Biochemistry of Pulmonary Emphysema

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Contents

8. Proteinase Inhibitor Candidates for Therapy of Enzyme-Inhibitor Imbalances

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Rationale for Therapeutic Use of Proteinase Inhibitors

Proteinase inhibitors are important regulators of proteolytic processes in the healthy organism as well as potent protectors against destructive proteolysis in various diseases.

Our knowledge on their functional role arises either from congenital or acquired deficiencies in endogenous proteinase inhibitor proteins.

Best known examples are the predisposition for lung emphysema formation because of inherited α_1PI deficiency[1] and severe bleeding disorders (disseminated intravascular coagulation, DIC) due to massive consumption of antithrombin III (AT III) e.g. in septic shock.[2]

The major target enzyme of α_1PI is the lysosomal elastase from neutrophils. This digestive proteinase is thought to play a crucial role in degradation of lung elastin fibres and thus emphysema development if it is released extracellularly from accumulating neutrophils into lung tissue over many years without being effectively inhibited due to lack of α_1PI.

Severe deficiency in α_1PI can be caused also by inflammatory events, at least locally.

For example, strong stimulation of phagocytes accumulating (e.g. neutrophils, monocytes) or present (e.g. macrophages) in an infectious or traumatic focus leads to generation and extracellular release of numerous oxidants and lysosomal digestive enzymes. α_1PI is rapidly inactivated by both types of substances, e.g. oxidants like hydrogen peroxide together with myeloperoxidase[3] as well as cysteine and metalloproteinases.[4,5]

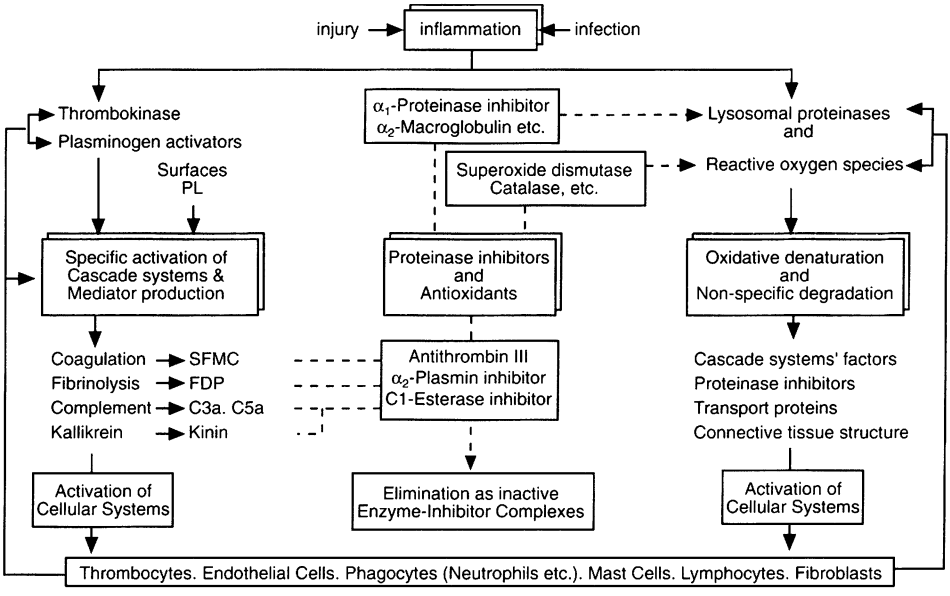

Fig. 1. Proteolysis-induced pathomechanisms in inflammatory processes. Activation of proteinase cascade systems and liberation of lysosomal proteinases concomitantly with reactive oxygen species may cause massive consumption of proteinase inhibitors which protect the organism against excessive system-specific proteolysis (see cascade systems) and unspecific proteolytic degradation by lysosomal enzymes.

