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INHIBITION OF HUMAN α-, β- AND γ-THROMBIN BY mono-, bis-, tris- AND tetra-BENZAMIDINE STRUCTURES: THERMODYNAMIC STUDY†

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The inhibitory effect of mono-, bis-, tris- and tetra-benzamidine structures (benzamidine, DAPP, TAPB and TAPP, respectively) on the catalytic properties of human α-, β- and γ-thrombin (α-, β- and γ-thrombin, respectively) was investigated (between pH 2.0 and 7.0, $I = 0.1$ M; $T = 37.0 \pm 0.5$°C). The affinity of DAPP, TAPB and TAPP for α- and β-thrombin is higher than that found for benzamidine association around neutrality, converging in the acidic pH limb; in constrast, benzamidine, DAPP, TAPB and TAPP show the same value of the association inhibition constant (K_i; M^{-1}) for γ-thrombin over the whole pH range explored. On lowering the pH from 5.5 to 3.0, the decrease in affinity for benzamidine binding to α-, β- and γ-thrombin, as well as for DAPP, TAPB and TAPP association to γ-thrombin reflects the acidic-pK shift, upon inhibitor binding of a single ionizing group. On the other hand, values of K_i for DAPP, TAPB and TAPP binding to α- and β-thrombin appear to be modulated by the acidic-pK shift, upon inhibitor association, of two equivalent proton-binding residues over the same pH range. By considering molecular models of the serine proteinase : inhibitor complexes, the observed binding behaviour of benzamidine, DAPP, TAPB and TAPP to α-, β- and γ-thrombin has been related to the inferred stereochemistry of the enzyme : inhibitor contact region(s).

KEY WORDS: Human α-thrombin, human β-thrombin, human γ-thrombin, benzamidine, 1,3-bis-(*p*-amidinophenoxy)-propane, 1,3-bis(*p*-amidinophenoxy)-2-(*p*-amidinophenoxymethyl)-2-ethyl-propane. 1,3-bis-(*p*-amidinophenoxy)-2,2-bis(*p*-amidinophenoxymethyl)-propane, serine proteinase : inhibitor complex formation, thermodynamics.

†This paper is dedicated to Prof. Alessandro Ballio on the occasion of his 70th birthday.

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Abbreviations: α-thrombin, human α-thrombin; β-thrombin, human β-thrombin; γ-thrombin, human γ-thrombin; DAPP, 1,3-bis(*p*-amidinophenoxy)-propane; TAPB, 1,3-bis(*p*-amidinophenoxy)-2-(*p*-amidinophenoxymethyl)-2-ethyl-propane; TAPP, 1,3-bis(*p*-amidinophenoxy)-2,2-bis(*p*-amidinophenoxymethyl)-propane; Bz-Phe-Val-Arg-*p*-NA, *N*-α-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide (S-2160).

INTRODUCTION

Thrombin is a serine proteinase which plays a central role in the coagulation and thrombogenetic processes; thus, beside catalyzing the fibrinogen-to-fibrin conversion, thrombin activates, (i) platelets, interacting with a membrane-bound receptor, (ii) factor XIII, to stabilize fibrin polymers and, (iii) protein C, to inhibit coagulation.[1] Therefore the possibility of selectively influencing thrombin action by specific inhibitors appears of considerable interest in view of their potential therapeutic value as drugs.[2]

The development of most synthetic competitive inhibitors of serine proteinases acting on cationic substrates so far has been based on the discovery of the effectiveness of benzamidine as an anti-trypsin agent by Mares-Guia and Shaw 1965.[3] Significant progress in the development of this class of compounds has been brought about by the finding that the strength and specificity of the inhibitory effect also depend on the remainder (in addition to the benzamidine moiety) of the chemical structure. Thus, synthetic benzamidine derivatives of N-α-arylsulfonyl-4-amidinophenylalanine show K_i values[4] ranging between $1.0 \times 10^5\,M^{-1}$ and $1.0 \times 10^9\,M^{-1}$. Next, bis-, tris- and tetra-benzamidine structures are generally stronger inhibitors of serine proteinases than monobenzamidine compounds.[5-8] Among benzamidine derivatives, DAPP, TAPB and/or TAPP inhibit, *in vivo*, the growth of sarcoma 180 implanted in hybrid mice,[9] and as well as a human melanoma cell line transplanted in nude mice,[10] and display *in vitro* a strong antitumor activity on a variety of cell lines.[11-13] Next, this class of compounds has been reported to inhibit, *in vitro*, haemostasis and clot lysis at different steps[9,14] as well as hydrolysis of esters and anilides of amino acids catalyzed by serine proteinases.[8]

