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Human Leukocyte Elastase Inhibitors: Designed Variants of Human Pancreatic Secretory Trypsin Inhibitor (hPSTI)

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Summary

Variants of human secretory trypsin inhibitor were constructed with the aim of producing inhibitors specific for human leukocyte elastase. Models of the hPSTI/HLE and hPSTI/chymotrypsin complexes were generated by computer aided protein design and used to plan better HLE inhibitors. This resulted in the production of the strongest and most specific inhibitors of HLE known.

Introduction

Human leukocytic elastase (HLE) is a potent intracellular proteinase stored in lysosomes of polymorphonuclear granulocytes. Excessive extracellular release of HLE occurs after severe injuries or in diseases like septic shock, acute respiratory distress syndrom and burn shock. In these clinical situations the plasma level of HLE, complexed with α_1-proteinase inhibitor, and corresponding degradation of hemostatic factors, correlate with mortality (1-4). In experimental models of sepsis and emphysema, synthetic elastase inhibitors (5) and eglin c of medicinal leech (recombinant protein) (6), have been proven to be therapeutically useful. However, a proteinase inhibitor of human origin would be preferable in order to avoid allergic reactions when a prolonged or repeated therapy is indicated. Therefore, it was undertaken to design an inhibitor for HLE based on the structure of human pancreatic secretory trypsin inhibitor.

Abbreviations:
CAPD, computer aided protein design; HLE, human leukocyte elastase; PSTI, pancreatic secretory trypsin inhibitor; hPSTI, human PSTI; Nan, 4-nitroanilide.

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We report the computer aided design and production of the most specific and strongest HLE inhibitors known (K_i = $5x10^{-12}$ M) in the hope that such compounds may find use in the investigation of and possibly treatment of the clinical situations referred to above.

Methods

The construction of synthetic PSTI genes and their expression has been described (7,8). Site-specific mutagenesis with short oligonucleotide primers (9,10) was used to construct variant PSTIs in a new vector (pMAMPF) (10) which can be propagated in either plasmid or single strand phage form. The vector uses the OmpA-signal sequence for secretion, and synthesis is controlled by a lambda P_L promoter. Dideoxy sequencing on single strand phasmid DNA (11) and protease inhibitor tests (7,12,13) were carried out as described. K_{on} and K_{off} measurements were performed according to published methods (14-16). The BRAGI (CAPD) program is described elsewhere (17,18).

Results

Inhibitory specificities of the PSTI variants

The native hPSTI with Lys in position P_1 is a highly potent inhibitor for trypsin and completely inactive towards chymotrypsin- and elastase-like proteinases (19). Replacing the Lys in P_1 by Leu (variant PSTI-7 in Table 1) causes a drastic change of the specificity: this variant no longer inhibits trypsin-like

PSTI variant	P_1	P'_1	K_i (Ch) [M]	K_i (HLE) [M]	K_i (Ch)/K_i (HLE)
PSTI-3	Y	E	$1.6x10^{-11}$	$>10^{-7}$	$>1.6x10^{-4}$
PSTI-5	V	E	$3.1x10^{-7}$	$1.5x10^{-11}$	$2.1x10^{4}$
PSTI-4	L	E	$2.4x10^{-11}$	$3.7x10^{-11}$	$6.5x10^{-1}$
PSTI-7	L	I	$8.0x10^{-9}$	$2.5x10^{-11}$	$3.3x10^{-2}$

Table 1. Effects of the nature of P_1 and P'_1 residues in PSTI on the dissociation constants of the complexes with either HLE or bovine chymotrypsin (Ch). All variants have Arg in position P'_3.

