The selectivity of statine-based inhibitors against various human aspartic proteinases

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The interactions of five human enzymes (renin, pepsin, gastricsin, cathepsin D and cathepsin E) and the aspartic proteinase from *Endothia parasitica* with several series of synthetic inhibitors were examined. All of the inhibitors contained the dipeptide analogue statine or its phenylalanine or cyclohexylalanine homologues in the P₁–P₁' positions. The residues occupying the peripheral sub-sites (P₄ to P₃') were varied systematically and inhibitory constants were determined for the interactions with each of the proteinases. Inhibitors were elucidated that specifically inhibited human renin and did not affect any of the other human enzymes or the fungal proteinase. With suitable selection of residues to occupy individual sub-sites, effective inhibitors of specific human aspartic proteinases may now be designed.

INTRODUCTION

The human body is a source of at least five distinct aspartic proteinases (reviewed in ref. [1]). Pepsin and gastricsin together play an important role in the digestion of proteins in the stomach, and cathepsin D, a lysosomal proteinase, is a key enzyme in the intracellular degradation of proteins. Renin is the (highly specific) first enzyme in a multi-regulated proteolytic cascade that ultimately produces the vasopressor agent angiotensin II. The least-well-characterized enzyme is cathepsin E (which is synonymous with the electrophoretically slow-moving proteinase or erythrocyte membrane aspartic proteinase [2]). Although the function of this proteinase is not clear [1], it has a strategic location within a number of cells and tissues in the human body [3].

The active-site cleft of all of these enzymes is considered to be a long deep groove that extends for about 3 nm (30 Å). It appears to be capable of accommodating as many as nine amino acid residues of a substrate or inhibitor in the S₆–S₃' sub-sites with a distinct preference for cleavage to occur between hydrophobic residues occupying the S₁–S₁' sites [4,5]. Thus the differences in specificity and activity of the various enzymes may be attributed to discrete alterations in some or all of these sub-sites comprising the cleft. Partial characterization of the active sites of some of the enzymes has been reported previously with the use of synthetic chromogenic substrates [5,6]. In order to evaluate the preferences of individual sub-sites in each enzyme in more detail, a systematic investigation was therefore carried out with the five known human aspartic proteinases (together with the proteinase from the fungus *Endothia parasitica*, since it has been used frequently as a facsimile of human

renin [7]) with several series of synthetic inhibitors. These inhibitors were originally designed on the polypeptide sequence of human angiotensinogen adjacent to the peptide bond cleaved by renin. At their core the inhibitors all contained the unusual amino acid statine {which acts as a non-hydrolysable transition-state analogue of the two residues contributing the scissile peptide bond (in P₁–P₁') in the substrate [8]} or its phenylalanine (AHPPA) or cyclohexylalanine (ACHPA) analogues. Statine itself is the active moiety in pepstatin, the naturally occurring inhibitor of all aspartic proteinases first discovered by Umezawa and co-workers [9].

Thus several series of inhibitors with the general structure

Acyl-His-Pro-P₃-P₂-(Sta/AHPPA/ACHPA)-P₂'-P₃'-NH₂

were compiled, and the residues occupying the various sub-sites were varied systematically in order to determine the effect upon interaction with each of the proteinases.

MATERIALS AND METHODS

The sources of the homogeneous proteinase preparations used were as described previously [5].

Human pepsin, gastricsin, cathepsin E, cathepsin D and *Endothia* proteinase were assayed at 37 °C in 0.1 M-sodium formate buffer, pH 3.1. Ionic strength was maintained constant at 0.1 by the addition of NaCl. The hydrolysis of synthetic chromogenic substrates was monitored spectrophotometrically at 300 nm as described previously [2,5,6]. The substrates used for each enzyme were as follows:

Human pepsin:

Lys-Pro-Ile-Glu-Phe-Phe(-4-NO₂)-Arg-Leu

Abbreviations used: Boc, t-butoxycarbonyl; Etoc, ethoxycarbonyl; Sta, statine (4-amino-3-hydroxy-6-methylheptanoic acid); AHPPA, 4-amino-3-hydroxy-5-phenylpentanoic acid; ACHPA, 4-amino-5-cyclohexyl-3-hydroxypentanoic acid; 1-NA, naphth-1-ylalanine; 2-NA, naphth-2-ylalanine; Abu, α-aminobutyryl; Acn, acetamido; Orn-Ac, δ-N-acetylorithine; Iva, isovaleryl; Ibu, isobutyryl; HPhe, homophenylalanine; HCys, homocysteine.