In order to shed more light on the inhibitory mechanism of these (poly)benzamidine derivatives, whose pharmacological effects are so widely distributed,[8-14] the influence of pH (between pH 2.0 and 7.0; $I = 0.1$ M) on the values of the association inhibition constant (K_i, M^{-1}) for the binding of benzamidine, DAPP, TAPB and TAPP to human α-, β- and γ-thrombin has been investigated at 37.0 ± 0.5°C; similar experiments were performed previously on bovine α-thrombin.[8] The analysis of the molecular models of α-, β- and γ-thrombin : inhibitor complexes has allowed the binding behaviour of benzamidine, DAPP, TAPB and TAPP to be related to the stereochemistry of the enzyme : inhibitor contact region(s).

EXPERIMENTAL

Materials

Human α-, β- and γ-thrombin were purified from commercial enzyme preparations (from Sigma Chemical Co., St. Louis, MO, USA) as detailed previously.[15] Bz-Phe-Val-Arg-*p*-NA and benzamidine (from Sigma Chemical Co., St. Louis, MO. USA) were of analytical grade and used without further purification. DAPP, TAPB and TAPP were synthesized according to the published methods.[16-18] The characterization of α-, β- and γ-thrombin, Bz-Phe-Val-Arg-*p*-NA, benzamidine, DAPP, TAPB and TAPP has been reported elsewhere.[6,15-18]

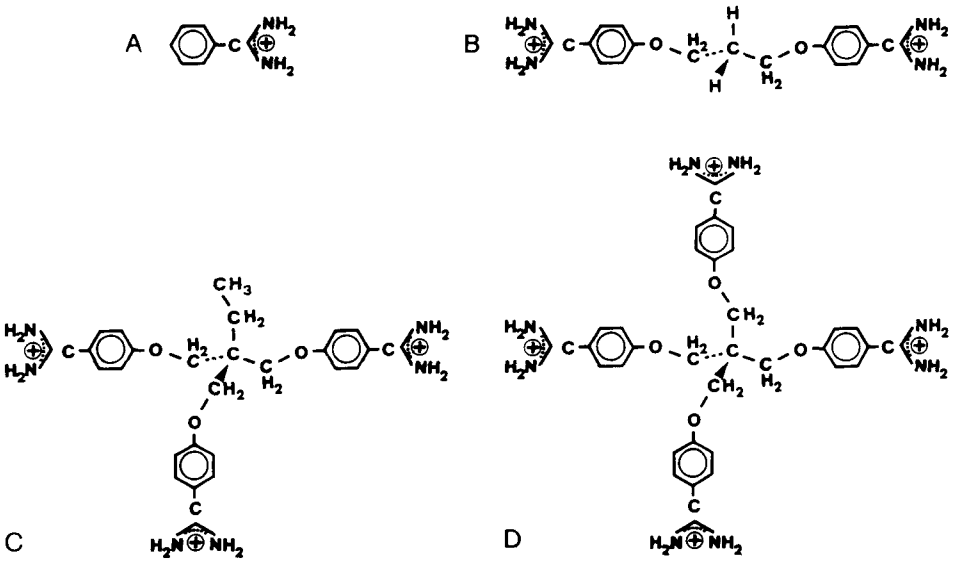

FIGURE 1 Chemical structures of benzamidine (A), DAPP (B), TAPB (C) and TAPP (D).

Determination of the Association Inhibition Constant for the Proteinase : Inhibitor Complex Formation

Values of the association inhibition constant (K_i: M^{-1}) benzamidine, DAPP, TAPB and TAPP binding to α-, β-, and γ-thrombin were determined between pH 2.0 and 7.0 (I = 0.1 M), at 37 ± 0.5°C, by the assay system using Bz-Phe-Val-Arg-*p*-NA as substrate.[6] Values of K_i were obtained using the graphical method proposed by Ascenzi *et al.*[19]; an average error value of ±8% was evaluated for K_i values.

