constant (K_{cat}) for substrate hydrolysis 225-fold. The proteinase cleaves off an 11-amino acid peptide from the *C*-terminus of the precursor to virion protein VI, pVI. This peptide (pVIc) then binds ($K_d = 5$ nM) to the enzyme that generated it forming a ternary complex with 15800

Table 1 Pathologies Associated with Proteinases

Proteinase	Mechanism	Class	Pathology
Thrombin	Coagulation	Sp	MI, Stroke, PTCA, UA, septic shock
Fxa	Coagulation	Sp	MI, DVT
UPA	Fibrinolysis	Sp	Cancer
Renin	Angiotensin 1 formation	Ap	Hypertension
Human neutrophil elastase	Inflammation	Sp	Pulmonary disease
HIV-1 protease	HIV replication	Ap	AIDS
Plasmepsin I, II	*Plasmodium falciparum* metabolism	Ap	Malaria
Picornovirus Proteinases	RNA virus replication	Tp	Common cold
Proteosome proteinases	Protein metabolism, inflammatory response	Tp	Cancer, inflammation
Caspase (ICE)	Apoptosis	Tp	Rheumatoid arthritis
ACE	Angiotensin II formation	Zinc Mp	Hypertension
TACE	Tumour α-necrosis factor release	Zinc Mp	Arthritis, MS
MMP-1	Fibroblast collagenase degradation	Zinc Mp	Inflammation, peridontal disease
MMP-2	Gelatinase	Zinc Mp	Cancer, MS, angiogenesis
MMP-3 (stromelysin)	Matrix degradation	Zinc Mp	Cancer, arthritis
MMP-7 (matrilysin)	Matrix degradation	Zinc Mp	Cancer, arthritis
Neutral endopeptidase	ANP release	Zinc Mp	Hypertension

Class: Sp = serine proteinase; Ap = Aspartic proteinase; Tp = thiol proteinase; Mp = metalloproteinase.
ANP = atrial natriuretic polypeptide.
DVT = deep vein thrombosis; MI = myocardial infarction; MS = multiple sclerosis; PTCA = percutaneous transluminal coronary angioplasty.

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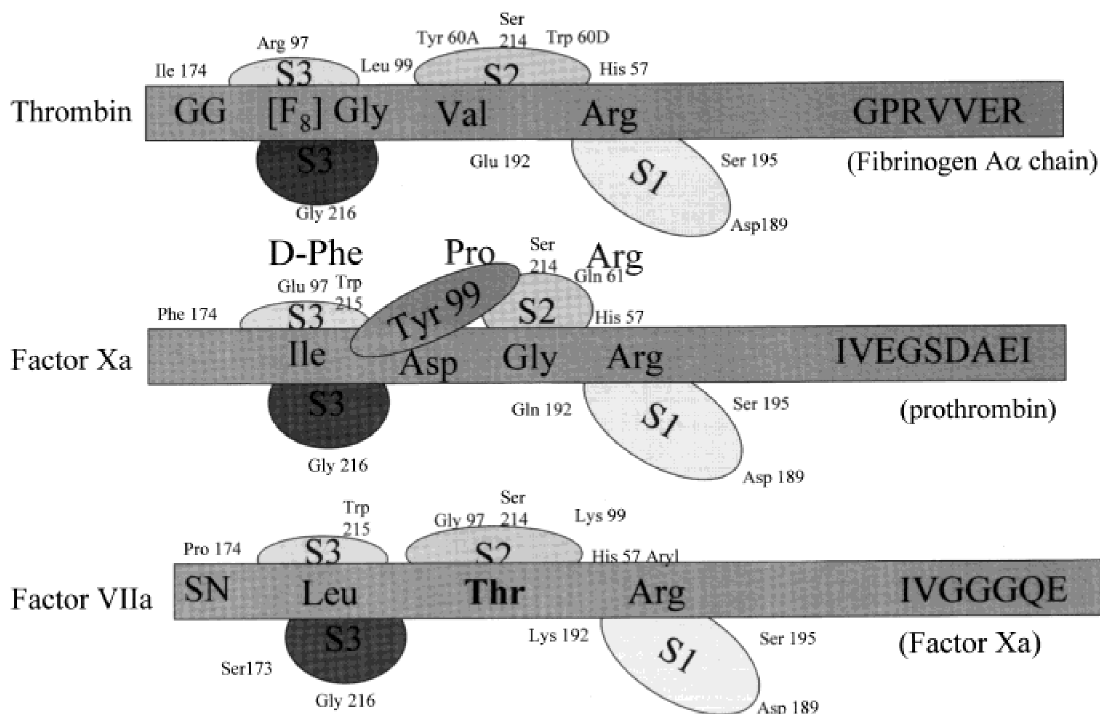

