Design of kallidin-releasing tissue kallikrein inhibitors based on the specificities of the enzyme's binding subsites

Fernanda. C. V. PORTARO*, Maria H. S. CEZARI*, Maria A. JULIANO*, Luiz JULIANO*, Adrian R. WALMSLEY† and Eline S. PRADO*‡

*Department of Biophysics, Universidade Federal de São Paulo – Escola Paulista de Medicina, Rua Três de Maio 100, São Paulo 04044-020, Brazil, and

†Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH, U.K.

The tissue kallikrein inhibitors reported in the present work were derived by selectively replacing residues in *N*^z-substituted arginine- or phenylalanine-pNA (where pNA is *p*-nitroanilide), and in peptide substrates for these enzymes. Phenylacetyl-Arg-pNA was found to be an efficient inhibitor of human tissue kallikrein (K_i 0.4 μ M) and was neither a substrate nor an inhibitor of plasma kallikrein. The peptide inhibitors having phenylalanine as the P₁ residue behaved as specific inhibitors for kallidin-releasing tissue kallikreins, while plasma kallikrein showed high

affinity for inhibitors containing (*p*-nitro)phenylalanine at the same position. The K_i value of the most potent inhibitor developed, Abz-Phe-Arg-Arg-Pro-Arg-EDDnp [where Abz is *o*-aminobenzoyl and EDDnp is *N*-(2,4-dinitrophenyl)-ethylenediamine], was 0.08 μ M for human tissue kallikrein. Progress curve analyses of the inhibition of human tissue kallikrein by benzoyl-Arg-pNA and phenylacetyl-Phe-Ser-Arg-EDDnp indicated a single-step mechanism for reversible formation of the enzyme–inhibitor complex.

INTRODUCTION

Kallikreins are highly specific kininogen-processing serine proteinases found in plasma and in various animal tissues [1]. Tissue kallikreins are species-specific, and very similar amino acid sequences for kallikreins from different animal species have been reported [2]. Tissue kallikreins (EC 3.4.21.35) and plasma kallikreins (EC 3.4.21.34) are distinct enzymes, both in their chemical and in their enzymic properties [3]. The latter release bradykinin from kininogen by the cleavage of a Lys–Arg and an Arg–Ser bond [4], while the former, with the exception of the rat enzyme [5], cleave a Met–Lys and an Arg–Ser bond to release Lys-bradykinin [6]. These cleavage specificities have been shown to be determined by secondary enzyme–substrate interactions [7].

The first evidence for an extended binding site for tissue kallikreins was obtained from the X-ray crystal structure of the aprotinin–(pig tissue kallikrein complex) [8]. Details of the extension of this binding site for cleavage of Arg–Ser and Met–Lys bonds were obtained from studies on the hydrolysis by human tissue kallikrein of oligopeptides having the sequence of human kininogen flanking these susceptible Arg–Ser and Met–Lys bonds [9].

Studies by Fiedler et al. [10], Ketner and Shaw [11], Prado et al. [12] and Araújo-Viel et al. [13] on the specificities of tissue kallikrein subsites S₃–S'₂ (nomenclature of Schechter and Berger [14]) resulted in the development of better substrates for these enzymes, including the chromogenic substrate D-Pro-Phe-Arg-pNA (where pNA is *p*-nitroanilide), which is also susceptible to plasma kallikrein [10], and the internally quenched fluorogenic substrate Abz-Phe-Arg-Ser-Arg-EDDnp [where Abz is *o*-aminobenzoyl and EDDnp is *N*-(2,4-dinitrophenyl)-ethylenediamine], which discriminates between tissue and plasma kallikreins [15]. In recent work [16] we have reported that

substitution of arginine by phenylalanine in these structures leads to substrates specific for the kallidin-releasing tissue kallikreins, i.e. they are resistant to rat tissue and plasma kallikreins, as well as to other arginyl hydrolases.

The aim of the present work was to develop inhibitors for kallidin-releasing tissue kallikreins. The interest in these inhibitors derives from evidence implicating the kallikrein/kinin system in a variety of physiological as well as pathological processes [17]. The modifications introduced in the sequences of tissue kallikrein substrates were based on previous [7] and new [16] data on the interactions of the enzyme S₃–S'₃ subsites with specific amino acid residues, which were shown to result in low K_m and k_{cat} values and/or a higher specificity for the kallidin-releasing kallikreins.