As neutrophil elastase has its proteolysis optimum at slightly alkaline, i.e. physiological pH, this enzyme degrades effectively structural elements such as basal membranes, elastin and collagen fibres, fibronectin and proteoglycans as well as all kinds of humoral protein factors including the proteinase inhibitors regulating the plasmatic enzyme cascade systems (clotting, fibrinolysis, complement)[6] in the absence of inhibitors (Fig. 1).

In this way a local inflammatory process with an isolated impaired organ function due to e.g. oedema formation may become a generalized systemic inflammation leading finally to multiple organ failure and even death.[6]

Excessive consumption or destruction of proteinase inhibitors and especially of α_1PI during an inflammatory process is, therefore, a most critical event enabling the propagation of the manifold pathomechanisms inducible by proteinases which are "out of control" by their natural antagonists, the proteinase inhibitor proteins. As the natural sources for their preparation from human material are very restricted, the design of highly effective inhibitory proteins on the basis of human proteinase inhibitor molecules by molecular modelling and their production by recombinant DNA technology is the most promising approach at present to get the quantities necessary for proteinase inhibition therapy in future.[7]

Inhibitor Candidates

General comments and overview

Numerous efforts to design synthetic proteinase inhibitors (including such for neutrophil elastase) for therapeutic purposes have been not very successful so far.[8,9] The major problems such synthetic compounds are concerned with are:

1. sufficient restriction of the inhibitory specificity to avoid undesired side effects;
2. rapid elimination from all compartments of the organism.

Proteinase inhibition therapy suitable for a wider medical application has to be oriented primarily on the "functional design" of the natural endogenous inhibitors and, in special cases, on the biochemical conditions of the disease state. For example: Inhibitors designed to interfere with proteinases of the humoral cascade systems (clotting and kallikrein-kinin pathway, fibrinolysis, complement) should react either highly specifically with a certain proteinase - many of them with different functions are closely related - or resemble in their inhibition spectrum the endogenous serpins, which presumably have adopted "ideal" properties during evolution. Inhibitors designed to block lysosomal digestive proteinases (if released extracellularly) should not interfere at all with the intracellular protein breakdown, i.e. the elimination function of the phagocytes (reticuloendothelial system).

Hence, they should not be taken up into phagolysosomes or, if this occurs, they must be sensitive to oxidative and/or proteolytic inactivation in the digestive vacuole. The same holds true if such inhibitors are used for long term therapy to enable their proper inactivation, especially if their target proteinase should be, in addition to intracellular processes, involved also in an extracellular function, e.g. in penetration of phagocytes through glycoprotein membrane layers.[10,11]

On the other hand, under severe inflammatory conditions (e.g. multiple injuries, septicaemia, isolated or multiple organ failure like ARDS or MOF) phagocytes may produce high amounts of oxygen free radicals, hydrogen peroxide etc. and even discharge their lysosomal contents;[6] in such dramatic pathological events oxidation-resistant proteinase inhibitors might be much more effective as "anti-inflammatory drugs".[7] Further, to minimize the risk of a response of the immune system, clinically administered inhibitors should resemble as closely as possible the endogenous inhibitor proteins. Inhibitor candidates which according to our opinion are most suitable for proteinase inhibition therapy as discussed above or elsewhere[7] are listed in Table I.

α_1 Proteinase Inhibitor (α_1PI)

The predominant natural antagonist of neutrophil elastase, which is already

Table I. Inhibitor candidates of human origin suitable for proteinase (neutrophil elastase etc.[a]) inhibition therapy

α_1 **PI**	native form	- glycoprotein	-oxidizable
	r-variants	- proteins	-oxidation resistant
MPI	native form	- miniprotein	-oxidizable
	r-variants	- miniproteins	- oxidation resistant

Aprotinin (miniprotein) homologous domains[b] (no.) in:
1. Inter-α-trypsin inhibitor complex as Bikunin (2)
2. Alzheimer amyloid protein precursor Pre A4 (1)
3. Lipoprotein-associated coagulation inhibitor (3)

Kazal-type inhibitors (miniproteins): r-variants of:
1. Pancreatic secretory trypsin inhibitor, PSTI
2. Seminal acrosin-trypsin inhibitor, HUSI-II

[a] and/or cathepsin G and/or mast cell chymase; [b] recombinant variants; r = recombinant

therapeutically given to α_1PI-deficient individuals with lung emphysema, can be isolated from normal human blood only in limited quantities.

