RESEARCH COMMUNICATION

Benzyloxycarbonyl-D-Phe-Pro-methoxypropylboroglycine: a novel inhibitor of thrombin with high selectivity containing a neutral side chain at the P1 position

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Thrombin, the blood-clotting enzyme, is a serine proteinase with trypsin-like specificity and is able to cleave Arg-Xaa peptide bonds but only in a very limited number of substrates (and sites therein). For the prevention and treatment of thrombosis the control of thrombin activity is a key target, and a variety of synthetic inhibitors have been introduced recently, all of which have a positive charge at the P1 site. We report the synthesis of the first example of a new class of inhibitor containing a neutral

side chain at the P1 site, the peptide benzyloxycarbonyl-D-Phe-Pro-methoxypropylboroglycine. The peptide is a potent inhibitor of thrombin [K_i (limiting) = 7 nM] and is highly selective for its target enzyme in respect of other serine proteinases. This may be expected to confer considerable advantage in terms of specificity of action and reduced toxicity over conventional, positively charged, inhibitors.

INTRODUCTION

Thrombin is the central bioregulatory enzyme in blood-clotting functioning during all stages at the fluid to the cellular level. For this reason the development of synthetic thrombin inhibitors has been a primary aim [1] in the search for new antithrombotic drugs, and various research groups have endeavoured to design inhibitors that bind tightly to the active site of the enzyme [2]. To date all of these inhibitory molecules have contained a positively charged group which interacts at the S1 site of thrombin, binding to the negatively charged carboxylic group of Asp-189 located at the base of the specific pocket [3].

The amino acid sequence of fibrinogen preceding the bond split by thrombin has been found to be of special importance for the affinity between thrombin and its substrate [4–6]. By mimicking crucial features of this sequence, a favourable tripeptide sequence was determined that led to the synthesis of a chromogenic substrate for thrombin, namely benzoyl (Bz)-Phe-Val-Arg *p*-nitroanilide (pNA) [7,8]. This sequence showed the importance of Phe at the P3 site, and by further modifications a tight-binding hydrophobic peptide was developed, leading to introduction of the highly sensitive and specific chromogenic substrate *H*-D-Phe-Pro-Arg-pNA [9]. Using this sequence the potent thrombin inhibitors *H*-D-Phe-Pro-Arg-H [10], *H*-D-Phe-Pro-Arg-chloromethane (CH₂Cl) [11] and *H*-D-Phe-Pro-boro-Arg pinanediol [12] were readily designed.

As noted above, all of the amide substrates and active-site-directed inhibitors of thrombin described in the literature are based upon the placement of an arginine or arginine analogue [13]; positively charged groups at the P1 position, which presumably bind to the S1 site of the enzyme (thrombin shows less specificity in P1 when catalysing the hydrolysis of activated ester

substrates [14]). The present paper arises from our observation that Z-D-Phe-Pro-methylpropylboroglycine (boroMpg) pinanediol ester (Scheme 1 below; IV), in which the boroMpg residue is a C-terminal P1 amino acid analogue with a neutral side chain, is a potent thrombin inhibitor.

MATERIALS AND METHODS

The synthesis of the inhibitor is outlined in Scheme 1. The boronic acid group of the starting material (I) is protected by (+)-pinanediol. It was prepared as described by Matteson et al. [15] and purified by the isolation of the hydrochloride (II). The dipeptide was coupled to II via an active ester to generate III, and in the last step the substitution of the methoxy group for the terminal bromine was achieved by the use of methanol and free guanidine. The inhibitor (IV) was purified by flash chromatography on a silica-gel column with toluene/ethyl acetate (1:1, v/v) as eluent. The mass spectrum and the proton and ¹³C-n.m.r. spectra were in agreement with the structure.

The influence of the inhibitor on clotting times was measured using normal citrated human plasma (pooled from ten donors) in an automatic coagulometer. Thrombin time was measured upon addition of 1.5 NIH (National Institutes of Health) units of human α -thrombin (courtesy of Dr. J. Fenton, New York Department of Health, Albany, NY, U.S.A.), activated partial thromboplastin time (APTT) was measured using a soluble activator (Auto APTT, General Diagnostics) according to manufacturer's instructions. In each case, inhibitor was added at various concentrations (4–5 doubling dilutions) to prewarmed plasma in round-bottomed polystyrene tubes. After preincubation for 2 min the coagulation process was initiated by addition of thrombin or, in the case of the APTT, by addition of soluble

Abbreviations used: Z, benzyloxycarbonyl; Bz, benzoyl; pNA, *p*-nitroanilide; CH₂Cl, chloromethane; APTT, activated partial thromboplastin time; u-PA, urokinase-type plasminogen activator; PPBA, acetyl-D-Phe-Pro-boro-Arg; t-PA, tissue-type plasminogen activator.

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Scheme 1 Synthesis of compound IV

The starting material (I) was prepared according to the method of Matteson et al. [15] and purified by isolation of the hydrochloride (II). The dipeptide was coupled to II via an active ester to generate III. In the final step the methoxy group was substituted by use of methanol and guanidine (IV).