Figure 1 Long oblong (green) represents the sequence (three letter amino acid codes) at the cleavage site of the principal natural substrate (in parenthesis) of the proteinase. For thrombin 'F_8' indicates that the residue in position 8 of the cleavage sequence of fibrinogen occupies the 'S2'site during cleavage. Semicircles represent specificity sites of the enzyme in the classical nomenclature (Schecter and Berger [8]). Numbered residues correspond to the residues of the proteinase (chymotrypsin numbering).

fold rate enhancement activating virion particles with a 50 equivalence [10].

Threonine Proteases

Threonine proteinases that constitute some of the 14 active sites of the proteosome 20S unit [11] are involved in the intracellular degradation of proteins in eukaryotic cells, leading to both removal of proteins for housekeeping and presentation of epitopes for immune response. Crystal structures of the bacterial equivalent proteosome have been solved [12]. Uniquely, the threonine is here an *N*-terminal residue, utilizing both its amino and carboxyl as nucleophiles, with possible stabilization by adjacent Lys, Glu and Gly (as the oxyanion pocket) residues.

Thiol/Cysteine Proteinases

Thiol/Cysteine proteinases include papain, ICE (interleukin-1 converting enzyme, now called caspase-1), calpains and proteinases from the picornoviruses (including hepatitis A virus, poliovirus and rhinovirus). Thiol proteinases are structurally distinct, but generally use a similar catalytic mechanism to that of serine proteinases (Table 1).

Aspartic Proteinases

Aspartic proteinases utilize the carboxyl residue groups of two aspartic acids as the catalytic group [13]. The endopeptidases of this class include pepsin, renin, HIV-1 and -2 proteases, the intracellular Cathepsin D, plasmepsin I and II of the malarial *Plasmodium falciparum* and they are produced by the *Candida* yeast strains.

Metalloproteinases

Metalloproteinases utilize zinc co-ordinated to three groups from the enzyme and an 'activated' water molecule as the catalytic centre. Since the

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mechanism has an intermediate with the neo-*N*-terminus co-ordinated to the enzyme hence the primed binding sites provide the specificity for the binding to the enzyme. These include carboxypeptidase A, Angiotensin converting enzyme and tumour necrosis factor-α convertase.

Neutral Endopeptidase and the Large Matrix Metalloproteinase Class

Matrix metalloproteinases (MMPs) are major modulators of the cellular environment, effecting localized proteolytic degradation of the extracellular matrix (ECM), the release and processing of growth factors and the modification of cell-cell interactions. MMPs are generally secreted as proenzymes and association with the cell surface or ECM components is an important feature of their regulation, modulating their relationship with activators, inhibitors and substrates [14].

Small Molecule Proteinase Inhibitors

For catalysis of cleavage of a peptide bond by a proteinase, there is a loss of free energy from the loss of entropy due to desolvation and loss of translational and rotational degrees of freedom balanced to the enthalpic gain of specific interactions and burying of hydrophobic surface of the ligand [15]. The most useful tool for assessing these conformational and thermodynamic balances is computational modelling however this is limited for complete systems to Newtonian rather than quantum mechanics for macromolecules. For this reason, it typically has an accuracy of ± 3 kcal mol^{-1} which is equivalent to an error of one–two orders of magnitude in predicting the affinity of a ligand for its receptor [16].

