Peptidyl vinyl sulphones: a new class of potent and selective cysteine protease inhibitors

S₂P₂ specificity of human cathepsin O2 in comparison with cathepsins S and L

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Peptidyl vinyl sulphones are a novel class of extremely potent and specific cysteine protease inhibitors. They are highly active against the therapeutically important cathepsins O2, S and L. The highest k_{inact}/K_i values exceed $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cathepsin S and $10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cathepsins O2 and L. To study the primary specificity site of the novel human cathepsin O2 and the effectiveness of this novel class of inhibitors, a series of peptidyl vinyl sulphones with variations in the P₂ residue was synthesized. Leucine in the P₂ position was proven to be the most effective residue for cathepsin O2 and also for cathepsins S and L.

Cathepsins O2 and S share a decreased accessibility towards P₂ hydrophobic non-branched residues such as aminohexanoic acid (norleucine), methionine and oxidized methionine, but are distinguished by their different affinity towards phenylalanine in the P₂ position. In contrast, cathepsin S accepts a broader range of hydrophobic residues in its S₂ subsite than cathepsins O2 and L. The primary specificity-determining subsite pocket S₂ in cathepsin O2 appears to be spatially more restricted than those of cathepsins S and L.

INTRODUCTION

Most of the irreversible and reversible inhibitors of cysteine proteases such as aldehydes, halomethyl ketones, acyloxymethyl ketones, diazomethyl ketones, and peptidyl sulphonium salts contain a carbonyl moiety [1–7] that reacts with the thiol of the active-site cysteine residue. Exceptions are peptidyl epoxides [8,9], peptidyl nitriles [10] and *N*-peptidyl-*O*-acylhydroxylamines [11]. Liu and Hanzlik [12] recently described Michael acceptors as a class of potential protease inhibitors. However, the second-order rate constants of inactivation of papain with these compounds were only in the range of $1\text{--}30 \text{ M}^{-1} \cdot \text{s}^{-1}$ and did not promise a potent inactivation of mammalian cysteine proteases. We have developed peptidyl vinyl sulphones as extremely potent and specific cysteine protease inactivators of human cathepsins [13]. The structure of peptidyl vinyl sulphones ($\text{R}_n\text{-NH-CH(R}_1\text{)-}\Psi(\text{CH=CH-SO}_2\text{-R}_x)$) permits variation in the P as well as P' region of the inhibitors, thus allowing selective control of their affinity and reactivity towards the enzyme.

Human cysteine proteases such as cathepsins B, L, S and O2 are implicated in the pathogenesis of several diseases characterized by tissue-damaging processes including rheumatoid arthritis [14], lung emphysema [15], tumour invasion [16,17] and Alzheimer's disease [18]. Cathepsins are thought to be involved in the pathological degradation of extracellular matrix proteins like collagen [19], proteoglycans [20] and elastin [21]. As such, specific inhibition of these proteases represents a potentially important therapeutic strategy. Of particular interest is the design of potent inhibitors for a novel cysteine protease, cathepsin O2, that is predominantly expressed in osteoclasts, and which may play a central role in bone remodelling and bone pathology [22,23]. It could be shown that cathepsin O2 displays a high collagenolytic activity and that the protease exhibits a significantly higher pH stability at neutral pH than cathepsin L [23a].

In the present paper we report the potent inactivation of

cathepsins O2, S and L and discuss the influence of P₂-residue variation on the specificity and efficacy of peptidyl vinyl sulphones.

EXPERIMENTAL

Enzymes and substrates

Recombinant human cathepsins O2 (EC 3.4.22.-) and S (EC 3.4.22.27) were produced in *Spodoptera frugiperda* (Sf9) cells using the baculovirus expression system and purified as described (Brömme and McGrath, unpublished work; [23a]). Recombinant human cathepsin L (EC 3.4.22.15) was a generous gift from J. S. Mort (Shriner's Hospital for Crippled Children, Montreal, Quebec, Canada). All proteases used were electrophoretically homogeneous and their molarity was determined by E-64 titration [24].