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Human gastricsin:

Lys-Pro-Val-Val-Phe-Phe(-4-NO₂)-Arg-Leu

Cathepsin D, cathepsin E and the *Endothia* proteinase:

Pro-Pro-Thr-Ile-Phe-Phe(-4-NO₂)-Arg-Leu

The kinetic parameters K_m and V_{max} for substrate hydrolysis were estimated from a plot of $[S]/v$ against $[S]$. For each determination the initial rates (v) were measured with at least six values of the initial substrate concentration ($[S]$) within an appropriate range. These values, together with the estimates for K_m and V_{max} , were entered into a non-linear curve-fitting programme, based on the equation:

$$v = V_{max} \cdot [S] / (K_m + [S])$$

as described previously [2]. The estimated precision of the values obtained was in the range 5–10%.

Estimates of the inhibition constants (K_i) were determined by using the Dixon construction [10], and in every case a competitive mode of inhibition was observed. If the ratio of $[E]_t/K_i$ was less than 0.2 (where $[E]_t$ is the total active enzyme concentration), the Michaelis–Menten equation was assumed to be valid. In these cases precise K_i values were computed by using a non-linear curve-fitting program based on the equation:

$$v = V_{max} \cdot [S] / ([S] + K_m (1 + [I]/K_i))$$

The K_i values obtained had a standard error of approx. 10%. If the ratio of $[E]_t/K_i$ was between 0.2 and 10, the Michaelis–Menten equation was assumed to be inappropriate, so that K_i values were then calculated according to the method of Henderson [11].

The active concentration of each of the above five enzyme preparations was determined by titration against a solution of isovaleryl-pepstatin whose concentration had been pre-determined by amino acid analysis [2].

Endogenous renin in human plasma was measured by means of a radioimmunoassay for angiotensin I by using a commercial kit (Clinical Assays, Cambridge, MA, U.S.A.) at 37 °C in 0.1 M-sodium phosphate buffer, pH 7.4, as described previously [12]. IC₅₀ values (i.e. concentrations causing 50% inhibition) for the interaction of inhibitors were also derived as described

previously [12]. Purified human kidney renin (approx. 1000 Goldblatt units/mg) [13] was occasionally assayed instead with purified human-plasma-derived angiotensinogen (approx. 21 µg of AI/mg) [14] as substrate. In the latter assay the inhibitor compounds dissolved in degassed *NN*-dimethylformamide were assayed in 100 µl of 0.1 M-sodium phosphate buffer, pH 6.0, containing 3 mM-EDTA, 2.4 mM-phenylmethane-sulphonyl fluoride, 1 mg of bovine serum albumin/ml, 0.32 nM-renin and 0.5 µM-angiotensinogen (approx. $0.5 \times K_m$) for 1 h at 36 °C. After incubation, the samples were heated at 95 °C for 3 min followed by quantification of angiotensin I by radioimmunoassay (Travenol/Genentech clinical assay kit; IC₅₀ of assay 3 ng of AI/ml). Under these conditions less than 2% of the added substrate was utilized. No detectable effect of the added *NN*-dimethylformamide (<0.5%) was noted in the reaction. Following an estimation of the IC₅₀ for each inhibitor with the use of a broad concentration range, 12 different concentrations of each inhibitor around the estimated IC₅₀ were run for the final inhibitory potency determined by non-linear regression analysis. The IC₅₀ values should be considered to be reproducible to ± 30%.