Proteinases have evolved the orientation of their active site residues to bind tightly to the transient chemical species that occurs during amide bond cleavage. For efficient catalysis, the strongest interactions must be with this transition state species, given excess substrate [17,18] and so chemical analogues of this species have been designed. For serine proteinases, the first transition state is related to the tetrahedral carbon that decays to the acyl enzyme intermediate.

Peptide boronic acids are highly effective reversible inhibitors of serine proteinases, since the adduct is a transition state analogue where an aminoboronic acid or Boro(aa) corresponds to the replacement of the CO_2 of the equivalent amino acid by BO_2. Specific, tight binding boronic acid based inhibitors have been reported for elastase (K_i, 0.25 nM), chymotrypsin (K_i, 0.16 nM) and cathepsin G (K_i, 21 nM) [19]. For example, the following compounds, Z-D-Phe-Pro-BoroIrg-OPin [20], (1) Z-D-Dpa-Pro-BoroIrg-Opin (2) and H-D-Phe-Pro-boro-Arg-OH have comparable initial K_i for thrombin (initial K_i for thrombin 1.5, 2.8 and 1.2 nM, respectively, [21]) and, when pre-incubated with thrombin, also form a tighter complex (K_i final for compound Z-D-Phe(1), Z-D-Dpa(2), and H-D-Phe-Pro-boroArg-OH, 4, 65 and 4 pM, respectively).

For compounds (1) and (2) additionally, the isothiouronium side chain at P1 is analogous to arginine, of DUP714, in its ability to salt bridge to Asp-189. It is interesting to note that the recently reported crystal structure of DUP-714 (Ac-D-Phe-Pro-boroArg-OH) with thrombin [22] shows the ε-N to be a hydrogen bond acceptor, to a water molecule shared with Gly-219 of the S1 specificity pocket, a role that could be shared by the sulphur atom of Z-D-Phe-Pro-BoroIrg-OPin (1,), consistent with our observation that no loss of binding energy occurs due to this replacement.

It was shown (Deadman J, Chino N, Scully M., unpublished results) that the ubiquity of this S1 specificity pocket interaction leads to the inhibition of other trypsin-like serine proteinases which play a therapeutically vital role in *in vivo* thrombolysis (Plasmin, t-PA and Urokinase) abrogating the activity of the physiologically important fibrinolytic system. Z-D-Phe-Pro-BoroIrg-OPin (1) acted in the low nanomolar range as a potent inhibitor of urokinase mediated clot dissolution and of t-PA mediated activation of plasminogen [20]. By changing the P3 group from Z-D-Phe to Z-D-Dpa it is possible to achieve considerable modulation of the specificity towards thrombin giving IC_{50}s (concentration of inhibitor required to produce 50% inhibition of the enzyme) for inhibition of plasmin generation of 0.5 nM and 5 μM for the Z-D-Phe compounds (1) and Z-D-Dpa (2), respectively.

It is clear that the strength of the initial encounter complex formed with thrombin, as indicated by the initial K_i, compared to that of the other proteinases reflects the true selectivity of the compound as expected in a milieu of proteinases generated on initiation of coagulation in plasma. Time dependent inhibitors [31] can, given pre-incubation with thrombin, tighten to lower K_is. However compounds (1) and DUP-714, as seen from their tendency to inhibit equipotently in aPTT and TT assays [20], fail to discriminate between mixtures of enzymes. DUP-714 is a potent, slow, tight-binding inhibitor with

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many serine proteinases, e.g. thrombin, Factor Xa, t-PA and, from final, pre-incubated K_is, should retain selectivity for thrombin, (final K_{iPla}/K_{ithr} 120), and yet this compound has been shown to prolong fibrinolysis *in vivo*) [23]. Similarly, compound (1) inhibits plasminogen activation by t-PA, and prolongs urokinase mediated clot dissolution [20]. This is most consistent with compound (1) equilibrating between enzymes to a degree best described by its initial K_i (K_{iUK}/K_{iThr} 12, and for DUP-714 initial K_{ipla}/K_{iThr} 6), as if no preincubation could occur (final K_{iUK}/K_{ithr} 24).