The substrates, Z-Phe-Arg-NH-Mec and Z-Val-Val-Arg-NH-Mec (-NH-Mec is 4-methyl-7-coumarylamide), were obtained from Bachem, Inc. (Switzerland).

Synthesis of peptidyl vinyl sulphones

Mu-Leu-Hph- $\Psi(\text{CH=CH-SO}_2\text{-Ph})$ and Mu-Phe-Hph- $\Psi(\text{CH=CH-SO}_2\text{-Ph})$ were synthesized as described in [13]. (The following abbreviations are used throughout: Mu-, 4-morpholinecarbonyl; Hph, homophenylalanine; $\Psi(\text{CH=CH-SO}_2\text{-Ph})$, vinyl sulphone.) The carbonyl group in Hph is replaced by the vinyl moiety (Figure 1).

Peptidyl vinyl sulphones were prepared through mixed-anhydride coupling of *t*-butyloxycarbonyl (Boc)-protected amino acids with the toluene sulphonate salt of (S,E)-3-amino-5-phenyl-1-phenylsulphonyl-1-pentene, abbreviated as TsOH.Hph- $\Psi(\text{CH=CH-SO}_2\text{-Ph})$. Acid deprotection of the Boc groups with

Abbreviations used: Mu-, 4-morpholinecarbonyl; Z-, benzyloxycarbonyl; Boc-, tert-butyloxycarbonyl; -NH-Mec, 4-methyl-7-coumarylamide; $\Psi(\text{CH=CH-SO}_2\text{-Ph})$, vinyl sulphone; Ph, phenyl; Hph, homophenylalanine; Ahx, aminohexanoic acid; MetO₂, methionine dioxide; MCPBA, *m*-chloroperoxybenzoic acid.

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Figure 1 Mu-Leu-Hph-Ψ(CH=CH-SO₂-Ph)

4 M HCl in dioxane, followed by treatment with 4-morpholinecarbonyl chloride (Mu-Cl), in the presence of triethylamine, afforded the title compounds [P₂ residues of aminohexanoic acid ('norleucine'; Ahx), Ile, Val, Ala, Gly, Met; methionine dioxide (MetO₂) at P₂ was obtained by oxidizing the Met derivative with 2.2 equivalents of *m*-chloroperoxybenzoic acid (MCPBA)]. TsOH.Hph-Ψ(CH=CH-SO₂-Ph) itself was prepared in four steps from Boc-Hph-OH (Synthetech): (i) formation of the *N,O*-dimethylhydroxamate; (ii) reduction to the aldehyde; (iii) Wadsworth–Emmons coupling with diethyl phenylsulphonylmethylenephosphonate; and (iv) removal of the Boc group with anhydrous toluenesulphonic acid in ether to give the salt [13].

All compounds were characterized by TLC and NMR. NMR spectra were recorded on a Jeol Eclipse 270 MHz instrument. All ¹H-NMR data of this and subsequent examples are reported as δ values in p.p.m. relative to an internal tetramethylsilane standard in C²HCl₃ and peak assignments are in italic typeface. The following abbreviations are used: s, singlet; d, doublet; dd, double doublet; t, triplet; q, quartet; br, broad; m, multiplet. An asterisk (*) denotes that a signal is obscured or buried under another resonance. TLC was performed on each sample using the following systems: A, ethyl acetate/CH₂Cl₂ (1:1, v/v); and B, ethyl acetate/CH₂Cl₂ (1:4, v/v). Visualization was accomplished by means of a UV light at 254 nm, followed by ninhydrin, Bromocresol Green, or *p*-anisaldehyde stain. Yields listed represent the yield of the last coupling step.

Mu-Ahx-Hph-Ψ(CH=CH-SO₂-Ph)

The yield was 34%.

TLC data: solvent system A; *R_f* = 0.36.