MATERIALS AND METHODS

Synthesis of peptidyl-pNAs and peptides

Previously described procedures were used for the synthesis, purification and analysis of *N*^z-substituted arginine- and phenylalanine-pNAs [18] and of the intramolecularly quenched fluorogenic peptides having Abz and EDDnp as a donor–acceptor pair at the N- and C-termini of the peptides respectively [15]. Peptides with arginine-NH₂ as the C-terminal residue were synthesized by solid-phase peptide synthesis as described previously [19]. The final deprotected peptides were purified by semi-preparative HPLC using an Econosil C-18 column (10 μ m; 22.5 mm $\times$ 250 mm) and two solvent systems: A, trifluoroacetic acid/water (1:1000, v/v); B, trifluoroacetic acid/acetonitrile/water (1:900:100, by vol.). The column was eluted at a flow rate of 5 ml/min with a 10 (or 30)–50% (or 60%) gradient of

Abbreviations used: Abz, *o*-aminobenzoyl; Bz, benzoyl; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; MCA, 7-amino-4-methylcoumarin; pNA, *p*-nitroanilide; Pac, phenylacetyl; OEt, ethyl ester.

‡ To whom correspondence should be addressed.

system B over 30 or 45 min. Analytical HPLC was performed using a C-R7A Shimadzu UV/visible detector and a Shimadzu RF-535 fluorescence detector, coupled to an Ultrasphere C-18 column (5 μ m; 4.6 mm $\times$ 150 mm) which was eluted with solvent systems A and B at a flow rate of 1.7 ml/min and a 10–80 % gradient of solvent B over 15 min. The HPLC column eluates were monitored by absorbance at 220 nm and by fluorescence emission at 420 nm following excitation at 320 nm. These HPLC conditions were also used for the identification of the products of enzymic hydrolyses and for assays of the resistance of the inhibitors to cleavage by the kallikreins. Amino acid analyses were carried out in a Beckman 6300 amino acid analyser following hydrolysis in 6 M HCl with 5 % phenol at 110 °C for 48 h.

Enzymes

Homogeneous human and rat tissue kallikreins were generously provided by Professor Julie Chao (Medical University of South Carolina, Charleston, SC, U.S.A.). Pig pancreatic kallikrein (1956 kallikrein units/mg of protein), prepared by Bayer AC (Wuppertal, Germany), was kindly supplied by Dr. F. Fiedler (University of Munich, Germany). Dr. C. A. M. Sampaio (Department of Biochemistry, Escola Paulista de Medicina, São Paulo, Brazil) kindly supplied the sample of human plasma kallikrein isolated as described previously [20]. The molar concentrations of the enzyme solutions were determined by active-site titration with 4-nitrophenyl 4-guanidinobenzoate [21].

Enzyme assays

The hydrolysis of the peptidyl-pNAs at 30 °C in 0.05 M Tris/HCl, pH 9.0, containing 1 mM EDTA was measured photometrically as previously described [22] by determining the amount of *p*-nitroaniline released after the addition of enzyme to 2 ml of reaction buffer. Hydrolysis was carried out for 5–10 min, and initial hydrolytic rates were calculated from the slope of the time-dependent absorbance curves extrapolated to zero time.

Hydrolysis of the fluorogenic peptide substrates at 30 °C in 0.02 M Tris/HCl, pH 9.0, containing 1 mM EDTA was monitored by measuring the fluorescence at λ_{em} = 420 nm and λ_{ex} = 320 nm in a Hitachi F-2000 spectrofluorimeter, as previously described [15].

The concentrations of the peptide substrate solutions were determined from the concentrations of the products of their total hydrolysis by trypsin or chymotrypsin, as previously described [7,16]. The concentrations of the inhibitor solutions were determined by amino acid analysis.

Inhibition studies

The chromogenic (D-Pro-Phe-Phe-pNA) and fluorogenic (Abz-Phe-Arg-Ser-Ser-Arg-EDDnp) substrates for human tissue kallikrein (K_m values of 10.8 and 0.19 μ M respectively at 30 °C and pH 9.0) were used to assay the peptide inhibitors. In the case of the *N*^ω-substituted arginine- and phenylalanine-pNAs, the fluorogenic substrate could not be used because of the quenching effect of these compounds on the fluorescence of the hydrolysis products. The type of inhibition was determined from Lineweaver–Burk plots as described by Segel [23]. Four inhibitor concentrations were used with each of three substrate concentrations. The substrate concentration intervals used corresponded to 1–10 $\times K_m$, and the limit of substrate hydrolysis was 10 %. The K_i values were calculated from Dixon plots [24] and/or from the relationships $K_i = K_i(\text{app})/(1 + [S]/K_m)$ and $v_o/v_i = 1 + [I]/K_i(\text{app})$, as described by Nicklin and Barrett [25], where v_o and v_i

are the initial velocity in the absence and presence respectively of the inhibitor.