The following rational describes the structure function of this relationship. When blood clots are formed at the bleeding site of a controlled dermal incision, there is a rapid generation of thrombin activity [24], the concentration of enzyme reached during this pulse is of the order of 100–200 nM, the enzyme being inhibited by plasma proteinase inhibitors (especially antithrombin III) over 3–4 min. Clearly, plasma concentrations of synthetic inhibitor would be needed to titrate the thrombin generated, and these concentrations will be above the initial K_i for inhibition of other plasma serine proteinases for a compound such as Ac-D-Phe-Pro-BoroArg (DUP-714), which has an initial K_i 5 nM for Plasmin.

Several groups have recognized the significance of selectivity for thrombin, compared to the trypsin-like fibrinolytic enzymes for a potential antithrombotic agent [25].

The initial association of a proteinase and its ligand (substrate or inhibitor) is limited to the collisional diffusion rate (approximately 10^{-8} s^{-1}), and it must be considered that the both components may exist free in solution as several conformations, with hydration and possible hydrophobic collapse [26] of residues. This folding phenomenon has also been shown to play a role in the interaction of small molecule synthetic inhibitors with proteinases. For example, the 'collapsed conformation' that DUP-714 adopts even when free in solution, as identified by the nuclear magnetic resonance (NMR) [27] as when finally bound to its target proteinase thrombin, means that the P3 group is compressed against the P2 proline, and, while this is a favoured configuration for interaction with thrombin, for other proteinases it is compact and so there is less steric effect to deter interaction. Ro 42-6240 [28], a non-peptide thrombin inhibitor, is also believed when free in solution to adopt a conformation, stabilized by intramolecular bonding, that is favoured for binding to thrombin. It is also likely that compound (1), which contains the same structural unit D-Phe-Pro as DUP-714, also adopts a compact conformation, hence its lack of specificity in the initial encounter complex with serine proteinases.

It should be noted that, when isolated enzymes are used for discussions of specificity of enzyme inhibitors, it is important to consider that IC_{50}s are not usually directly comparable for a given inhibitor between enzymes in different assays [29]. This arises because IC_{50} is defined by Equation (1) [30], which is dependent on the substrate used, and, as in this case for serine proteinase inhibitors, different substrates of optimum K_m with the target enzyme must generally be used.

$$IC_{50} = \left(\frac{1}{2}E_t + K_i\right) + \frac{K_i S}{K_m} \quad (1)$$

where E_t is the total enzyme concentration (M), K_m is the Michaelis constant, S is the substrate concentration and K_i is the inhibition constant.

A novel class of peptide boronic acid inhibitors of thrombin has been described containing the motif (D)-Phe-Pro- but in which arginine is replaced at the P1 site by a neutral grouping. Less potent at binding thrombin than DUP-714, they are generally more specific and in particular we noted that they were less able to inhibit the fibrinolytic system than the positively charged compounds [32].

Discovery of Novel Small Molecule Serine Proteinase Inhibitors

The urokinase-type plasminogen activator (uPA) is an interesting target for anti-metastatic therapy. A new class of low molecular weight, hydrophobic derivatives of 4-aminomethyl-phenylguanidine have recently been described [33]. Crystallographic analysis indicated that the substituents of the *p*-amino group occupy the S′ binding site, presenting an interesting option for analogue design (vide supra, [9]).

Mast cell tryptase inhibitors have been designed [34] based on 6A,6D-dideoxy-6A,6D-diamino-β-cyclodextrin as rigid template to bridge between subunits A/D or B/C of the tetrameric enzyme and to orientate the binding heads (3-(aminomethyl)-benzenesulfonyl-glycine) to interact with the Asp-189 residue of the S1 site. The bivalent inhibitor has an enhanced K_i compared to that of the binding head (0.002 μM, 26.0 μM, respectively), which is an example of multivalency in inhibitor design.