¹H-NMR data: δ_H [(²H)chloroform] 0.6–1.0 (3 H, m, CH₃CH₂CH₂CH₂); 1.2–2.0 (8 H, m*, PhCH₂CH₂CH, CH₃CH₂CH₂CH₂, CH₃CH₂CH₂CH₂ and CH₃CH₂CH₂CH₂); 2.5–2.7 (2 H, m, PhCH₂CH₂CH); 3.2–3.4 [4 H, m, OCH₂CH₂N (Mu)]; 3.5–3.7 [4 H, m, OCH₂CH₂N (Mu)]; 4.2–4.4 (1 H, q, *J* 7.2 Hz, PhCH₂CH₂CH); 4.5–4.8 (1 H, m, CH₃CH₂CH₂CH₂CH); 5–5.1 [1 H, d, *J* 7.7 Hz, *NH* (Mu)]; 6.35–6.5 (1 H, d, *J* 15.1 Hz, *trans* CH=CHSO₂Ph); 6.8–6.95 (1 H, dd, *J* 15.1 Hz and 5 Hz, *trans* CH=CHSO₂Ph); 7–7.8 (11 H, m*, *NH*, and aromatic).

Mu-Ile-Hph-Ψ(CH=CH-SO₂-Ph)

The yield was 34%.

TLC data: solvent system A; *R_f* = 0.32.

¹H-NMR data: δ_H [(²H)chloroform] 0.7–1.1 [8 H, m*, CH₃CH₂CH(CH₃)CH, CH₃CH₂CH(CH₃)CH and CH₃CH₂CH(CH₃)CH]; 1.4–1.5 [1 H, m, CH₃CH₂CH(CH₃)CH]; 1.7–1.9 (2 H, m, PhCH₂CH₂CH); 2.4–2.6 (2 H, m, PhCH₂CH₂CH); 3.2–3.4 [4 H, m, OCH₂CH₂N (Mu)]; 3.5–3.7

[4 H, m, OCH₂CH₂N (Mu)]; 4.1–4.2 (1 H, t, *J* 8.2 Hz, PhCH₂CH₂CH); 4.5–4.7 [1 H, m, CH₃CH₂CH(CH₃)CH]; 5–5.1 [1 H, d, *J* 7.7 Hz, *NH* (Mu)]; 6.35–6.5 (1 H, d, *J* 15.1 Hz, *trans* CH=CHSO₂Ph); 6.8–6.95 (1 H, dd, *J* 15.1 Hz and 5.7 Hz, *trans* CH=CHSO₂Ph); 7–7.8 (11 H, m*, *NH*, and aromatic).

Mu-Val-Hph-Ψ(CH=CH-SO₂-Ph)

The yield was 57%.

TLC data: solvent system B; *R_f* = 0.15.

¹H-NMR data: δ_H [(²H)chloroform] 0.7–1 [6 H, m, (CH₃)₂CHCH]; 1.7–2.1 [3 H, m*, PhCH₂CH₂CH and (CH₃)₂CHCH]; 2.4–2.6 (2 H, m, PhCH₂CH₂CH); 3.2–3.4 [4 H, m, OCH₂CH₂N (Mu)]; 3.5–3.7 [4 H, m, OCH₂CH₂N (Mu)]; 4–4.2 (1 H, t, *J* 7.9 Hz, PhCH₂CH₂CH); 4.5–4.7 [1 H, m, (CH₃)₂CHCH]; 5–5.1 [1 H, d, *J* 8.4 Hz, *NH* (Mu)]; 6.4–6.5 (1 H, d, *J* 15.1 Hz, *trans* CH=CHSO₂Ph); 6.8–6.95 (1 H, dd, *J* 15.1 Hz and 5.4 Hz, *trans* CH=CHSO₂Ph); 7–7.9 (11 H, m*, *NH*, and aromatic).

Mu-Ala-Hph-Ψ(CH=CH-SO₂-Ph)

The yield was 94%.

TLC data: solvent system B; *R_f* = 0.10.