The synthesis of most of the inhibitors used has been described previously in detail [12,15–17]. New compounds reported in this paper were prepared at Merck according to the methods used in ref. [15]. They were commonly characterized by elemental analysis, n.m.r. and m.s. as detailed in ref. [15]. Purities of greater than 97% were confirmed by h.p.l.c. and t.l.c.

RESULTS

In order to maintain compatibility with previous investigations, values of K_i were determined primarily at pH 3.1 for the interactions of all the inhibitors with human pepsin, gastricsin, cathepsin D, cathepsin E and the *Endothia* proteinase. However, a competitive mode of inhibition was always observed. Renin assays were performed primarily at pH 7.4 (and occasionally at pH 6.0).

The nature of the resistant transition-state analogue occupying the central P₁–P₁' positions was first examined

Table 1. Inhibition constants for the interaction of several aspartic proteinases with synthetic inhibitors containing different substituents in the P₁–P₁' positions

All measurements except those involving renin were carried out at 37 °C in 0.1 M-sodium formate buffer, pH 3.1. Human plasma renin was assayed at 37 °C in 0.1 M-sodium citrate/phosphate buffer, pH 7.4, and inhibition was measured as IC₅₀ values. Substrates used were as described in the Materials and methods section.

	K_i or IC ₅₀ (nM)					
	Renin	Pepsin	Gastricsin	Cathepsin D	Cathepsin E	<i>Endothia</i> proteinase
Iva-His-Pro-Phe-His-P ₁ -P ₁ '-Leu-Phe-NH ₂						
Sta	13	900	550	150	70	90
AHPPA	2	1900	300	600	100	90
ACHPA	0.2	2200	860	230	60	420
Boc-Phe-His-P ₁ -P ₁ '-Leu-Phe-NH ₂						
Sta	140	2160	640	35	6	10
ACHPA	2	2800	2350	140	200	2200

Table 2. Inhibition constants for the interaction of several aspartic proteinases with synthetic inhibitors containing different substituents in the P₄ position

All measurements were made as described in the legend to Table 1.

	<i>K_i</i> or IC ₅₀ (nM)					
	Renin	Pepsin	Gastricsin	Cathepsin D	Cathepsin E	<i>Endothia</i> proteinase
P₄-Phe-His-ACHPA-Leu-Phe-NH₂						
Boc	2	2800	2350	140	200	2200
Etoc	0.8	2100	1600	210	20	> 13 000
Acetyl	3	1500	1100	550	60	2650
Longer (= Iva-His-Pro)	0.2	2200	860	230	60	420
P₄-Phe-His-ACHPA-NH-2(S)-methylbutyl						
Acetyl	42	16 000	14 500	9200	1500	6600
Boc	19	13 000	15 000	800	1150	650

Table 3. Inhibition constants for the interaction of several aspartic proteinases with synthetic inhibitors containing different substituents in the P₃ position

All measurements were made as described in the legend to Table 1.

	<i>K_i</i> or IC ₅₀ (nM)					
	Renin	Pepsin	Gastricsin	Cathepsin D	Cathepsin E	<i>Endothia</i> proteinase
Iva-His-Pro-P₃-His-Sta-Leu-Phe-NH₂						
Phe	13	900	550	150	70	90
1-NA	11	950	600	200	20	130
2-NA	100	1200	1500	500	50	100
HPhe	0.14	40	30	40	9	120
Boc-His-Pro-P₃-His-Sta-Leu-Phe-NH₂						
Phe	5	1000	440	70	8	40
Ala	27 000	80	660	> 5000	1450	2650

in both long (filling nine sub-sites; upper part of Table 1) and shorter (filling seven sub-sites; lower part of Table 1) inhibitors. For pepsin, gastricsin and cathepsin D, the size of the side chain (which occupies the S₁ pocket in the enzymes) would seem of little consequence, since comparable *K_i* values (within 2–4-fold) were obtained for each enzyme with all substituents in both series. However, a very clear preference was displayed by renin in that the ACHPA-containing inhibitors were bound very much more tightly, as has been reported previously by us and by others [12,15,18]. Cathepsin E and the *Endothia* proteinase displayed a distinct preference for the leucine-type side chain of statine in the shorter inhibitors. By contrast, in the longer inhibitors a much less pronounced effect was observed, suggesting that energy derived from interactions at the remote S₆ and S₅ sub-sites can offset relatively unfavourable interactions in the region close to the focal point of the inhibitor, as has been described previously [7].