Proteinase : Inhibitor Molecular Models

The molecular models of α-, β- and γ-thrombin inhibited by DAPP, TAPB and TAPP were prepared on the basis of the atomic coordinates of the human α-thrombin : hirudin complex,[20] kindly provided by Dr R. Huber and Dr W. Bode (Max Planck Institut für Biochemie, Martinsried, FRG). The models for DAPP, TAPB and TAPP inhibitor molecules were prepared on the basis of their chemical structures (see Figure 1), using the program NEMESIS.[21] In order to obtain an initial docking between the inhibitor and the enzyme, the molecular model of the benzamidine inhibited bovine β-trypsin[22–24] was properly fitted to the three-dimensional structure of α-thrombin by means of a least-squares procedure. Benzamidine was assumed to bind with comparable geometries in the specificity S_1 subsite of bovine β-trypsin and α-, β- and γ-thrombin. Accordingly, one benzamidine moiety of the inhibitor molecules was fitted to the benzamidine transformed coordinates in the S_1 pocket of α-, β- and γ-thrombin, positioning one "head" of the inhibitor with respect to the enzyme. Subsequently, allowing for the proper rotational degrees of freedom of the inhibitor molecules, and keeping the first benzamidine moiety in the S_1 pocket fixed, possible orientations for the remaining benzamidine substituents of DAPP, TAPB, TAPP were explored, using the program FRODO.[25]

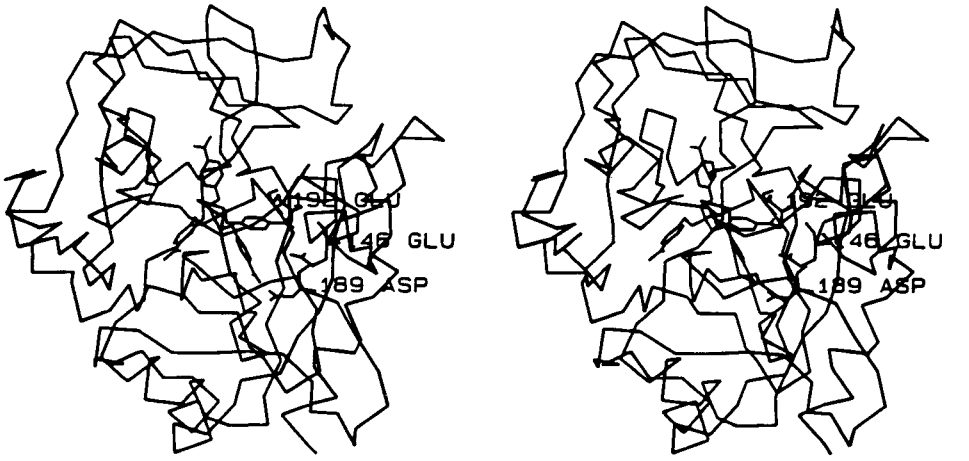

FIGURE 2 A general view of the model proposed for the α-thrombin : TAPP interaction, displaying the inhibitor bound to the enzyme active site region. The side chains of residues Glu-146, Asp-189, and Glu-192 have been drawn in order to highlight the potential electrostatic interactions stabilizing inhibitor binding.

The preliminary α-thrombin : TAPP complex model obtained according to this procedure was subsequently subjected to energy minimization using the program X-PLOR.[26] Firstly, a rigid-body refinement of the complex was run, considering the enzyme and the inhibitor as independent molecules, and minimizing the interaction energy between the two structures. Secondly, the enzyme : inhibitor interaction energy was minimized allowing for conformational readjustments in the whole TAPP molecule, and in the loops Leu144–Trp148 and Arg187–Ser195 of the proteinase. A total of 500 minimization cycles were run, with electrostatic charges located on residues Glu-146, Asp-189 and Glu-192 of α-thrombin, and on the two amidino groups of TAPP which resulted as their potential electrostatic partners in the enzyme : inhibitor complex formation, during the preliminary steps described above.

All the modeling and refinement cycles were run in the absence of water molecules; the model of α-thrombin alone had been subjected to 100 cycles of X-PLOR energy-minimization prior to docking of the inhibitor, in order to release possible conformational strains related to the crystal structure from which it was derived. The model of the α-thrombin : TAPP complex is shown in Figures 2 and 3.