¹H-NMR data: δ_H [(²H)chloroform] 1.3 (3 H, m, CH₃CH); 1.8–2.0 (2 H, m, PhCH₂CH₂CH); 2.4–2.6 (2 H, m, PhCH₂CH₂CH); 3.2–3.4 [4 H, m, OCH₂CH₂N (Mu)]; 3.5–3.7 [4 H, m, OCH₂CH₂N (Mu)]; 4.3–4.4 (1 H, m, PhCH₂CH₂CH); 4.6–4.7 (1 H, m, CH₃CH); 5–5.1 [1 H, d, *J* 7.2 Hz, *NH* (Mu)]; 6.4–6.5 (1 H, d, *J* 15.1 Hz, *trans* CH=CHSO₂Ph); 6.8–6.95 (1 H, dd, *J* 15.1 Hz and 4.9 Hz, *trans* CH=CHSO₂Ph); 7–7.9 (11 H, m*, *NH*, and aromatic).

Mu-Gly-Hph-Ψ(CH=CH-SO₂-Ph)

The yield was 72%.

TLC data: solvent system A; *R_f* = 0.12.

¹H-NMR data: δ_H [(²H)chloroform] 1.8–2.0 (2 H, m, PhCH₂CH₂CH); 2.4–2.6 (2 H, m, PhCH₂CH₂CH); 3.2–3.4 [4 H, m, OCH₂CH₂N (Mu)]; 3.5–3.7 [4 H, m, OCH₂CH₂N (Mu)]; 3.8–3.9 (2 H, d, *J* 4.9 Hz, NHCH₂CO); 4.6–4.7 (1 H, m, PhCH₂CH₂CH); 5.5 [1 H, br t, *NH* (Mu)]; 6.4–6.5 (1 H, d, *J* 15.1 Hz, *trans* CH=CHSO₂Ph); 6.8–6.95 (1 H, dd, *J* 15.1 Hz and 4.9 Hz, *trans* CH=CHSO₂Ph); 7–7.9 (11 H, m*, *NH*, and aromatic).

Mu-Met-Hph-Ψ(CH=CH-SO₂-Ph)

The yield was 99%.

TLC data: solvent system A; *R_f* = 0.32.

¹H-NMR data: δ_H [(²H)chloroform] 1.8–2.2 (7 H, m*, CH₃SCH₂CH₂CH, CH₃SCH₂CH₂CH and PhCH₂CH₂CH); 2.4–2.7 (4 H, m, PhCH₂CH₂CH and CH₃SCH₂CH₂CH); 3.2–3.4 [4 H, m, OCH₂CH₂N (Mu)]; 3.5–3.7 [4 H, m, OCH₂CH₂N (Mu)]; 4.4–4.5 (1 H, q, *J* 7.2 Hz, PhCH₂CH₂CH); 4.55–4.7 (1 H, m, CH₃SCH₂CH₂CH); 5–5.1 [1 H, d, *J* 7.4 Hz, *NH* (Mu)]; 6.35–6.5 (1 H, d, *J* 15.1 Hz, *trans* CH=CHSO₂Ph); 6.8–6.9 (1 H, dd, *J* 15.1 Hz and 5.2 Hz, *trans* CH=CHSO₂Ph); 7–7.8 (11 H, m*, *NH*, and aromatic).

Mu-MetO₂-Hph-Ψ(CH=CH-SO₂-Ph)

The yield was 81%.

TLC data: solvent system A; *R_f* = 0.09.

¹H-NMR data: δ_H [(²H)chloroform] 1.8–2.0 (2 H, m, PhCH₂CH₂CH); 2.2–2.4 (2 H, m, CH₃SO₂CH₂CH₂CH); 2.6–2.8

Figure 2 Progress curves of the cathepsin O2-catalysed hydrolysis of Z-Phe-Arg-NH-Mec in the presence of the inhibitor Mu-Leu-Hph-Ψ(CH=CH-SO₂-Ph)

The substrate concentration was 2.5 μM and the following inhibitor concentrations were used: ○, 50 nM; ●, 40 nM; ▽, 30 nM; ▼, 20 nM; □, 10 nM and ■, 5 nM.