enzymes but is an excellent inhibitor of chymotrypsin and HLE. As can be seen from the data in Table 1, dramatic alterations in specificity in relation to inhibition of chymotrypsin or HLE can be obtained by varying the P_1 residue (amino acid 18): changing Tyr to Val causes a change in relative specificity of **eight orders of magnitude**. Leu at P_1 yields inhibitors that have high affinities to both chymotrypsin and HLE. The affinity for chymotrypsin is reduced 30-fold by changing Glu (P'_1) to Ile (see also Table 2). The effects on the kinetic constants of the various P_1 residues may be explained by steric hindrance in the substrate binding pocket (S1) as can be deduced from the model given below. Initially no K_i value below 10^{-10} M was obtained in the absence of Arg at P'_3. This could be accounted for in the models by hydrogen bonds present between this Arg residue and the proteases, whereas Asp or Asn are far from contact with any protease groups. That the relative contributions of individual side chains are not simply additive is seen by the effect of the P'_3 residue on chymotrypsin binding affinity with either Ile (6-fold increase) or Glu (830-fold increase) present at P'_1 (Table 2).

PSTI variant	P'_3	P'_1	K_i (Ch) [M]	Ki (HLE) [M]
PSTI-1	N	I	5.0×10^{-8}	5.0×10^{-11}
PSTI-7	R	I	8.0×10^{-9}	2.5×10^{-11}
PSTI-6	N	E	2.0×10^{-8}	2.5×10^{-10}
PSTI-4	R	E	2.4×10^{-11}	3.7×10^{-11}

Table 2. The effect of arginine as the P'_3 residue (P_1 = Leu; P'_{11} = Asp). Abbreviations are the same as in Table 1.

A selection of data obtained with over 24 PSTI variants is given in the Tables 1, 2 and 3. Conclusions derived from some two thousand kinetic measurements are briefly summarised as follows: None of the variants tested inhibited the kallikreins, thrombin or plasmin; proline at P_2 position reduced the affinity to chymotrypsin; aspartic acid or asparagine at position P'_{11} has had little effect; proline, alanine, serine or glycine at position P'_{14} had negligible effects although asparagine at position P'_{15} appears to be hydrogen bonded to C-alpha atoms in the inhibitory loop of all PSTI variants as deduced from the PSTI-3 and -4 crystal structure (unpublished results).

A model of complexes of PSTI variants with HLE or chymotrypsin

Using the BRAGI program (18) and the known tertiary structure of porcine PSTI bound to bovine trypsinogen (20), a model was created which simulated the tertiary structure of human PSTI and its variants (17). Similarly a model of the tertiary structure of HLE was simulated on the basis of the known structure of porcine pancreatic elastase (21) (Figure 1). The other PSTI variants were then modelled in this complex. The planning of more specific and stronger elastase inhibitors was moderately successful for the $P'_{1\,8}$ = aspartic acid derivative of PSTI-4 (Table 3), but unsuccessful for PSTI-4 derivatives containing glutamic acid at P_5 or P_6: The K_i value for the complex with HLE increased tenfold whereas the interaction with chymotrypsin was not effected. This failure to achieve a reliable prognosis probably results from the fact that water molecules were not included in the modelling. Results obtained with further rounds of model-based mutagenesis are shown in the lower part of Table 3.

In the case of PSTI-4A40 in which glutamic acid 40 is substituted for an alanine, a change in structure is postulated, in which