RESULTS AND DISCUSSION

For all the serine proteinase/inhibitor systems considered, the inhibition patterns were strictly competitive, and the complex formation conformed to simple equilibria, as indicated by the unitary value of the Hill coefficient ($= 1.00 \pm 0.02$). Moreover, values of K_i were independent of the serine proteinase as well as of the substrate concentration, and compared well with those reported in the literature.[6]

Data shown in Figure 4A and 4B indicate that the affinity of DAPP, TAPB and TAPP for α- and β-thrombin is higher than that observed for the proteinase : benzamidine complex formation around neutrality, but that values of the K_i converge

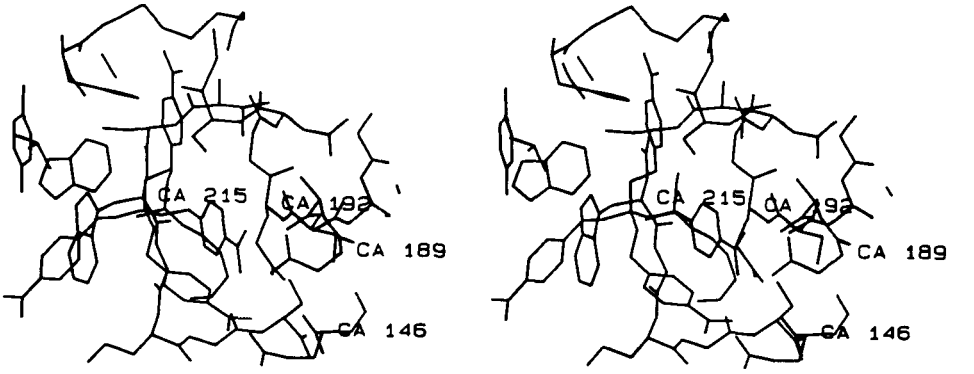

FIGURE 3 A detailed view of the location of the four benzamidine moieties of TAPP with respect to the α-thrombin surface notches and residues.

in the acidic pH limb. In constrast, benzamidine, DAPP, TAPB and TAPP bind to γ-thrombin with the same affinity (i.e., values of K_i) over the whole pH range explored (see Figure 4C).

On lowering the pH from 5.5 to 3.0, the decrease in affinity (i.e., in K_i values) for benzamidine binding to α-, β- and γ-thrombin as well as for DAPP, TAPB and TAPP association to γ-thrombin reflects, according to linkage relations,[27] the acidic-pK shift of a single ionizing group ($n = 1$; see Eq. (1)) on the serine proteinase : inhibitor complex formation. In constrast, DAPP, TAPB and TAPP binding to α- and β-thrombin appears to be modulated by two equivalent inhibitor-linked proton-binding

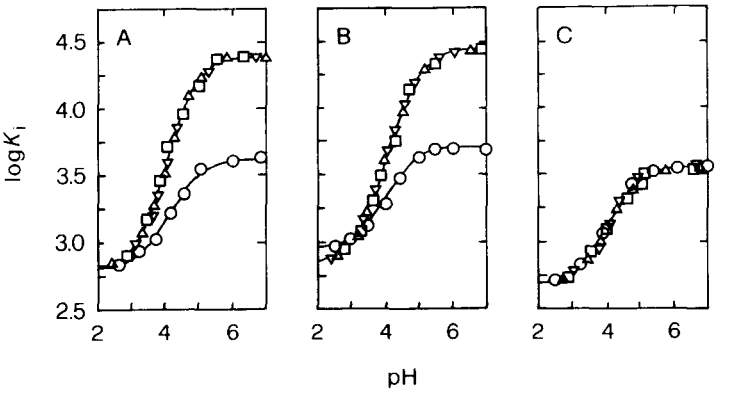

FIGURE 4 pH dependence of K_i (M^{-1}) for benzamidine (○), DAPP (△), TAPB (□) and TAPP (▽) binding to α-, β- and γ-thrombin (A, B, and C, respectively), at 37.0 ± 0.5°C. The unbroken lines, generated according to Eq. (1) with sets of parameters given in Table I, were obtained with an iterative non-linear least-squares curve fitting procedure, which also allowed ascribing an average error value of ±12% to 10^C, K_{UNL} and K_{LIG} values as the standard deviation.[8] The pH profile was explored using the following buffers: phosphate (pH 2.0 to 3.5), acetate (pH 3.5 to 6.0), phosphate (pH 6.0 to 7.0), all at $I = 0.1$ M (sodium salts). No specific ion effects were found using different buffers with overlapping pH values. For further details, see text.