Progress curve analysis of enzyme inhibition

The kinetics of the inhibition of human tissue kallikrein by Bz-Arg-pNA and Pac-Phe-Ser-Arg-EDDnp (inhibitors A and E-4 respectively; where Bz is benzoyl and Pac is phenylacetyl) were assessed using the progress curve method described by Bieth [26]. For these inhibition studies the hydrolysis of the fluorescent substrate D-Pro-Phe-Phe-MCA (where MCA is 7-amino-4-methylcoumarin) was monitored by measuring the fluorescence at λ_{em} = 380 nm and λ_{ex} > 420 nm in an Applied Photophysics stopped-flow instrument operated at 30 °C [27]. The concentrations of D-Pro-Phe-Phe-MCA and enzyme were fixed at 7.5 μ M ($0.1 \times K_m$) and 10 nM respectively, while the inhibitor concentration was varied from 50 nM to a concentration equivalent to the K_i for each inhibitor. In each case the progress curves were biphasic, with an initial exponential phase followed by a linear phase, and could be analysed by non-linear regression curve fitting, using the software supplied with the stopped-flow apparatus, to the following equation defining the rate of product (P) formation:

$$[P] = v_s t + [(v_z - v_s)/k](1 - e^{-kt}) \quad (1)$$

where v_z is the initial reaction velocity at zero time (t), v_s is the final steady-state reaction velocity and k is the pseudo-first-order rate constant for the approach to steady state [26]. Generally, the exponential phase lasted between 60 and 90 s, and the progress curves were analysed over 200 s, during which time less than 5 % of the substrate was depleted, as assessed by comparison with the final fluorescence level. Each progress curve analysed was the average of between five and ten traces generated in the stopped-flow instrument. The constant k is related to the association constant k_{ass} and the dissociation constant k_{diss} [26] by the equation:

$$k = k_{ass}[I]/(1 + [S]/K_m) + k_{diss} \quad (2)$$

RESULTS AND DISCUSSION

The tissue kallikrein inhibitors developed in the present work were divided into five series (A, B, C, D and E) according to the model substrate used for their design [Bz-Arg-pNA, D-Pro-Phe-Phe-pNA, Abz-Phe-Arg-Ser-Arg-EDDnp, Abz-Phe-Arg-Arg-Val-EDDnp and Abz-Phe-Phe-Ser-Arg-EDDnp respectively]. They were characterized as competitive inhibitors of human tissue kallikrein and shown to be resistant after a 1 h incubation at the conditions used for K_i determination ([human tissue kallikrein] = 1 nM; [I] = 0.1–50 μ M; 37 °C, pH 9.0). Increasing the concentrations of the inhibitors and the kallikrein to 150 μ M and 43 nM respectively, and the incubation time to 4 h, led to partial hydrolysis of Bz-Arg-pNA, Abz-Arg-pNA, Bz-Arg-NH₂ and Bz-Arg-OEt (OEt is ethyl ester). All the other compounds in Tables 1 and 2 were shown by HPLC analyses to be resistant to human tissue kallikrein.

Inhibition of kallikreins by *N*^ω-substituted Arg- or Phe-pNAs

Analogues of Bz-Arg-pNA (series A)

Bz-Arg-pNA, characterized as a poor substrate for human tissue kallikrein (K_m 3.5 mM and k_{cat} 0.085 s^{−1} at 37 °C, pH 9.0), behaved as a competitive inhibitor of this enzyme, as demonstrated by the Lineweaver–Burk plot for the hydrolysis of D-Pro-Phe-Arg-pNA. The K_i values calculated from the Dixon plot and from the $K_i(\text{app})$ were found to be 1.0 and 0.8 μ M respectively.