(2 H, m, PhCH₂CH₂CH); 2.9 (3 H, s, CH₃SO₂CH₂CH₂CH); 3.0–3.4 [6 H, m*, CH₃SO₂CH₂CH₂CH, and OCH₂CH₂N (Mu)]; 3.5–3.7 [4 H, m, OCH₂CH₂N (Mu)]; 4.4–4.7 (2 H, m*, PhCH₂CH₂CH and CH₃SO₂CH₂CH); 5.7–5.8 [1 H, d, *J* 6.9 Hz, NH (Mu)]; 6.4–6.6 (1 H, d, *J* 15.1 Hz, *trans* CH=CHSO₂Ph); 6.8–6.95 (1 H, dd, *J* 15.1 Hz and 5.2 Hz, *trans* CH=CHSO₂Ph); 7.0–7.8 (11 H, m*, NH, and aromatic).

Enzyme assays

The inactivation rates (k_{obs}) in the presence of substrate and for different inhibitor concentrations (eight to nine inhibitor concentrations) were determined in duplicate for cathepsins O2, S and L according to Tian and Tsou [25] and as previously described

[26]. The inactivation of proteinases with substrate-analogous inhibitors proceeds according to eqn. (1):

where EI, E-I, k_1 and k_{-1} represent the enzyme–inhibitor complex, the inactivated enzyme and the rate constants of non-covalent reaction steps respectively ($K_i = k_{-1}/k_1$). k_{inact} is the rate constant of the formation of covalent modified enzyme.

By fitting the rate constants obtained at different inhibitor concentrations to eqn. (2), the inactivation parameters $K_{i,\text{app}}$ and k_{inact} were obtained. The $K_{i,\text{app}}$ values were then corrected to zero substrate concentration using eqn. (3).

$$k_{\text{obs}} = k_{\text{inact}}[I]/K_{i,\text{app}} + [I] \quad (2)$$

$$K_i = K_{i,\text{app}}/(1 + [S]/K_m) \quad (3)$$

Inhibitors were prepared as stock solutions in dimethyl formamide, and added to buffer solutions of activated enzyme. The final concentration of organic solvent was less than 1 % in all assays.

Progress curves for the inactivation of the proteases were monitored at room temperature (22 °C) using a Perkin-Elmer luminescence spectrometer LS50B at an excitation wavelength of 380 nm, and with an emission filter of 450 nm.

The kinetic experiments were performed with a constant enzyme concentration in 100 mM sodium acetate buffer, pH 5.5, for cathepsin L (0.5 nM), and in 100 mM potassium phosphate buffer, pH 6.5, for cathepsins S (1.8 nM) and O2 (1 nM). All buffers contained 2.5 mM EDTA and 2.5 mM dithiothreitol. Substrates used were Z-Phe-Arg-NH-Mec (1 μM for cathepsin L; 2.5 μM for cathepsin O2) and Z-Val-Val-Arg-NH-Mec (5 μM for cathepsin S). The following K_m values were used as correction factors for the respective $K_{i,\text{app}}$ values: 0.6 μM for cathepsin L [27], 18 μM for cathepsin S [28] and 7.5 μM for cathepsin O2 [23a].

RESULTS AND DISCUSSION

Peptidyl vinyl sulphones are specific cysteine protease inhibitors of human cathepsins, some of which display second-order rate constants higher than 10⁷ M⁻¹·s⁻¹ [13]. This reflects a 10⁶-fold increase in second-order rate constants of inactivation when compared with the published inactivation constants for papain

Table 1 Inactivation of human cathepsins O2, S and L with peptidyl vinyl sulphones [Mu-X-Hph-Ψ(CH=CH-SO₂-Ph)]

Values are means ± S. D.