Therefore, various possibilities for its production by genetic engineering are presently under investigation.[12,13]

The same is true for an oxidation resistant artificial mutant of α_1PI, Val^{358} α_1PI,[14,15] as well as for a naturally occurring mutant (and variants thereof), Arg^{358} α_1PI, which is a strong inhibitor of thrombin and plasma kallikrein[15,16] (Table II). However, as long as these α_1PI homologues cannot be produced in sufficient

Table II. Residues in the reactive site region and inhibitory specificity of serpins

Serpin or variant	Major target enzyme	Residues in positions					
		P_2	P_1	P'_1	P'_2	P'_3	P'_4
α_1 PI (α_1 AT)	neutrophil E	Pro	**Met**	Ser	Ile	Pro	Pro
r-variant*	neutrophil E	Pro	**Val**	Ser	Ile	Pro	Pro
α_1 PI-Pittsburgh	thrombin	Pro	**Arg**	Ser	Ile	Pro	Pro
Antithrombin III	thrombin	Gly	**Arg**	Ser	Leu	Asn	Pro

AT = antitrypsin; E = elastase; *oxidation resistant, cf. Table I

quantities in the natural glycoprotein form, their suitability in pharmacological terms (distribution within the organism, elimination rate, etc.) and a possible immune response against the carbohydrate chain free, "naked" proteins has to be carefully considered.

Mucus proteinase inhibitor (MPI)

The mucus proteinase inhibitor MPI, also known as antileucoprotease (ALP) or secretory leucocyte proteinase inhibitor (SLPI), is the predominant natural antagonist of neutrophil elastase in all mucous secretions of the organism.[17] It represents together with α_1PI the main antiprotease shield of the upper airways (molar ratio of MPI=α_1PI > 1) and the lung (MPI=α_1PI < 1).[7]

Despite the presence of 8 disulphide bridges within the molecule (Fig. 2), the

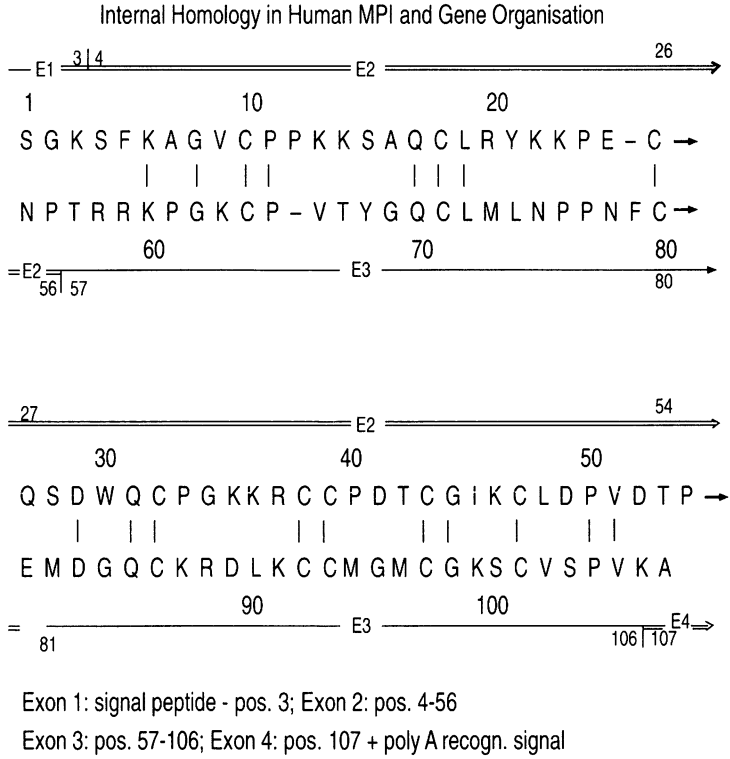

Fig. 2. Primary structure, internal sequence homology and genomic organization of the human mucus proteinase inhibitor MPI.
The exon-intron organization reflects the two structural homologous domains of the MPI molecule. Present evidence suggests that it is the C-terminal domain which is inhibitorily active against neutrophil elastase and cathepsin G as well as chymotrypsin and trypsin (P_1= Leu^{72}, P_1'= Met^{73})