In keeping with this, direct comparison of a longer ACHPA-containing inhibitor (long enough to occupy sub-sites S₆ and S₅) with three shorter derivatives (filling only as far as the S₄ sub-site) indicated (upper part of Table 2) that the *Endothia* proteinase was inhibited

effectively only when the P₆ and P₅ positions were filled, whereas the other enzymes were insensitive towards such manipulations.

With regard to the P₄ position itself, renin, pepsin and gastricsin were not influenced significantly by the nature of the substituent (upper and lower parts of Table 2) whereas cathepsin D appeared to interact better with a t-butoxycarbonyl substituent than with the smaller acetyl group (upper and lower parts of Table 2). This is consistent with previous observations [19,20]. An ethoxycarbonyl substituent in this position seemed to ensure the most effective inhibition of cathepsin E (upper part of Table 2).

For investigations into the requirements for the P₃ position, two statine-containing series were examined. In the first series of inhibitors (upper part of Table 3) only substitution of phenylalanine by its homophenylalanine analogue containing an additional methylene group (thus introducing greater flexibility of the side chain) had a significant effect. The inhibitory potency towards human renin was substantially better, but parallel improvements were observed also towards the other human enzymes (but not the *Endothia* proteinase), so that no gain in selectivity was achieved by this substitution.

Table 4. Inhibition constants for the interaction of several aspartic proteinases with synthetic inhibitors containing different substituents in the P₂ position

All measurements were made as described in the legend to Table 1.

	<i>K_i</i> or IC ₅₀ (nM)					
	Renin	Pepsin	Gastricsin	Cathepsin D	Cathepsin E	<i>Endothia</i> proteinase
Boc-His-Pro-Phe-P ₂ -Sta-Leu-Phe-NH ₂						
His	5	1000	440	70	8	40
Phe	76	120	6	230	< 1	~ 1
Ibu-His-Pro-Phe-P ₂ -Sta-Leu-Phe-NH ₂						
His	2	5600	1000	40	50	190
Ala	144	80	40	50	10	20
Abu	66	40	60	80	< 1	~ 1
Tyr	14	15	40	270	10	15
Ibu-His-Pro-Phe-P ₂ -Sta-Leu-NH-[CH ₂] ₂ -S-Acm						
Cys(Acm)	220*	35	35	10	5	80
HCys(Acm)	670*	20	450	1	10	200

* Inhibition measured with purified kidney renin.

Table 5. Inhibition constants for the interaction of several aspartic proteinases with synthetic inhibitors containing different substituents in the P₂' and P₄' positions

All measurements were made as described in the legend to Table 1.

	<i>K_i</i> or IC ₅₀ (nM)					
	Renin	Pepsin	Gastricsin	Cathepsin D	Cathepsin E	<i>Endothia</i> proteinase
Iva-His-Pro-Phe-His-Sta-P ₂ '-Phe-NH ₂						
Orn	170	3300	6400	2160	20	330
Orn-Ac	110	2600	1700	115	70	400
Leu	13	900	550	150	70	90
Ile	2	870	850	210	30	35
Ibu-His-Pro-Phe-Lys-Sta-Leu-Phe-P ₄ '						
OMe	1330*	100	180	10	10	15
GlyOMe	1850*	350	170	15	2	5

* Inhibition measured with purified kidney renin.

The *K_i* values obtained for Boc-His-Pro-Phe-His-Sta-Leu-Phe-NH₂ (lower part of Table 3) interacting with the enzymes were rather similar to those obtained (upper part of Table 3) with the closely related Iva-His-Pro-Phe-His-Sta-Leu-Phe-NH₂ (differing only in the acylating group in the P₆ position) with the exception once again of cathepsin E, where the potencies differed by almost an order of magnitude.