X	Human cathepsin O2			Human cathepsin S			Human cathepsin L		
	k_{inact} (s ⁻¹)	K_i (μM)	k_{inact}/K_i (M ⁻¹ ·s ⁻¹)	k_{inact} (s ⁻¹)	K_i (μM)	k_{inact}/K_i (M ⁻¹ ·s ⁻¹)	k_{inact} (s ⁻¹)	K_i (μM)	k_{inact}/K_i (M ⁻¹ ·s ⁻¹)
Gly	0.009 ± 0.001	145 ± 36	60	0.12 ± 0.04	46 ± 24	2600			380*
Ala	0.011 ± 0.002	23 ± 2	480	0.04 ± 0.01	7 ± 2	5600			210*
Val	0.033 ± 0.007	5.6 ± 0.2	5900	0.13 ± 0.04	1.6 ± 0.6	81 300	0.061 ± 0.019	4.2 ± 19	14 500
Ile	0.043 ± 0.012	3.4 ± 1.2	12 600	0.21 ± 0.05	0.4 ± 0.1	525 000	0.029 ± 0.006	4.7 ± 1.2	6200
Leu	0.08 ± 0.01	0.11 ± 0.01	727 000	0.19 ± 0.08	0.013 ± 0.006	14 600 000	0.039 ± 0.019	0.12 ± 0.01	325 000
Ahx	0.07 ± 0.01	1.6 ± 0.5	44 000	0.15 ± 0.01	0.015 ± 0.002	10 000 000	0.029 ± 0.006	2.2 ± 0.2	13 200
Met	0.03 ± 0.001	0.42 ± 0.15	71 000	0.07 ± 0.01	0.005 ± 0.002	14 000 000	0.029 ± 0.008	4.8 ± 2.2	6000
MetO ₂	0.014 ± 0.003	14 ± 5	1000	0.09 ± 0.02	0.031 ± 0.011	2 903 000	0.006 ± 0.001	12 ± 6	500
Phe	0.25 ± 0.04	36 ± 5	6900	0.09 ± 0.03	0.018 ± 0.009	5 000 000	0.028 ± 0.006	0.11 ± 0.01	255 000

* Below saturation concentrations.

Figure 3 Effects of the surface area (in Å²) of the P₂ inhibitor side chain on the inactivation parameters k_{inact} (s⁻¹), K_i (M) and k_{inact}/K_i (M⁻¹·s⁻¹) of (a) human cathepsin S and (b) human cathepsin O2

The surface areas of amino acid side chains were taken from Chothia [31] and the single-letter amino acid code is used to indicate each data point as follows: G, Gly; A, Ala; L, Leu; I, Ile; M, Met; F, Phe.

[12]. The reaction with the target cysteine protease is irreversible. No recovery of activity was observed after removal of excess inhibitor by gel filtration [13]. Typical progress curves for the cathepsin O2-catalysed hydrolysis of Z-Phe-Arg-NH-Mec in the presence of the inhibitor Mu-Leu-Hph-Ψ(CH=CH-SO₂-Ph) are shown in Figure 2.

Neither binding to nor inactivation of serine proteases was observed with peptidyl vinyl sulphones even when bearing complementary binding determinants [13].

In the present study we compared the S₂P₂ specificity of human cathepsins O2, S and L. For this purpose we synthesized a series

Figure 4 Effect of the P₂ residue in Mu-X-Hph-Ψ(CH=CH-SO₂-Ph) inhibitors on the inactivation of human cathepsins O2, S and L

Amino acids are designated below the bars by single-letter amino acid code (see Figure 2). MO, methionine dioxide; nL, norleucine (aminohexanoic acid).

of compounds with the general structure Mu-X-Hph-Ψ(CH=CH-SO₂-Ph) (X = Gly, Ala, Val, Leu, Ile, Ahx, Met, MetO₂ and Phe). Cathepsin S was most efficiently inhibited displaying second-order rate constants as high as 10⁷ M⁻¹·s⁻¹ (Table 1). Cathepsins O2 and L were inhibited approximately one to three orders of magnitude less than cathepsin S. A lower K_i (10- to 100-fold) and a higher k_{inact} (2- to 7-fold) for cathepsin S account for these differences. The generally more efficient inactivation of cathepsin S by this class of inhibitors contrasts with inhibition by previously described N,O-diacylhydroxamates which are better inhibitors of cathepsin L than cathepsin S [26,29]. This may reflect differences in the intrinsic activity of the proteases and in the geometry of the active-site residues between the cysteine proteases of the papain family.