Hepatitis C virus encodes the protease NS3, and the inhibition of this protease is promising

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clinically. Potent peptide inhibitors derived from the P'-region [8] of the cleaved substrate have been described. For serine proteases, such as NS3, the P'-region of the substrate is not used for ground-state binding to the enzyme [9], however the S' region of NS3 has pockets which might be exploited for binding. S'-binding ligands, non-cleavable decapeptides spanning P6–P4′, display a range of interactions different from those used by the substrate and represent a novel class of NS3 inhibitors. [35]. Binding was increased to give an inhibitor of IC_{50} < 200 pM by introduction of the previously optimized P-region, change of the P4′ residue, and combinatorial optimization of positions P2′–P3′.

In a further approach, NS3 protease was screened by aldehyde 'transition state' analogue inhibitors from a 'mix and split' library of 960 peptide aldehydes for mappings subsite specificity [36].

Emerging Proteinase Targets for Small Molecule Inhibitors and Combinatorial Library Synthesis

The threonine proteinase activity of proteasome can be studied by use of small molecule inhibitors containing 'transition state analogue' groups that target the catalytic site. A variety of synthetic peptide inhibitors (peptidyl aldehydes, boronic acids and vinyl sulphones), as well as some known anti-tumour and antibiotic compounds, have been found to inhibit various peptidase activities of proteasomes and protein degradation by them [11].

Inhibition of Cysteine/Thiol Proteinases by Small Molecule Inhibitors

Caspases as an enzyme class have been the subject of intense study as potential clinical targets for their roles in inflammatory diseases [37,38] (Table 1). For example ICE, which is a cysteine protease that activates the inflammatory mediator of monocytes, IL-1β, by hydrolysis of pro-IL-1β. The catalytic dyad of Cys285 and His237 can be complexed by aldehydic groups, which achieve high potency by generating transition state analogue complexes, such as Ac-Trp-Glu-His-Asp-CHO ($K_i = 56$ pM) [39]. Recently these studies have been extended to be the subject of rational drug design using modelling and combinatorial techniques to generate focused libraries. This accelerates the drug development process by producing candidates that are not only potent and selective, but have good pharmacological characteristic like the orally bioavailable Vertex compound HMR3480/VX-740, which is now the subject of clinical trials [36].

Metalloproteinase Inhibition by Synthetic Ligands

Combinatorial chemistry has been used for rapid screening of small molecule peptide based metalloproteinase inhibitors containing the 'transition state inhibitor' phosphinic group to identify several potent and highly selective inhibitors of different zinc metalloproteases [40–43]. *In vivo* screening of these compounds has shown that the N-selective inhibitors of ACE can block the metabolism of the haematopoietic regulator peptide Ac-SDKP, without interfering with the metabolism of angiotensin I. These observations suggest that *in vivo* activities mediated by the two active sites of ACE can dissociated [43]. RXP407, a phosphinic peptide, is the first potent inhibitor of angiotensin I converting enzyme able to differentiate between its two active sites [44].

Potent phosphinic peptide inhibitors have also been developed for matrixins, a family of enzymes involved in both physiological and pathological processes [45] with *in vivo* activity. Treatment of subcutaneous (s.c.) C26 tumour-induced mice by daily intraperitoneal (i.p.) injection of this inhibitor slowed the growth of primary tumours.

Endopeptidase EC 3.4.24.15 (EP 24.15) is a thermolysin (TLN)-like metalloendopeptidase, which is expressed widely especially in the brain, pituitary and testis, and may play a role in neuropeptide regulation. The vasoldilator peptide bradykinin (RPPGFSPFR) is cleaved at the F-S bond by EP24.15. Inhibitors of the enzyme of μM affinity, of the order of five–ten fold weaker than the substrate, have been made by incorporation of unnatural β-amino acids (e.g. β-G) [46].

For example, studies of N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-amino benzoate (CFP) have shown it to be cleaved (Ala-Tyr bond) by EP24.11 in plasma. While incorporation of β-amino acids adjacent to the scissile bond can indeed stabilize the peptides against cleavage by EP 24.11 and still allow inhibition of EP 24.15 [47].