Table 1 Inhibition of human tissue (hK1) and plasma (hpKK) kallikreins by *N*²-substituted Arg- or Phe-pNAs, -anilides and -amides

Assays were carried out at 30 °C in, 20 mM Tris/HCl, 1 mM EDTA, pH 9.0, with D-Pro-Phe-Arg-pNA as substrate. Abbreviation: n.d., not determined.

Inhibitor no.	Compound	K_i (μ M)	
		hK1	hpKK
Series A			
A	Bz-Arg-pNA	1.0	Substrate*
A-1	Bz-Phe-pNA	3.0	5.5
A-2	Abz-Arg-pNA†	2.0	Substrate
A-3	Abz-Phe-pNA	9.5	9.5
A-4	Pac-Arg-pNA	0.4	n.i./n.h.‡
A-5	Pac-Phe-pNA	0.8	n.i./n.h.‡
A-6	Pac-(ρ NO ₂)Phe-pNA	0.7	0.8
A-7	Bz-Arg-anilide†	4.0	Substrate
A-8	Bz-Phe-anilide	10.0	120
A-9	Bz-(ρ NO ₂)Phe-anilide	3.4	0.2
A-10	Bz-Arg-NH ₂ †	80.0	n.d.
A-11	Bz-Phe-NH ₂	n.i./n.h.§	n.d.
A-12	Bz-Arg-COEt†	10.0	n.d.
Series B			
B	D-Pro-Phe-Phe-pNA	Substrate	n.i./n.h.‡
B-1	Bz-Phe-Phe-pNA	0.4	130
B-2	Abz-Phe-Phe-pNA	12.4	n.d.
B-3	Bz-Phe-(ρ NH ₂)Phe-pNA	n.i./n.h.§	n.d.
B-4	Bz-Gly-Phe-pNA	2.1	55
B-5	Bz-Gly-(ρ NO ₂)Phe-pNA	1.0	2.8

* $K_m = 130 \mu$ M and $k_{cat} = 15.5 \text{ s}^{-1}$ at pH 8.0 and 37 °C.

† Hydrolysed by hK1 at [substrate] = 130 μ M and [enzyme] = 43 nM.

‡ No inhibition of hpKK by A-4, A-5 or B at 150 μ M; no hydrolysis of A-4, A-5 or B by hpKK at [E] = 50 nM and [S] = 70 μ M.

§ No inhibition of hK1 by A-11 or B-3 at 100 μ M.

In contrast, Bz-Arg-pNA was shown to be a substrate for human plasma kallikrein (K_m 0.13 mM and k_{cat} 15 s⁻¹ at 37 °C, pH 8.0). The analogue compound, Bz-Phe-pNA (A-1), possessed similar K_i values for both tissue and plasma kallikreins (Table 1). The Arg- and Phe-pNAs with Pac as the N-terminal group (A-4 and A-5) had the lowest K_i values for human tissue kallikrein in this series, and were neither inhibitors nor substrates of human plasma kallikrein under the conditions described in Table 1. In contrast, Pac-(*p*NO₂)Phe-pNA (A-6) had similar K_i values for both kallikreins, while a lower value for the plasma enzyme was shown for Bz-(*p*NO₂)Phe-anilide (A-9). The K_i values in Table 1 also show that Bz-Arg-pNA and Bz-Phe-pNA are better inhibitors of human tissue kallikrein than the corresponding anilides, esters and, particularly, amides. Bz-(*p*NO₂)Phe-anilide (A-9) was a more efficient inhibitor of tissue kallikrein than was Bz-Phe-anilide (A-8), and was also more active against plasma kallikrein, as demonstrated by the 600-fold lower K_i value (Table 1). For the N-terminal side residues (P₂ position), the order of increasing K_i values was phenylacetylated > benzoylated > *o*-aminobenzoylated residues. The lower K_i values for Pac-Arg-pNA (A-4) and Pac-Phe-pNA (A-5) in comparison with Bz-Arg-pNA (A) and Bz-Phe-pNA (A-1) respectively suggest that the α -methylene group in the Pac radical elongates the molecule, allowing the benzyl group to reach the S₂ subsite and thus favouring the inhibitor–enzyme interaction.