TABLE I

Values of C, pK_{UNL} and pK_{LIG} for benzamidine, DAPP, TAPB and TAPP binding to α-, β- and γ-thrombin (T = 37.0 ± 0.5°C; I = 0.1 M)[a]

Proteinase	Inhibitor	C	pK_{UNL}	pK_{LIG}
α-Thrombin	Benzamidine	3.62	4.50	3.70
	DAPP	4.40	4.40	3.60
	TAPB	4.40	4.40	3.60
	TAPP	4.40	4.40	3.60
β-Thrombin	Benzamidine	3.70	4.40	3.65
	DAPP	4.45	4.50	3.70
	TAPB	4.45	4.50	3.70
	TAPP	4.45	4.50	3.70
γ-Thrombin	Benzamidine	3.55	4.45	3.60
	DAPP	3.55	4.45	3.60
	TAPB	3.55	4.45	3.60
	TAPP	3.55	4.45	3.60

[a]Values of C, pK_{UNL} and pK_{LIG} were determined by curve fitting from Eq. (1). Data in Figure 4. An average error value of ± 12% was evaluated, as the standard deviation, for 10^C, K_{UNL} and K_{LIG} values according to the iterative non-linear least-squares curve fitting procedure (see Figure 4).

groups (n = 2; see Eq. (1)). These models lead to the following expression (Eq. (1)):[8]

$$\log K_i = C - \log \left\{ \frac{([H^+] + 10^{-pK_{UNL}})^n}{([H^+] + 10^{-pK_{LIG}})^n} \right\} - \log \left\{ \frac{(10^{-pK_{LIG}})^n}{(10^{-pK_{UNL}})^n} \right\} \quad (1)$$

where C is a constant that corresponds to the alkaline asymptote of the log K_i, and pK_{UNL} and pK_{LIG} are pK values of the proton dissociation equilibrium constants for the inhibitor-free (K_{UNL}) and the inhibitor-bound (K_{LIG}) serine proteinase, respectively. Equation (1) has been used to generate the unbroken lines shown in Figure 4 with the choice of parameters given in Table I; the agreement with the experimental data is fully satisfactory.

Values of pK_{UNL} and pK_{LIG} fitting benzamidine, DAPP, TAPB and TAPP binding to α-, β- and γ-thrombin are closely similar for the three species investigated (see Table I), and are in agreement with those of amino acid residue(s) modulating (between pH 3.0 and 5.5) catalytic, inhibitor binding and spectral properties of serine proteinases acting on cationic substrates.[28,29]

The model building experiments performed indicate that, in the case of the four benzamidine substituted inhibitor TAPP, a limited region around the active site of the enzyme can be productively exploited for interactions, once the first benzamidine moiety is properly fitted into the S_1 subsite. This restriction is mainly due to the "notched" nature of the α-, β- and γ-thrombin binding site, characterized by well defined surface clefts, shown to be efficiently fitted by different low molecular weight inhibitors.[30–33]

Concerning the specificity of mono-, bis-, tris- and tetra-benzamidine inhibitors in α-, β- and γ-thrombin recognition, the energy refined model shows, as expected, the conventional orientation and interactions of the inhibitor's benzamidine subunit in the S_1 subsite of the enzyme. In particular, an ion pair interaction between Asp-189 and the inhibitor amidino group, as well as a hydrogen bond to the Gly-219 carbonyl oxygen are compatible with the location of the S_1 benzamidine substituent[22,33] (see Figure 3). Thus, the proteinase ionizable group affecting benzamidine binding to the three species of thrombin, as well as the γ-thrombin : DAPP, : TAPB and : TAPP

complex formation, can be identified with the Asp-189 residue, present at the proteinase S_1 subsite. Similarly, one of the two ionizations affecting DAPP, TAPB and TAPP binding to a α- and β-thrombin can be assigned to the Asp-189 residue, known to interact with the amidino group of benzamidine in the S_1 subsite of serine proteinases acting on cationic substrates.[34]