A stark contrast was obtained, however, when phenylalanine was replaced (lower part of Table 3) by alanine. Renin, cathepsin D, cathepsin E and the *Endothia* proteinase were dramatically less able to interact with the inhibitor containing this substitution, whereas binding to gastricsin was not changed by the replacement and pepsin showed a clear preference for the smaller alanine side chain at this position. This is entirely consistent with our previous observations [5] with chromogenic substrates with alanine as the preferred residue for pepsin in the P₃ position and further underscores the primary importance

of contacts in the S₃ pocket in governing interactions in these aspartic proteinases [6,21,22].

The effects of substitution in the P₂ position are described in Table 4. In the first series (upper part of Table 4) it becomes clear that replacement of the naturally occurring histidine residue in the angiotensinogen sequence with phenylalanine diminished the potency towards human renin by approximately one order of magnitude but substantially improved the interactions with the other enzymes except cathepsin D (see below). This trend was reflected with the second series (middle part of Table 4), where histidine in the P₂ position was, in general, not favourable for interactions with the enzymes other than renin. In particular, introduction of a bulky aromatic tyrosine residue in this position decreased the binding once again to cathepsin D. This influence was exploited further (lower part of Table 4) to produce very tight-binding inhibitors of cathepsin D through introduction of flexible aliphatic side chains

Table 6. Inhibition constants for the interaction of several aspartic proteinases with synthetic inhibitors varying in the P₃' position

All measurements were made as described in the legend to Table 1.

	<i>K_i</i> or <i>IC</i> ₅₀ (nM)					
	Renin	Pepsin	Gastricsin	Cathepsin D	Cathepsin E	<i>Endothia</i> proteinase
	Iva-His-Pro-Phe-His-Sta-Ile-P ₃ '-NH ₂					
His	6	6000	2200	> 10000	90	15
Phe	2	870	850	210	30	35

terminating in a neutral acetamido function (but also blocked with a different function at the C-terminus). The homocysteine-containing peptide is one of the most potent inhibitors of human cathepsin D ever described, but as is readily obvious from Table 4 (lower part) it is not selective in that it is also fairly effective as an inhibitor of cathepsin E and pepsin.

Substitutions in the P₂' and P₄' positions towards the C-terminal end of the inhibitors are described in Table 5. Replacement of hydrophobic leucine/isoleucine in P₂' (upper part of Table 5) with a positively charged ornithine or neutral δ -N-acetylornithine decreased the potency towards renin substantially but had much less effect on pepsin and gastricsin and indeed very little effect at all on cathepsin E. Thus all of the enzymes would appear to bind a hydrophobic residue best in this position, although cathepsin D was less able to accommodate the positive charge of the ornithine residue. Insertion of an additional amino acid (glycine; lower part of Table 5) into the P₄' position did not alter the *K_i* values obtained significantly. Thus interactions at this remote location would appear to have relatively little influence. This is in keeping with findings from crystallographic investigations [23,24].

With retention of an isoleucine residue in the P₂' position, the effect of substitution in the P₃' position was examined (Table 6). The presence of a hydrophobic phenylalanine residue was generally beneficial and particularly so for cathepsin D. Replacement of the aromatic side chain of phenylalanine with a 4-pyridinyl substituent (compound A in Fig. 1) resulted in an inhibitor with excellent potency against human renin and considerably diminished efficacies towards all of the other human enzymes except for cathepsin D (Table 7). Truncation of the substituent in this P₃' position (compound B in Fig. 1) generated an inhibitor that produced strong inhibition of human renin at nanomolar concentrations yet had virtually no effect on any of the other proteinases (Table 7).