natural mature form of the miniprotein MPI can be produced already in sufficient quantity and purity to start investigation of its therapeutic effectiveness in animal models[18] and in patients suffering from emphysema and cystic fibrosis (Synergen, Boulder/Colorado, USA). For first therapeutic approaches to elucidate its potential anti-inflammatory effectiveness, an oxidation resistant MPI variant is also available (Grünenthal GmbH, Stolberg/Rheinland, Germany). In this variant the four Met residues present in the second domain, which is responsible for neutrophil elastase and cathepsin G inhibition (Fig. 2), have been exchanged by aliphatic amino acid residues without impairment of the inhibitory activity.[19]

Aprotinin homologues

The miniprotein aprotinin from bovine mast cells, a proteinase inhibitor with rather low specificity, has been used in medical therapy uncritically for a long time to treat numerous diseases[20] before clinically clearly effective dosages were applied more recently.[21,22]

Proteinase inhibition therapy with higher dosages of aprotinin proved to be especially valuable in open heart surgery with extracorporeal circulation whereby blood loss and transfusion requirement could be highly significantly reduced most probably due to effective plasmin inhibition.[23,24]

Polypeptide domains which are structurally closely related to bovine aprotinin have been found to occur also in human high molecular mass protein complexes or proteins (Table I). The "Bikunin" molecule present in the protein complex of the inter-alpha-trypsin inhibitor consists essentially of two aprotinin-like domains, the N-terminal one (D_1) being responsible for inhibition of neutrophil elastase and the C-terminal domain (D_2) for trypsin inhibition.[25,26] Exchange of the two Met residues in the reactive site region of the human Bikunin domain D_1 by Leu residues in the bovine molecule (Table III) leads to a dramatic increase in the affinity to neutrophil elastase and cathepsin G.[26] Hence, artificial mutants of the human Bikunin molecule with high specificity and strong affinity to a certain target proteinase like the neutrophil elastase may be designed - e.g. by comparison with naturally occurring mutants or by molecular modelling of the structure of the inhibitor in its complex with the proteinase - and finally produced by recombinant DNA techniques.[27] A similar approach should be possible with the aprotinin-like domain(s) present in Alzheimer amyloid protein precursor PreA4,[28] and lipoprotein-associated coagulation inhibitor.[29]

Kazal-type Inhibitors

Kazal-type inhibitors comprise a family of miniproteins (single inhibitory domain) or proteins (composed of several such domains: multiheaded) with primary structures similar to the sequence of bovine pancreatic secretory trypsin

Table III. Reactive site residues in the N-terminal domains (D_1) responsible for chymotrypsin and neutrophil elastase inhibition of the human and bovine Bikunin molecules. The C-terminal domains (D_2) responsible for trypsin inhibition have identical sequences in this region for both species

	Subsite positions					
Domain	P_3	P_2	P_1	P'_1	P'_2	P'_3
human D_1	Pro	Cys	**Met**	Gly	**Met**	Thr
bovine D_1	Pro	Cys	**Leu**	Gly	**Leu**	Phe
D_2	Pro	Cys	**Arg**	Ala	Phe	Ile

inhibitor first described by Kazal;[30] they are widely distributed in vertebrates.[31,32] In the human organism two Kazal-type inhibitors, each of them single-headed, have been identified unequivocally by amino acid sequence analysis. The first was pancreatic secretory trypsin inhibitor, PSTI,[33] and more recently the trypsin-acrosin inhibitor HUSI-II (human seminal acrosin inhibitor II) which occurs in male genital tract organs and secretions.[34,35]