Neutral endopeptidase (NEP, EC 24.11) belongs mechanistically to the zinc metalloproteinase class of the bacterial protease TLN. This has been studied by site directed mutagenesis, crystalographic data and molecular modelling [48] or plurifunctional inhibitors have been developed. Several types of zinc chelators are used in inhibitors of this enzyme, a number of which are in clinical trial.

Tetanus and Botulinium toxins express zinc peptidases [49,50] and are also a target for inihbitors.

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PROTEINASE INHIBITORS: REGULATION OF FUNCTION BY PHYSIOLOGICAL NATURAL PRODUCT INHIBITORS

Inhibition of Serine Proteinases by Serpins

The coagulation proteases maintain the haemostatic balance by acting as a cascade of inactive, zymogen forms. In the cascade, the catalytic proteinases demonstrate high selectivity (K_m) for their target substrate zymogen, but generally low proteolytic activity (K_{cat}). To achieve attenuation at each step of the cascade requires assembly of complexes on the platelet surface that act to increase the local concentration of the proteinase and, therefore, its apparent catalytic efficiency and, by allosteric changes to directly stimulate proteolysis.

The activated proteinase are tightly regulated, by availability of the receptors, for example expression of the cofactor of FVIIa the macroglobulin tissue factor on endothelial cells, and its activation by the 'flip-flop' mechanism of PS exposure [50]. Additionally, the half-lives of the circulating protease are controlled by circulating inhibitors, such as the kazal based TFPI for FVIIa/TF and FXa. More recently, proteolytic degradation has also been shown to play a role in potentiation of thrombosis e.g. tissue associated fibrinolysis inhibitor.

Serpins can be highly selective, for example the plasminogen activator inhibitor or more general inhibitors such as alpha-2 macroglobulin or bovine lung-protein aprotinin, and may also require allosteric and template activation as in the inhibition of thrombin by the ATIII-heparin complex, which reduces thrombins plasma $t_{1/2}$ to 15 s, while Fxa is inhibted by ATIII and a pentasaccharide (LMWH).

The plasminogen activation system of serine proteinases in particular is implicated in pericellular proteolysis, plasmin having the capacity to hydrolyse components of the ECM both directly and indirectly, via activation of MMPs. The broad specificity of the proteinase plasmin is regulated through binding of the two plasminogen activators to specific cellular receptors: uPA to the glycolipid-anchored membrane protein uPAR and tPA to a type II membrane protein. These binary complexes interact with membrane-associated plasminogen reduce the K_m for plasminogen activation and protect the proteinases from their cognate serpin inhibitors [51].

For the serpin bovine pancreatic trypsin inhibitor (BPTI) and serine proteases, a large part of the contact surface area is made by the P1 residue side chain. Association energies determined for chymotrypsin and elastase were compared with data sets for other inhibitor families allowed estimates of the interscaffolding additivity, i.e. whether the free energies of association of P1 mutants of different inhibitors are independent of the type of inhibitor scaffolding [52]. The results show that, in many cases, introduction of the same mutations into P1 position produces similar energetic effect in different inhibitor structures.

Analogues of the Kazal based serine proteinase inhibitors have been synthesized by chemoselective ligation into the natural protein of ester bonds (–COO–) and/or a reduced peptide bonds (–CH_2NH–) in the binding loop region of turkey ovomucoid protein third domain (OMTKY3) and eglin c. Head-to-tail backbone cyclization was also performed on BPTI and eglin c in an attempt to understand functional consequences of protein cyclization [53].

The Bowman Birk family of serine proteinase inhibitor proteins (BBIs) have a symmetrical structure with two reactive site loops [54]. Nonapeptides constrained by disulphide bonds are mimics of the BBI 'bait' loop and libraries of peptides [55] have been shown to retain nM K_i's. [56]. Retention of activity in the synthetic peptides compared to the BBI's has been shown by NMR to be the result of achieving families of conformations that map to the X-ray structures of the full length proteins.