Analogues of D-Pro-Phe-Phe-pNA (series B)

The substitution of a Bz group for D-proline (compound B-1) in

Table 2 K_i values for the inhibition of human tissue kallikrein by peptide substrate analogues

Assays were carried out at 30 °C in 20 mM Tris/HCl, 1 mM EDTA, pH 9.0, with Abz-Phe-Arg-Ser-Arg-EDDnp as substrate and enzyme concentrations of the order of 0.1 nM. The substrate for tissue kallikrein gave the following kinetic parameters [16]: series C, K_m 0.06 μ M, k_{cat} 0.45 s⁻¹; series D, K_m 0.04 μ M, k_{cat} 1.01 s⁻¹; series E, K_m 0.45 μ M; k_{cat} 1.65 s⁻¹. Abbreviation: Cap, ζ NH₂-caproyl.

Inhibitor no.	P ₃	P ₂	P ₁	P' ₁	P' ₂	P' ₃	P' ₄	K_i (μ M)
Series C								
C	Abz	Phe	Arg	Arg	Val	EDDnp	—	Substrate
C-1	Abz	Phe	Arg	Arg	Pro	EDDnp	—	0.7
C-2	Abz	Phe	Arg	Arg	Pro	Arg	EDDnp	0.08
C-3	Bz	Phe	Phe	Arg	Pro	Arg	NH ₂	0.5
C-4	Bz	Phe	(<i>p</i> NH ₂)Phe	Arg	Pro	Arg	NH ₂	1.1
C-5	Pac	Phe	Phe	Arg	Pro	Arg	NH ₂	0.3
C-6	Cap	Phe	Phe	Arg	Pro	Arg	NH ₂	1.5
C-7	D-Arg	Phe	Phe	Arg	Pro	Arg	NH ₂	3.2
Series D								
D	Abz	Phe	Arg	Ser	Arg	EDDnp	—	Substrate
D-1	Abz	Phe	Arg	Pro	Arg	EDDnp	—	0.6
D-2	Abz	Phe	Arg	Pro	Arg	NH ₂	—	0.6
D-3	Bz	Phe	Arg	Pro	Arg	NH ₂	—	0.3
Series E								
E	Abz	Phe	Phe	Ser	Arg	EDDnp	—	Substrate
E-1	Bz	Phe	Phe	Ser	Arg	EDDnp	—	0.1
E-2	Bz	Phe	Phe	Ser	Arg	NH ₂	—	0.3
E-3	Bz	Phe	(<i>p</i> NO ₂)Phe	Ser	Arg	NH ₂	—	0.2
E-4	—	Pac	Phe	Ser	Arg	EDDnp	—	0.7

D-Pro-Phe-Phe-pNA, a recently described substrate that is specific for kallidin-releasing tissue kallikreins [16], resulted in a more potent human tissue kallikrein inhibitor (B-1) which also inhibited plasma kallikrein but with a 325-fold higher K_i value (Table 1). The preference of subsite S₃ for Bz compared with Abz was demonstrated by the higher K_i value of Abz-Phe-Phe-pNA (B-2) in comparison with Bz-Phe-Phe-pNA (B-1). Bz-Phe-(*p*NH₂)Phe-pNA (compound B-3) did not behave as an inhibitor of human tissue kallikrein. As expected from the well known specificity of the tissue kallikrein S₂ subsite for hydrophobic amino acid residues [27], the substitution of phenylalanine at position P₂ by glycine resulted in a higher K_i value (compound B-4). In agreement with the above observations with compounds Pac-(*p*NO₂)Phe-pNA (A-6) and Bz-(*p*NO₂)Phe-anilide (A-9), the introduction of an NO₂ group at the *para* position of phenylalanine, as in Bz-Gly-(*p*NO₂)Phe-pNA (compound B-5), resulted in a significant decrease in the K_i values for the inhibition of plasma kallikrein. These data suggest that the charge distribution around the NO₂ group and/or one of the resonance forms in the nitrophenyl ring induce a very favourable fitting of this radical into the S₁ subsite, particularly of plasma kallikrein.

Peptide inhibitors of human tissue kallikrein

Analogues of Abz-Phe-Arg-Arg-Val-EDDnp (series C)

This peptide was chosen as one of the model substrates for design of inhibitors because of the favourable interactions of the tissue kallikrein S₂ subsite with phenylalanine [28] and of the S₁ and S'₁ subsites with arginine [15]. Valine was replaced by proline in peptide C-1 (Table 2), since it was previously demonstrated that arginyl bonds having proline in the P'₂ position are resistant to cleavage by kallidin-releasing tissue kallikreins [15]. Based upon data on the specificity of the tissue kallikreins S'₃ subsite [16], an