Due to the extensive studies of M. Laskowski et al.[31,32] on structure-function relationships of Kazal-type avian ovomucoid domains, a large data base for predictable alterations of the inhibitory affinity and specificity by suitable substitutions of a single or a few residues in the reactive peptide sequence is available. In view of this knowledge the human PSTI which inhibits strongly its natural antagonist, the pancreatic trypsin, was chosen for a protein design project.[36] Our aim was to develop inhibitors with high affinity against human neutrophil

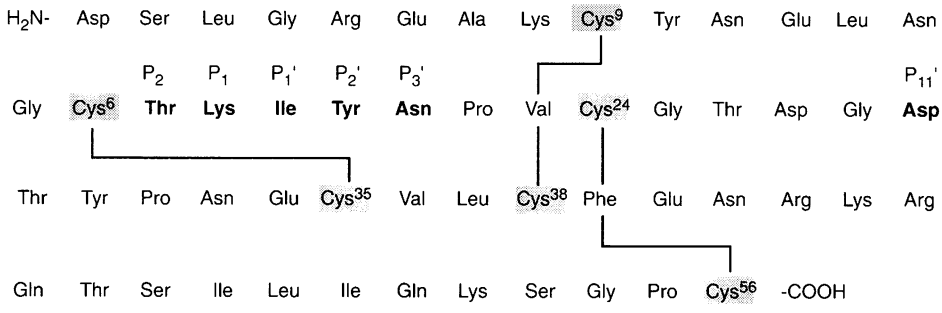

Fig. 3. Primary structure of the human pancreatic secretory trypsin inhibitor, PSTI, with subsite positions (residues) in most intimate contact to the enzyme(s) in the complex and disulphide bridges as indicated.

Table IV. Inhibitory specificity of PSTI variants expected on the basis of M. Laskowski's structure-function algorithm of Kazal-type ovomucoid inhibitors

PSTI variant	Subsite position						Specificity for
	P_2	P_1	P'_1	P'_2	P'_3	P'_{11}	
PSTI n	Thr	Lys	Ile	Tyr	Asp	*Asn*	T
PSTI 0	Thr	Lys	Ile	Tyr	Asn	Asp	T
PSTI 1	Thr	**Leu**	Ile	Tyr	Asn	Asp	E and Ch
PSTI 2	Thr	**Leu**	Ile	Tyr	Asp	*Asn*	E and Ch
PSTI 3	Thr	**Tyr**	*Glu*	Tyr	*Arg*	Asp	E
PSTI 4	Thr	**Leu**	*Glu*	Tyr	*Arg*	Asp	E and Ch
PSTI 5	Thr	**Val**	*Glu*	Tyr	*Arg*	Asp	E
PSTI 6S	Thr	**Leu**	*Glu*	Tyr	Asn	Asp	E and Ch
PSTI 7	Thr	**Leu**	Ile	Tyr	*Arg*	Asp	E and Ch
PSTI 8	Thr	**Val**	*Glu*	*Leu*	Asn	Asp	E
PSTI 9	Thr	**Val**	*Glu*	*Leu*	*Arg*	Asp	E
PSTI 10	*Pro*	**Lys**	Ile	Tyr	Asp	*Asn*	T
PSTI 11	*Pro*	**Leu**	*Glu*	Tyr	*Arg*	Asp	E and Ch
PSTI 12	*Pro*	**Val**	*Glu*	Tyr	*Arg*	Asp	E
PSTI 13	Thr	**Ile**	*Glu*	Tyr	Asn	Asp	E?
PSTI 14	Thr	**Arg**	*Glu*	Tyr	Asn	Asp	T
PSTI 15	Thr	**Phe**	*Glu*	Tyr	Asn	Asp	Ch and C-G
PSTI 16	Thr	**Ala**	*Glu*	Tyr	Asn	Asp	E
PSTI 17	Thr	**Val**	Ile	Tyr	Asn	Asp	E
PSTI 18	Thr	**Ile**	Ile	Tyr	Asn	Asp	E?
PSTI 19	Thr	**Val**	Ile	Tyr	Asp	*Asn*	E

n = natural native inhibitor; C-G = cathepsin G; Ch = chymotrypsin; E = elastase; T = trypsin

elastase and cathepsin G with PSTI as model compound.[37,38]