Table 1 Potency of synthetic thrombin inhibitors in clotting assays

The concentrations of inhibitor (μM) required to double clotting time of normal human plasma are shown. Values were calculated from curves prepared from clotting times of plasma containing different dilutions of inhibitor (see the Materials and methods section).

Inhibitor	Inhibitor concn. (μM)	
	Thrombin time	APTT
Compound	2.8	3.4
PPBA	0.66	0.45

Table 2 K_i values (in μM) for blood serum serine proteinases at pH 7.4

All inhibitions were competitive, except for that of plasmin by IV, which shows mixed inhibition. The two K_i values for the inhibition of plasmin by IV, marked by *, reflect the effect of inhibitor on the y-intercept and the slope of the Lineweaver-Burk plot. K_i values listed for PPBA were done under initial-rate conditions where only rapid inhibition is observed. Some of these have been studied by Kettner et al. [17] and are known to exhibit tighter binding that, in the presence of saturating substrate concentrations, is manifested only after preincubation of inhibitor and enzyme. The K_i value listed for the prompt inhibition of thrombin by PPBA (marked by †) is that determined by Kettner et al. [17] using human thrombin. Conditions: 0.1 M sodium phosphate buffer, pH 7.4, containing 10 $\mu\text{g}/\text{ml}$ albumin at 27 °C. Porcine plasmin was assayed with D-Val-Leu-Lys-pNA, human kidney urokinase and bovine Factor Xa were assayed with Bz-Ile-Glu-Gly-Arg-pNA, and bovine thrombin was assayed with D-Phe-Pip-Arg-pNA. Each K_i value is the result from at least six kinetic runs.

Enzyme	K_i (μM)	
	Compound IV	PPBA
Thrombin	0.022 ± 0.002	$0.00079 \pm 0.0017^\dagger$
Factor Xa	12 ± 2	0.059 ± 0.009
Urokinase	28 ± 5	0.011 ± 0.002
Kallikrein	77 ± 20	0.81 ± 0.12
Plasmin	2.5 ± 0.2 (k_{cat})* 24 ± 4 (k_{cat}/K_m)	0.023 ± 0.003

activator and after 5 min CaCl_2 to a final concentration of 10 mM. Clotting times were recorded in triplicate for each concentration of inhibitor, and a curve was constructed using the

mean values relating inhibitor concentration to clotting time. The concentration of inhibitor required to double the clotting time was estimated from each curve.

Enzyme activity (at concentrations up to 0.5 nM) was measured in 0.1 M sodium phosphate buffer, pH 7.4, containing 10 $\mu\text{g}/\text{ml}$ BSA at 27 °C. Porcine plasmin was assayed with D-Val-Leu-Lys-pNA; porcine pancreatic kallikrein was assayed with substrate Bz-Pro-Phe-Arg-pNA, human kidney urokinase and bovine Factor Xa were assayed with Bz-Ile-Glu-Gly-Arg-pNA, and bovine thrombin was assayed with D-Phe-Pip-Arg-pNA. Enzymes were obtained from Sigma and substrates from Kabi Pharmacia. Inhibition type was assessed by the patterns of three classes of plot: $1/v$ against $1/[S]$ for various [I]; $1/v$ against [I] for various [S]; and $[S]/v$ against [I] at various [S]. The K_i values were estimated by weighted least-squares and non-linear regression analysis of the data using the equation for linear competitive inhibition:

$$v = \frac{V_{\text{max}}}{1 + \frac{K_m}{[S]} \left(1 + \frac{[I]}{K_i} \right)}$$

In estimating the effect of inhibitors on the time required for urokinase-type plasminogen activator (u-PA)-mediated clot dissolution, an aliquot (0.1 ml) of a solution of human fibrinogen (Grade L; Kabi Pharmacia; 2 mg/ml in 0.05 M sodium phosphate buffer, pH 7.0) was clotted by the addition of 50 μl of human α -thrombin (final concn. 1.5 NIH units/ml) in round-bottomed polystyrene tubes (5 cm $\times$ 0.3 cm). The concentration of plasminogen in the fibrin clot was 0.06 μM . After incubation at 37 °C for 15 min the clot was overlaid with a solution (0.5 ml) containing u-PA (50 $\mu\text{g}/\text{ml}$; Abbott Laboratories) and various concentrations of inhibitor. The time to complete dissolution of the clot (lysis time) was estimated visually. In another series of investigations, aliquots of normal citrated human plasma or fibrinogen (0.2% w/v) were added to 96-well flat-bottomed plates and clotted by addition of 5 μl of human thrombin (final concn 1.5 NIH units/ml). After 30 min incubation at 37 °C, 50 μl of a solution of tissue-type plasminogen activator (t-PA, Kabi Pharmacia) or urokinase was added together with inhibitor. The rate of lysis was monitored as the decrease in turbidimetric absorbance at 340 nm observed using a Molecular Devices Thermomax microplate reader.