Thus, from all of these studies on the individual residues contributing to an inhibitor, it becomes clear that, if, for example, a specific inhibitor of human renin is required, the nature of residues occupying the P₂' and P₃' positions in particular would appear to influence whether interaction would take place with the other human enzymes. Consequently, compounds C, D and E (Fig. 1) were examined. Compound C displayed moderate potency towards human renin and relatively weak effects on the other enzymes (Table 7). However, incorporation of either of the heterocyclic ring structures (compounds D and E) into the P₂'-P₃' positions resulted in still

Fig. 1. Chemistry of several compounds varying in the substituents in the P₂' and P₃' positions but with the general structure Boc-Phe-His-ACHPA-P₂'-P₃'

effective inhibition of plasma renin activity, yet had such negligible effects on the other enzymes that, particularly in compound E, the interactions were too weak to be measured (Table 7). Consequently, this inhibitor has essentially absolute specificity towards human renin.

The interactions of the inhibitors described in Table 7 were also examined against pepsin and the fungal proteinase at higher pH values (5.0 and 6.0). The latter value is essentially as high a pH as can be attained in practice and still retain the activity of these 'acid' proteinases [6]. However, no significant improvement was observed in

Table 7. Inhibition constants for the interaction of several aspartic proteinases with synthetic inhibitors of the structure Boc-Phe-His-ACHPA-P₂'-P₃'

All measurements were made as described in the legend to Table 1.

	<i>K_i</i> or IC ₅₀ (nM)					
	Renin	Pepsin	Gastricsin	Cathepsin D	Cathepsin E	<i>Endothia</i> proteinase
Compound A	0.4	8000	5500	600	630	200
Compound B	5	24000	22000	3000	3150	3000
Compound C	19	13000	15000	800	1150	650
Compound D	60	25000	>11500	9300	>11500	16300
Compound E	42	>42000	>14000	>14000	>14000	>14000

the very weak binding of any of these inhibitors for any of the enzymes under these conditions (results not shown).

DISCUSSION

All of the inhibitors described in this paper were based on the amino acid sequence adjacent to the scissile peptide bond attacked by renin in its naturally occurring substrate, angiotensinogen. Inhibitors with high potency towards renin were often poorly selective, however. Several compounds (Table 7) that sacrificed some potency towards renin by comparison with other inhibitors that have been described elsewhere [7,12,15,18,25–27] nevertheless were shown to have a high degree of selectivity for renin. Peptide inhibitors of this type still have a relatively high molecular mass, however, so that, despite the selectivity obtained *in vitro*, such compounds cannot be assumed to have the high oral potency and long half-life in the circulation that are pre-requisites for an anti-hypertensive agent [28].

Since detailed three-dimensional structures are not available for any of the human proteins, it is not appropriate here to attempt to account in detail at the molecular level for the distinctions in the interactions that were observed in the various sub-sites in the enzymes. By contrast, however, the structure of the proteinase from *Endothia parasitica* has been solved and refined to 0.21 nm (2.1 Å) resolution [7,24]. The structures of the complexes obtained by co-crystallization of this enzyme and two of the inhibitors described in this paper, namely

Boc-His-Pro-Phe-His-Sta-Leu-Phe-NH₂ and
Iva-His-Pro-Phe-His-ACHPA-Leu-Phe-NH₂,

have been determined by X-ray diffraction and reported recently [24]. The detailed contacts made by backbone and side-chain atoms in the inhibitors interacting in the appropriate sub-sites in the enzyme have been described therein, and the diminished affinity of the ACHPA-containing inhibitor (Table 1) is thereby accounted for.

The similarity in susceptibility of the *Endothia* proteinase and renin to inhibition by several compounds [23] has led a number of investigators to use the crystallographically defined structure of the *Endothia* enzyme as a model for human renin, and indeed structures for a considerable number (more than ten) of renin inhibitors complexed with this fungal proteinase have been solved (T. L. Blundell & J. B. Cooper, personal communication). The limitations of this approach are, however,