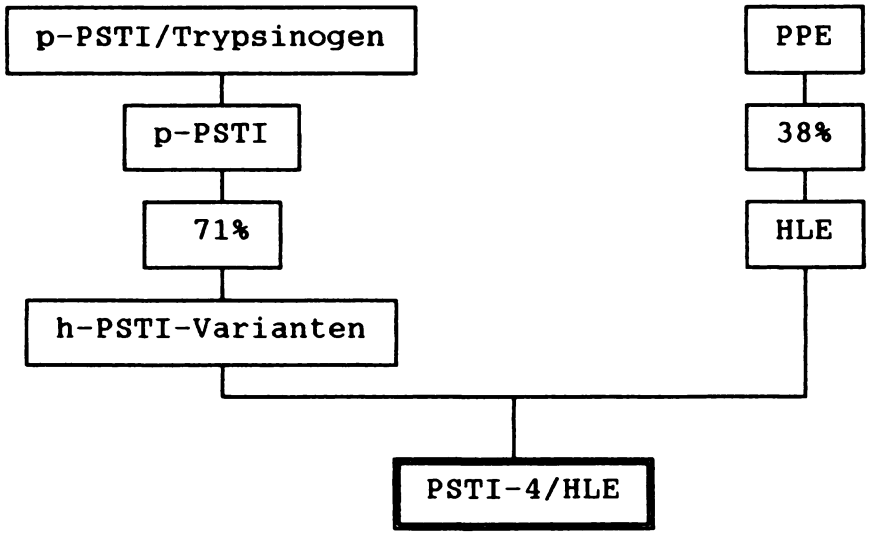

Figure 1. Schematic representation of the use of the "BRAGI" program to model tertiary structures and plan novel variants of human PSTI. Human PSTI was modelled from porcine PSTI (pPSTI) complexed with bovine trypsinogen (19). Human leukocyte elastase (HLE) was modelled from porcine pancreatic elastase (PPE; ref. 21). Homologies in primary sequence are shown as percentages. The final model of the PSTI variant complexed with HLE was obtained by superimposing the catalytic triads of the modelled HLE onto the trypsinogen complex, followed by further force field energy minimisation with the AMBER programme (22).

PSTI variant	P_1	P_1'	P_3'	Chymotrypsin K_i [M]	HLE K_i [M]	HLE K_{off} [s^{-1}]
PSTI-4D13	L	E	R	6.0×10^{-11}	2.5×10^{-10}	8.5×10^{-4}
PSTI-4D14	L	E	R	5.3×10^{-11}	8.7×10^{-10}	3.6×10^{-4}
PSTI-4D36	L	E	R	3.4×10^{-8}	3.1×10^{-11}	9.1×10^{-5}
PSTI-5D36	V	E	R	2.6×10^{-8}	7.3×10^{-12}	8.0×10^{-5}
PSTI-4A40	L	E	R	6.0×10^{-10}	7.0×10^{-12}	5.1×10^{-5}
PSTI-4F21	L	E	F	4.0×10^{-9}	5.2×10^{-12}	6.3×10^{-5}
PSTI-99	V	I	F	$>10^{-7}$	5.0×10^{-11}	$<10^{-6}$

Table 3. Kinetic constants obtained for variants proposed by CAPD as outlined in Figure 1. The apparent K_i values for the complexes with HLE have been corrected (15,16) since K_m of the chromogenic substrate was comparable to K_{off}, a fact that would influence the physiological effectiveness of the variants in the presence of natural substrates. This correction was not necessary for PSTI-99.

the loss of an internal hydrogen bond to lysine 43 would free it to form a hydrogen bond to HLE (e.g. to Asn 147 or the carbonyl backbone). Since this variant is one of the strongest elastase inhibitors known, it will be interesting, subsequent to X-ray crystallography, to see if the strategy had actually worked as planned. The choice of the aromatic residue phenylalanine at position 21 was predicted to give an excellent fit of this residue into a corresponding hydrophobic pocket (S3) on the surface of HLE. PSTI-99 represents the strongest and most specific (relative to chymotrypsin) HLE inhibitor known. The kinetics were measured in 1000 mM NaCl, 200 mM Tris·HCl (pH 8.0), 2 mM MetSuc-Ala-Ala-Pro-Val-Nan ($K_m = 5.3 \cdot 10^{-5}$, for HLE). At lower salt concentrations, e.g. 200 mM NaCl, the K_{off} values for the two inhibitors approach each other at $6 \cdot 10^{-5}$ M, supporting the involvement of a unique hydrophobic interaction in the case of PSTI-99, made stronger under conditions where hydration energies are increased. We speculate from our model that the hydrophobic contacts between HLE and PSTI-99 create a water-impermeable barrier to the entry of the water molecules used for cleavage of the serine-ester intermediate formed after nucleophilic attack during proteolysis. This would prevent cleavage of the inhibitor and strengthen the stability of the complex.