Variability in the inhibitory efficiency between the compounds is mainly controlled by their binding constants. Again, k_{inact} values vary only within one order of magnitude whereas binding constants range across three to four orders of magnitude (Table 1). The results suggest that increasing the surface area of the P₂ residue increases the reactivity (k_{inact}/K_i) of the inhibitors towards the target proteases. A plot of the log(k_{inact}/K_i) as a function of the surface area of the P₂ side chain displays a good linear correlation (slope 0.030±0.007) for cathepsin S (Figure 3a), whereas the same plot for cathepsin O2 reveals two clear exceptions from a linear dependence, phenylalanine and leucine (Figure 3b). Phenylalanine appears to be too bulky whereas the leucine residue indicates favourable effects on the binding.

The S₂P₂ specificity for cathepsins O2 and L is similar (Figure 4), which may reflect similarities in the S₂ subsite architecture. The crystal structure of a papain-inhibitor complex shows that the P₂ side chain of a bound inhibitor is adjacent to the side chains of residues 133 and 157 [30]. The putative residues in those positions are identical or similar in cathepsins O2 and L but different in cathepsin S (Table 2). Structure homology models of the human cathepsins O2, L and S (M. E. McGrath, unpublished

Table 2 Putative S₂ subsite-forming residues in human cathepsins O2, L and S

Residue numbers used are according to the papain numbering.

Residue	Cathepsin O2	Cathepsin L	Cathepsin S
67	Tyr	Leu	Phe
68	Met	Met	Met
133	Ala	Ala	Gly
157	Leu	Met	Val
160	Ala	Gly	Gly
205	Leu	Ala	Phe

work) suggest that the S₂ pocket in cathepsin S (Gly-133; Val-157) is significantly wider and deeper than those of cathepsins O2 and L (Ala-133; Leu- or Met-157, respectively). On the other hand, the Leu-157 residue in cathepsin O2 seems to restrict the size and depth of the S₂ binding pocket more than the appropriate methionine in cathepsin L.

The second-order rate constants of inactivation increase for all three enzymes, going from a glycine to a leucine (Table 1; Figure 4). However, hydrophobic unbranched residues, such as methionine and aminohexanoic acid, are well accepted only by cathepsin S. The 10- to 50-fold drop in the k_{inact}/K_i values for the inactivation of cathepsins O2 and L is mainly due to an increase in K_i which may indicate steric hindrance between the P₂ side chain and the S₂ binding pocket. Oxidation of the methionine, which results in a significant increase in bulkiness of the P₂ residue, decreases the second-order rate constants even further by factors of 10 and 70 for cathepsins O2 and L respectively. In contrast, cathepsin S is able to accommodate the oxidized methionine with only minimal loss in inhibitory efficacy. These observations clearly indicate that the binding pocket of cathepsin S is deeper and more spacious than those of cathepsins O2 and L and they are in accordance with the homology models of the S₂ binding sites for the three proteases.

A major difference between cathepsins O2 and L is the better acceptance of phenylalanine in the P₂ position by cathepsin L. The preferred specificity towards phenylalanine in P₂ by cathepsin L when compared with that of cathepsin O2 is also observed with substrates [23a].

In conclusion, peptidyl vinyl sulphones are very potent inhibitors of human cysteine proteases. They can be successfully used for subsite mapping and they have the potential to be developed as drugs against powerful cysteine proteases such as cathepsins S, O2 and L, which have been implicated in degenerative diseases.

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