indicated by the distinct effects observed, for example, with the shorter/ACHPA-containing inhibitors (Table 1) as well as with the evolution of specific inhibitors of renin (Table 7) that do not appear to interact with the *Endothia* proteinase to any significant extent. The nature of the substituents occupying the P₂' and P₃' positions would thus appear to have special significance in determining the degree of selectivity obtained. While this work was in progress, a number of other reports have appeared indicating also that inhibitors of renin with varying potencies can be produced by manipulation of the substituents in the P₂' and P₃' positions, e.g. in statine-containing [29,30], in ACHPA-containing [28,31,32] and in other [33–35] inhibitors. Some indication of the selectivity of action was usually included in these reports, but only towards non-human pepsin and cathepsin D. For example, the compound ES-6864 {*N*-[(2*R*)-3-morpholinocarbonyl-2-(1-naphthylmethyl)propionyl]-(4-thiazolyl)-*L*-alanylcyclostatine-(2-morpholinoethyl)amide}, which is similar to the final inhibitor described in Table 7 in also containing the morpholine ring substituent in positions P₂'-P₃', was shown to have IC₅₀ values greater than 10 µM towards pig pepsin and bovine cathepsin D [32]. Very recently the compound BW-175 ((2*S*,3*R*,4*S*)-4-{*L*-*N*-[(2*S*)-3-ethylsulphonyl-2-(1-naphthylmethyl)propionyl]-norleucyl}amino-5-cyclohexyl-1-morpholinopentane-2,3-diol), with a cyclohexyl side chain in the P₁ position and terminating also with a morpholino substituent, has been described as a potent inhibitor of human renin (3 nM) but required concentrations in excess of 25 µM to inhibit bovine cathepsin D or pig pepsin [36]. However, systematic investigations of the interactions with the other human aspartic proteinases have not been performed previously. Cathepsin E, in particular, has received very little consideration.

The avidity with which cathepsin E interacted with most of the inhibitors in the present study, almost irrespective of their content (except in positions P₂' and P₃'), was readily apparent. Cathepsin E might thus be considered as a rather 'promiscuous' enzyme, apparently able to accept almost any amino acid residue in any of the sub-sites with relative facility. Unfortunately, no primary or tertiary structural data are yet available for this enzyme, but it is present in surface epithelial cells in the human stomach and duodenum, in cells overlying Peyer's patches in the ileum and in neutrophils as well as in erythrocytes [3]. These strategic (non-lysosomal) locations may well have a bearing on the relatively poor

bio-availability that has been widely observed with a number of renin inhibitors [28,32,36]. By contrast, pepsin and gastricsin in the human stomach appear to pose very little of a problem in terms of interfering with an orally administered renin inhibitor. The relatively weak interactions that were observed with these two enzymes were readily overcome by suitable manipulation of the inhibitor structure.

A further feature to emerge from the present study was the effect on pepsin (Table 3) of introducing a (small) alanine residue into the P₃ position. This finding in itself was not unexpected, since the nature of the substituent occupying the S₃ pocket in aspartic proteinases has been documented previously to be of particular significance [5,6,21,22] in determining whether or not interaction will take place. However, it is noteworthy that substitution of the one phenylalanine residue by an alanine residue resulted in an inhibitor (Boc-His-Pro-Ala-His-Sta-Leu-Phe-NH₂) that was very potent (*K*_i 80 nM) and essentially specific for human pepsin despite its structure being based on the recognition sequence in angiotensinogen for cleavage by renin. What is all the more remarkable is that this level of potency was achieved despite the presence of a histidine residue in the P₂ position, which, in itself (upper and middle parts of Table 4), is highly unfavourable for pepsin interaction. The negative influence of histidine in the P₂ position for pepsin binding and the benefits for interaction with renin have been discussed recently by Maibaum & Rich [37].

Thus, with suitable selection of residues to occupy the other positions, it becomes feasible to design effective inhibitors, not just of human pepsin (e.g. for use as anti-ulcer compounds), but of the other human enzymes also. In addition, the proteinase that is essential for maturation of human immuno-deficiency viruses (HIV-1 and HIV-2) is strongly inhibited by a statine-based inhibitor and thus belongs unequivocally to the aspartic proteinase family [38]. A specific inhibitor of the viral proteinase(s) that did not interact with any of these human (host) enzymes might well have considerable value against AIDS.

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