Figure 1 Effects of Bz-Arg-pNA (a) and Pac-Phe-Arg-Ser-EDDnp (b) on k , the pseudo-first order rate constant governing human tissue kallikrein inhibition, at 30 °C and pH 9.0

The concentrations of Bz-Arg-pNA and Pac-Phe-Arg-Ser-EDDnp were determined by analysing the progress curves generated by stopped-flow mixing of equal volumes of the enzyme with the substrate/inhibitors. In each case the trace was biphasic, and the rate constant (k) could be determined by non-linear curve fitting to eqn. (1) (see the Materials and methods section). The line through the data points is the best-fit linear regression line for each data set.

arginine was introduced at the C-terminus. These replacements resulted in the inhibitor Abz-Phe-Arg-Arg-Pro-Arg-EDDnp (compound C-2) having the lowest K_i value of all the inhibitors developed in this study (Table 2). However, this peptide is known to be a good substrate for plasma kallikreins and other arginyl hydrolases [12]. The replacement of arginine by phenylalanine at the P_1 position of peptide C-2 resulted in peptide C-3, which is a less efficient inhibitor of the kallidin-releasing tissue kallikreins but is resistant to plasma kallikrein. We introduced a Bz instead of an Abz group at the N-terminus of peptides C-3 and C-4, since, as shown in Table 1, the K_i value for Bz-Phe-Phe-pNA (B-1) was 30-fold lower than that for Abz-Phe-Phe-pNA. In addition, in the peptides C-3 to C-7, EDDnp was replaced by NH_2 , because these peptides could be synthesized by solid-phase methods and this modification did not result in significant variation in the K_i values for tissue kallikrein inhibition (see below for compounds D-1 and D-2). In agreement with our data for peptide B-2 (Table 1), the K_i value for the inhibitor C-4 containing ($p\text{NH}_2$)Phe as the P_1 residue was higher than for the analogue that had Phe at this position. The peptide with a Pac group at the P_3 position (C-5) did not show any improvement in enzyme inhibition compared with peptide C-3. Further modifications at the P_3 position of this peptide, namely ϵNH_2 -caproyl

Table 3 Kinetic parameters for the inhibition of human tissue kallikrein by Bz-Arg-pNA and Pac-Phe-Ser-Arg-EDDnp

Inhibitor	k_{ass} ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	k_{diss} (s^{-1})	K_i (μM) [*]	K_i (μM) [†]
Bz-Arg-pNA	0.037 ± 0.001	0.045 ± 0.002	1.22 ± 0.07	1.0
Pac-Phe-Ser-Arg-EDDnp	0.054 ± 0.005	0.068 ± 0.003	1.26 ± 0.12	0.7

^{*} $K_i = k_{\text{diss}}/k_{\text{ass}}$

[†] Obtained from steady-state analysis of inhibition kinetics, as in Table 2.

(peptide C-6) and D-arginine (peptide C-7), resulted in less efficient inhibitors.

Analogues of Abz-Phe-Arg-Ser-Arg-EDDnp (series D)

This model peptide is the best peptide substrate described so far for human tissue kallikrein (K_m 0.04 μM ; k_{cat} 1.01 s^{-1} ; k_{cat}/K_m 25250 $\text{mM} \cdot \text{s}^{-1}$) [16]. The replacement of serine by proline at the P'_1 position led to the expected resistance of the Arg-Pro bond to hydrolysis by tissue kallikreins and other arginyl hydrolases. This change also resulted in an efficient inhibitor of human tissue kallikrein. The data in Table 2 confirm the preference of the S_3 subsite for the Bz group, and also indicate that there were no differences in the interaction of the S'_3 subsite with the amide and EDDnp groups.

Analogues of Abz-Phe-Phe-Ser-Arg-EDDnp (series E)

As previously reported [15], the substitution of Phe for Arg at the P_1 position of the peptide Abz-Phe-Arg-Ser-Arg-EDDnp resulted in a substrate that was less efficient with human tissue kallikrein (k_{cat}/K_m 3667 $\text{mM} \cdot \text{s}^{-1}$) but specific for kallidin-releasing tissue kallikreins. Substituting the Abz group in this peptide with Bz produced an inhibitor with a K_i value of 0.1 μM . For the analogue having ($p\text{NO}_2$)Phe in the P_1 position (E-3), the K_i value was not significantly different from that of peptide E-2. In contrast with above observations with Bz-Arg-pNA and Bz-Phe-pNA, the presence of Pac at the P_3 position (peptide E-4) resulted in an inhibitor with a higher K_i value.