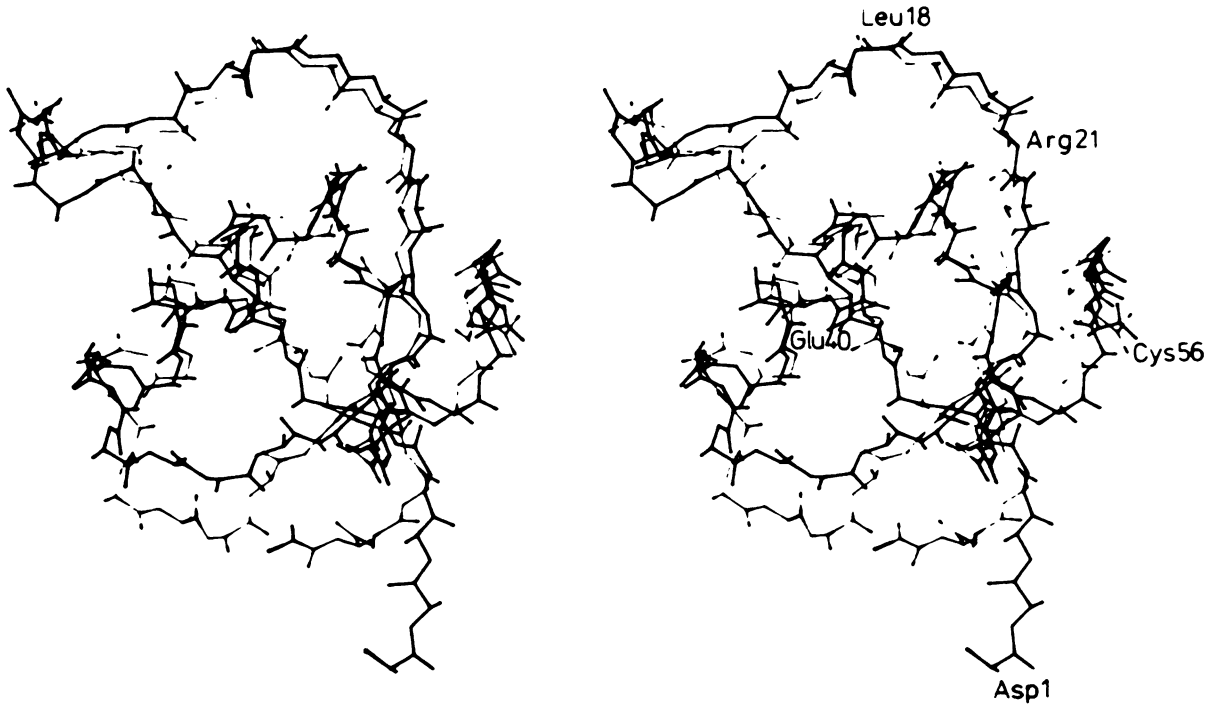

Figure 2. A comparison of the structure of PSTI-3 as predicted in the model (darker line: Figure 1) with the actual crystallographic structure of PSTI-3 as found in a complex with chymotrypsinogen (grey). To simplify the comparison only the backbone structure is shown.

Comparison between the model and actual structure for PSTI-3

A comparison of actual and modelled PSTI-3 structure is represented in Figure 2. Details of the crystallographic analysis are to be published elsewhere. The model represents a very good approximation to the actual structure of the internal and protease binding regions. An excellent superimposition is observed for the orientation of the side-chains, both in the core region and in the protease-binding region. Deviations in three exterior regions, near the amino terminus, can be partially rationalised by the fact that the structural prediction did not take account of solvent.

Conclusion

Elastase-specific inhibitors with inhibitory constants, comparable to the best known, had been generated by site-specific mutagenesis of the human trypsin-specific inhibitor PSTI. A structural model was generated for the protease/inhibitor complexes from proteins with analogous primary sequence. It was used to

account for the specificities of existing variants, and to predict further modifications that should optimise the specificity for HLE. The approach was validated.

It is hoped that the serine-protease inhibitors produced will provide a useful resource for the study of the function of proteases in the body and in particular in elucidating the role of HLE in clinical syndromes.

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