Tryptases are mast cell-specific serine proteinases that have been implicated as mediators in the pathogenesis of asthma and other allergic and inflammatory disorders. Their distinguishing features, the activity as a heparin-bound tetramer, narrow substrate specificity and resistance to plasma antiproteinases are perfectly explained by the crystal structure of human β2-tryptase. The tetramer consists of four quasi-equivalent monomers arranged in a flat frame-like structure. The active centers are directed towards a central pore whose narrow openings of $\sim 40 \times 15$Å restrict the access of macromolecular substrates and inhibitors. In particular, the plasma proteinase inhibitors are far too bulky to fit into the narrow pore [57]. The leech-derived tryptase inhibitor (LDTI), however, an 'atypical' Kazal-type inhibitor that is smaller than the classical members of this family, has just the size to allow two molecules to bind to two active centers of the tetramer, thus explaining experimental results showing $\sim$50% inhibition of the cleavage activity towards small chromogenic substrates. Multimeric LDTI-variants can even interact simultaneously with two monomers,

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resulting in a 100–1000-fold increased affinity and selectivity. These results emphasize the advantage of the principle of multivalency in molecular recognition processes and suggest that a bivalent or tetravalent approach is most promising for the design of highly potent and selective synthetic inhibitors for the tryptase tetramer [57].

The central role of the host serine endoprotease furin in the proteolytic activation of many pathogenic viruses (e.g. human cytomegalovirus [HCMV], infectious influenza A virus, HIV, Ebola virus) and bacteria (e.g. *Pseudomonas* exotoxin A and anthrax toxoid) makes this endoprotease a target for the development of antimicrobials.

α1-Antitrypsin Portland [58] the first potent and selective bio-engineered protein-based inhibitor of furin, has been shown to be an antipathogenic agent [59].

The prototypical member of the herpesviridae, HCMV, shows that the production of infectious virus is dramatically reduced by the exogenous addition of α1-PDX and it correlates with the ability of α1-PDX to block the furin-dependent processing of HCMV pro-gB. [59] Comparison to currently used antiherpetic agents shows that α1-PDX is ten-fold more effective in cell-culture models [59]. Studies with the recombinant haemagglutinin-precursor protein (HA0) of human H5N1 influenza A 'bird flu' virus demonstrated the ability of α1-PDX to block the furin-dependent activation of HA0 as well as preventing influenza A virus-induced cell–cell fusion activity *ex vivo*. Together with the lack of toxicity of α1-PDX, these results suggest that protein-based inhibitors of furin have potential as broad-based antiviral therapeutics.

Regulation of Matrix Metalloproteinases by TIMPS

MMPs are also regulated by a family of tissue inhibitors (TIMPs), and the role of TIMPS in these processes vary and can be explained by features of their structure and the interactions with specific MMPs [14].

Aspartic Proteinase Inhibition by Natural Products

Saccharomyces cerevisiae encodes a small (68 amino acid) protein inhibitor called IA3. [60] which is completely selective towards its target enzyme, the aspartic proteinase A. Structures were solved by X-ray crystallography for complexes of proteinase A with (1) a mutant protein form of IA3, and (2) a truncated, peptide form consisting only of residues 2–34, confirming that inhibitory activity resides in the *N*-terminal residues. Neither form of the free inhibitor had significant intrinsic secondary structure in solution but upon contact with the enzyme, residue 2–32 became ordered and adopted a classical α-helical conformation. This novel interaction indicates a mechanism whereby stabilization by the proteinase of a binding conformation of the inhibitor enhances specificity.

SUMMARY

The contributions in this meeting represent the state of the art in study of proteinases and their biological and therapeutic regulation. The near future of proteinase research will be fashioned by the new horizons of proteomics research [61], adding substance to the genomic data. Even new approaches in drug discovery, such as combinatorial chemistry, impact upon the understanding of the proteinase function, as with the discovery of a novel allosteric exosite in FVIIa by probing with libraries of ligands [62]. What is without question is that proteinases will remain at the forefront of understanding and intervention in human biochemistry and human disease pathology.

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JOHN DEADMAN

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