Progress curve analysis of the inhibition of human tissue kallikrein by Bz-Arg-pNA and Pac-Phe-Ser-Arg-EDDnp

For both inhibitors the pseudo-first-order rate constant (k) for the approach to the steady state was found to increase in a linear manner (Figure 1), indicating the occurrence of a single-step mechanism for reversible formation of the enzyme-inhibitor complex [26]. Under these circumstances, the rate constant k is related to the association (k_{ass}) and dissociation (k_{diss}) rate constants as described in eqn. (2).

The values of k_{ass} and k_{diss} were calculated from the data for the hydrolysis of 7.5 μM D-Pro-Phe-Phe-MCA, which occurs with a K_m of 70.8 μM and a k_{cat} of 12.5 s^{-1} . The K_i values for both inhibitors obtained from the ratio $k_{\text{ass}}/k_{\text{diss}}$ are in good agreement with those obtained from steady-state analyses of the inhibition kinetics, as shown in Table 3.

None of the peptide inhibitors of human tissue kallikrein in series C, D and E (Table 2) were able to inhibit human plasma kallikrein, even at concentrations 1000-fold higher than those used to determine the K_i values for human tissue kallikrein. With the exception of peptides C-1 and C-2, all of the peptides (each at 150 μM) in Table 2 were resistant to degradation by plasma kallikrein (56 nM) during a 4 h incubation at pH 9.0 and 37 °C.

The Arg-Pro bond of the D-series inhibitors was resistant to arginyl hydrolases, but the C-terminal amides NH_2 and EDDnp were cleaved by trypsin and plasmin.

The most efficient inhibitors of human tissue kallikrein designed in the present work (A-5, B-1, E-1 and E-4) were also assayed for their ability to inhibit pig tissue kallikrein; the K_i values found (0.9, 0.4, 0.5 and 0.7 μM respectively) were similar to those found for human tissue kallikrein (0.8, 0.4, 0.1 and 0.7 μM respectively). In contrast, these peptides were very inefficient inhibitors of rat tissue kallikrein, a bradykinin-releasing enzyme. The K_i values for Bz-Phe-Phe-pNA (B-1) and Abz-Phe-Arg-Pro-Arg-EDDnp (D-2) were, respectively, 375- and 70-fold higher for the rat than for the human enzyme. Bz-Phe-Phe-Arg-Pro-Arg- NH_2 (C-3), Bz-Phe-Phe-Ser-Arg-EDDnp (E-1) and Pac-Phe-Ser-Arg-EDDnp (E-4) did not behave as inhibitors of rat kallikrein, and the hydrolysis of these peptides could not be detected even after a 4 h incubation with this enzyme.

Okamoto et al. [29] have described PKSI-527 (*trans*-4-aminomethylcyclohexane carbonylphenylalanine-4-carboxymethylaniline) as a selective inhibitor of plasma kallikrein. We have confirmed this specificity and have demonstrated that PKSI-527 is also an inhibitor of rat tissue kallikrein with a K_i value of 1.1 μM . Szelke et al. [30] described a series of synthetic inhibitors of tissue and plasma kallikreins. The most potent and selective inhibitor for tissue kallikrein was D-Pro-Phe-D,L-Arg- CH_2 -(hexyl) $_2$, which had a K_i of 0.33 μM , 58-fold lower than the K_i for human plasma kallikrein.

In conclusion, the tissue kallikrein inhibitors described in the present paper belong to the first group of selective inhibitors of kallidin-releasing kallikreins to be reported, without detectable activity as plasma kallikrein substrates or inhibitors. The effects of these inhibitors have been screened in two biological systems, namely inhibition of the microvascular permeability induced by pig tissue kallikrein in rabbit skin *in vivo*, and inhibition of the relaxation of rabbit corpus cavernosum strips induced by this same enzyme. We chose to use Pac-Phe-Ser-Arg-EDDnp (E-4) for systematic biological assays, and this inhibitor was found to be the most active compound in both assays [31].