Location of the second benzamidine substituent, in the case of TAPB and TAPP, must comply also with the resulting position of the linked third (and fourth) substituent(s) of the inhibitor. From inspection of models, in the case of TAPP (the most sterically demanding inhibitor molecule of the series), the only location for a second benzamidine moiety avoiding sterical clashes between other parts of the inhibitor and the enzyme, coincides with the α-thrombin surface cleft between the Leu-60-Phe-60H loop and the Leu-40-Gly-43 region (the upper benzamidine in Figure 3). The benzamidine subunit thus located leaves unaltered the binding geometry at the S_1 site, but also does not provide evident specific interactions with the enzyme. Similarly a third benzamidine ring is orientated towards the solvent (in the lower left corner of Figure 3), and is 7 Å away from the "aryl" binding site of α-thrombin.[31,33] Based on modeling considerations, further movement of this benzamidine substituent towards Trp-215 seems to be precluded by loss of the enzyme : inhibitor interaction at the S_1 subsite and/or by sterical collisions at the entrance of the specificity pocket.

The fourth benzamidine substituent of TAPP, in the model developed, is located approximately halfway between the loop regions Leu-144–Trp-148 and Ala-190–Asp-194 of α-thrombin, such that the positive charge of the amidino group can potentially be balanced either by residue Glu-146 or by Glu-192 (see Figure 3). Allowing for proper flexibilities in both residues, the fourth benzamidine substituent is at hydrogen bonding distance from both carboxylates. On this basis, and taking into account the common stereochemistry of the inhibitors considered, the second ionizing group affecting DAPP, TAPB and TAPP binding to α- and β-thrombin can be tentatively assigned to residue Glu-146. Indeed, not only does Glu-146 meet the charge and steric requirements for productive interaction with the second benzamidine subunit of the inhibitor, once it is properly bound to the S_1 subsite of α-thrombin, but also it is the only negatively charged residue which can be reached by the inhibitor (under the restrictions and limitations of the model proposed) which is deleted in γ-thrombin,[30,35] for which a second ionizable group affecting DAPP, TAPB and TAPP binding is absent (see Figure 4). Loss of a potential inhibitor interaction with Glu-192, in the case of γ-thrombin, can be ascribed to alteration of the protein structure in this region upon loss of the extended Glu-127–Lys-149E γ-loop, and to the resulting substantial exposure of the fourth benzamidine ring to interaction with the solvent.

This interpretation agrees very well also with the pH independence, between pH 3.0 and 5.5, of thermodynamics for the association of cationic substrates and inhibitors to bovine α-chymotrypsin,[28,29] where the Asp-189 and the Glu-146 residues, whose ionization(s) affects the association of cationic ligands to α-, β- and γ-thrombin, are replaced by the seryl and the arginyl amino acid side chain, respectively (see Greer[24] and Bode *et al.*[30] for comparison).

The modulating effect of the γ-loop on DAPP, TAPB and TAPP binding to α-, β- and γ-thrombin is closely reminiscent of that observed for the serine proteinase : hirudin complex formation.[15,20,35–37] Indeed, the affinity of hirudin for γ-thrombin is lower than that observed for the α- and β-thrombin : inhibitor complex formation. In this respect, it may be recalled that β- and γ-thrombin (missing the β-domain, and both β- and γ-loops, respectively) show a reduced, or absent, coagulating activity, and

a lower affinity for macromolecular inhibitors and substrates, as well as for specific receptors when compared to the α-form of the serine proteinase.[1] Accordingly, the β- and γ-loops play a relevant role, through extensive interactions, in the stabilization of the serine proteinase : multifunctional substrate and inhibitor complexes.[1,37]

As a whole, the data here reported indicate that a single contribution, such as the binding of the first benzamidine moiety of DAPP, TAPB and TAPP, can be strong enough to give a powerful inhibition, but may not confere the desired specificity and capability of being modulated (e.g., by pH changes) for discriminating between close enzyme species (such as human α- β- and γ-thrombin). Next, additional binding sites (besides S_1, where the primary benzamidine moiety interacts) impose discrimination between human (present study) and bovine[8] α-thrombin (e.g., K_i values, for TAPP binding, are $2.2 \times 10^4\,M^{-1}$ and $3.6 \times 10^5\,M^{-1}$, respectively, between pH 6.0 and 8.0, at 37°C). Thus, a fine degree of selectivity in the association of synthetic inhibitors to enzymes may be obtained through proper interactions involving multiple binding clefts. From this view point, the association of multihead inhibitors to serine proteinases appears to be a powerful model in the study of multiple (pro)enzyme : inhibitor and : substrate recognition interactions.

Acknowledgements

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