The primary structure of PSTI and its subsite positions (residues) in most intimate contact with the target enzyme(s) in the complex are shown in figure 3. The primary inhibitory specificity of various artificial mutants of PSTI produced by recombinant DNA techniques is indicated in table IV.

In Table V, PSTI variants exhibiting highest affinity for the chosen target enzymes are listed together with the K_i values which reflect the influence of amino acid exchanges in certain subsite positions of the PSTI molecule on the affinity. Even further improvement of specificity and selectivity turned out to be possible by additional substitutions in position 21 or 36.[37]

At present, similar studies are being performed with the trypsin-acrosin inhibitor HUSI-II as model compound in other laboratories.

Table V. Inhibition of chymotrypsin (Ch) and neutrophil elastase (E) by r-PSTI variants

The effect of the P_1 and P'_1 residues (all with P'_3 = Arg)

PSTI variant	P_1	P'_1	K_i(Ch)	K_i(E)
PSTI-3	Tyr	Glu	1.6×10^{-11}	$>10^{-7}$
PSTI-5	Val	Glu	3.1×10^{-7}	1.5×10^{-11}
PSTI-4	Leu	Glu	2.4×10^{-11}	3.7×10^{-11}
PSTI-7	Leu	Ile	8.0×10^{-9}	2.5×10^{-11}

The effect of the P'_3 residue (P_1 = Leu; P'_{11} = Asp)

PSTI variant	P'_3	P'_1	K_i(Ch)	K_i(E)
PSTI-1	Asn	Ile	5.0×10^{-8}	5.0×10^{-11}
PSTI-7	Arg	Ile	8.0×10^{-9}	2.5×10^{-11}
PSTI-6	Asn	Glu	2.0×10^{-8}	2.5×10^{-10}
PSTI-4	Arg	Glu	2.4×10^{-11}	3.7×10^{-11}

K_i = dissociation equilibrium constant of the enzyme-inhibitor complex in mol/l

Table VI. Natural proteinase inhibitors in therapy

Indication	Applied	Use envisaged	Target enzymes
hyperfibrinolysis, shock states	bovine aprotinin		plasmin, plasma and tissue kallikrein
coagulopathy, DIC	antithrombin III	r-hirudin	thrombin
angioneurotic oedema	C1 inhibitor		plasma kallikrein, F XIIa, C1 esterase
emphysema	α_1PI		
inflammation (sepsis, ARDS, MOV, etc.)		r-α_1PI, r-eglin, r-MPI (ALP, SLPI)*	neutrophil elastase and cathepsin G

α_1PI = α_1 proteinase inhibitor; r = recombinant
*mucus proteinase inhibitor (ALP = antileucoprotease, SLPI = secretory leucocyte proteinase inhibitor)

Conclusion

The given data show clearly that miniprotein inhibitors with highest affinity and selectivity for certain proteinases including further desired properties (e.g. oxidation resistance) can be prepared. Such artificial mutants of natural regulators of proteinases are of great value for both biochemical investigations of structure-function relationships and therapeutic experimental and clinical studies. Hence, the modern methods of molecular modelling and biotechnology have provided us with suitable techniques to enable the design and production of inhibitors for effective proteinase inhibition therapy in the near future (Table VI). The major problem, however, which still has to be solved in this respect is the preparation of kilogram amounts of inhibitory drugs in highest purity for more extensive experimental and clinical studies. A successful outcome of these studies would imply the production of such inhibitors in sufficient amounts for common medical use.

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