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REFERENCES

- Fiedler, F. (1979) *Handb. Exp. Pharmacol.* **25**, (Suppl.), 103–161
- McDonald, R., Margolius, H. S. and Erdos, E. G. (1988) *Biochem. J.* **253**, 313–321
- Tschesche, H., Maus, G., Fiedler, F., Ehret, W., Hirschauer, C., Lemon, M. and Fritz, H. (1979) *Adv. Exp. Med. Biol.* **120A**, 245–260
- Movat, H. Z. (1979) *Handb. Exp. Pharmacol.* **25**, (Suppl.), 1–89
- Kato, H., Miyata, T., Hayashi, I., Oh-Ishi, S. and Iwanaga, S. (1985) *Biochem. Biophys. Res. Commun.* **127**, 289–295
- Pierce, J. W. and Webster, M. E. (1961) *Biochem. Biophys. Res. Commun.* **5**, 353–357
- Chagas, J. R., Hirata, I. Y., Juliano, M. A., Xiong, W., Wang, C., Chao, J., Juliano, L. and Prado, E. S. (1992) *Biochemistry* **31**, 4969–4974
- Bode, W., Chen, Z. and Bartels, K. (1983) *J. Mol. Biol.* **164**, 237–282
- Del Nery, E., Chagas, J. R., Juliano, M. A., Prado, E. S. and Juliano, L. (1995) *Biochem. J.* **312**, 233–238
- Fiedler, F., Hinz, H. and Lottspeich, F. (1986) *Adv. Exp. Med. Biol.* **198A**, 283–289
- Kettner, C. and Shaw, E. (1981) *Methods Enzymol.* **80**, 826–842
- Prado, E. S., Araujo-Viel, M. S., Juliano, M. A., Juliano, L., Stella, R. C. R. and Sampaio, C. A. M. (1986) *Biol. Chem. Hoppe-Seyler* **367**, 199–205
- Araújo-Viel, M. S., Juliano, M. A., Oliveira, L. and Prado, E. S. (1988) *Biol. Chem. Hoppe-Seyler* **369**, 397–401
- Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **127**, 157–162
- Chagas, J. R., Juliano, L. and Prado, E. S. (1991) *Anal. Biochem.* **192**, 419–425
- Chagas, J. R., Portaro, F. C. V., Almeida, P. C., Juliano, M. A., Juliano, L. and Prado, E. S. (1995) *Biochem. J.* **306**, 63–69
- Bhoola, K. D., Figueroa, C. D. and Worthy, K. (1992) *Pharmacol. Rev.* **44**, 1–80
- Juliano, M. A. and Juliano, L. (1985) *Braz. J. Med. Biol. Res.* **18**, 435–445
- Hirata, Y., Cezari, M. H. S., Nakaie, C. R., Boschcov, P., Ito, A. S., Juliano, A. M. and Juliano, L. (1994) *Lett. Peptide Sci.* **1**, 299–308
- Oliva, M. L., Grisolia, D., Sampaio, M. V. and Sampaio, C. A. M. (1982) *Agents Actions* **9**, 52–57
- Sampaio, C. A. M., Sampaio, M. U. and Prado, E. S. (1984) *Hoppe-Seyler Z. Physiol. Chem.* **365**, 297–302
- Oliveira, L., Araújo-Viel, M. S., Juliano, L. and Prado, E. S. (1987) *Biochemistry* **26**, 5030–5035
- Segel, I. H. (1974) *Enzyme Kinetics*, pp. 18–111, J. Wiley and Sons, New York
- Dixon, M. (1972) *Biochem. J.* **55**, 170–171
- Nicklin, M. J. H. and Barrett, A. J. (1984) *Biochem. J.* **223**, 245–253
- Bieth, J. G. (1995) *Methods Enzymol.* **248**, 59–84
- Basso, L. A., Engel, P. C. and Walmesley, A. R. (1995) *Eur. J. Biochem.* **23**, 603–615
- Fiedler, F. (1987) *Eur. J. Biochem.* **163**, 303–312
- Okamoto, S., Wanaka, K., Okumura, H., Okada, Y. and Katsimura, Y. (1991) *Chem. Pharm. Bull.* **39**, 2930–2936
- Szelke, M., Evans, D. M., Jones, D. M., Fawcett, L., Ashworth, D., Olsson, H., Featherstone, R. L. and Church, M. K. (1994) *Braz. J. Med. Biol. Res.* **27**, 1943–1947
- Bizeto, L., Antunes, E., Portaro, F. C. U., Juliano, M. A., Juliano, L., Prado, E. S. and de Nucci, G. (1996) *Immunopharmacology*, in the press