RESULTS AND DISCUSSION

At low concentrations, inhibitor ester (IV) is spontaneously hydrolysed when subjected to physiological conditions, and the free pinanediol does not interfere with the enzymic assay. The inhibition of thrombin assayed with D-Phe-Pip-Arg-pNA is competitive and strong, with a K_i of 22 nM at pH 7.4. As with previously used boronic acids [16], the inhibition of thrombin is strongly pH-dependent and shows a bell-shaped pH profile governed by pK values of 7.05 and 8.75, with a K_i (limiting) of 7 nM. Data comparing the properties of the boroMpg compound (IV) with that of the analogous boroArg compound, acetyl-D-Phe-Pro-boro-Arg pinanediol ester (PPBA) (a compound first

described by Kettner et al. [12]), are given in Tables 1 and 2. Table 1 shows that the arginine-containing compound is about 5–10-fold more potent than the boroMpg compound in doubling the *in vitro* clotting times. This is reflected in the K_i value for inhibition of thrombin by PPBA (Table 2; PPBA, 0.79 nM [12] at pH 7.4, compared with compound IV, 22 nM). However, compound IV was much more selective than PPBA in inhibiting thrombin over other haemostatic coagulation factors, the increase in K_i with each enzyme being between 15 and 32-fold higher with compound IV relative to the increase observed with PPBA (Table 2). Since a large difference was observed between compound IV and PPBA in the inhibition of plasmin and urokinase, the antifibrinolytic potency of the two inhibitors was tested (Tables 3 and 4). These results showed that PPBA was a potent inhibitor of clot lysis, unlike compound IV, both under conditions of assay in which the concentration of plasminogen is limiting the rate of digestion of fibrin (Table 3) and under conditions in which the concentration of plasminogen activator is limiting (Table 4). Thus, besides being an excellent thrombin inhibitor, PPBA will act also to inhibit plasmin-dependent destruction of fibrin monomer and clot dissolution increasing the tendency towards thrombosis [17]. In contrast, compound IV tends to act only as an anticoagulant.

Thrombin contributes to haemostasis by means of several bioregulatory reactions [18]. Besides its primary function to convert fibrinogen into fibrin, thrombin activates essential cofactors of the coagulation mechanism, Factors V and VIII, thereby potentiating its own formation from prothrombin in the manner of a positive-feedback mechanism. Thrombin also transforms Factor XIII into Factor XIIIa, which cross-links fibrin to give an insoluble network, and thrombin is also a potent activator of platelets. The activity of thrombin is controlled by natural inhibitors in plasma, the most important of which is the cofactor for heparin, antithrombin III. In individuals in which there is an imbalance of the haemostatic mechanism, with a risk of excessive prothrombin activation, there is a need for antithrombotic drugs. Of the currently used antithrombotic drugs, heparin and coumarin inhibit thrombin generation by different mechanisms and each has a number of drawbacks as therapeutic agents [19].

To summarize, the new peptidomimetic IV containing a P1 aminoboronic acid with a neutral side chain is a potent selective

Table 3 Effect of compound IV and PPBA upon the time required for u-PA-mediated clot dissolution

Aliquots of human fibrinogen solution (0.2%, w/v) were dispensed into round-bottomed tubes and clotted as described in the Materials and methods section. After incubation at 37 °C for 15 min the clot was overlaid with a buffered solution [0.025 M sodium phosphate/0.1 M NaCl/0.1% (w/v) poly(ethylene glycol), pH 7.0] containing u-PA (1.4 μ M) and increasing concentrations of inhibitor. Upon incubation at 37 °C the time to complete dissolution of clot was estimated visually. The results are expressed as estimated percentage lysis/h. The degree of inhibition is expressed with relation to the rate observed with the control value. Results (S.E.M.) are the means of two or three values.

Inhibitor	[I] (M)	Rate of dissolution (%/min)	Inhibition (%)
Control	0	2.5 $\pm$ 0.45	—
Compound IV	10 ⁻¹¹	2.5 $\pm$ 0.4	0
	10 ⁻¹⁰	2.5 $\pm$ 0.48	0
	10 ⁻⁹	1.25 $\pm$ 0.32	50
	10 ⁻⁸	1.11 $\pm$ 0.35	56
	10 ⁻⁷	1.0 $\pm$ 0.3	60
	10 ⁻⁶	1.0 $\pm$ 0.25	60
PPBA	10 ⁻¹¹	2.5 $\pm$ 0.42	0
	10 ⁻¹⁰	2.3 $\pm$ 0.39	8
	10 ⁻⁹	1.11 $\pm$ 0.24	56
	10 ⁻⁸	0.17 $\pm$ 0.10	93
	10 ⁻⁷	(no lysis at 24 h)	~ 100
	10 ⁻⁶	(no lysis at 24 h)	~ 100

Table 4 Effect of compound IV and PPBA upon the time required for u-PA- and t-PA-mediated clot dissolution

Aliquots of human citrated plasma or human fibrinogen solution (0.2%, w/v) were dispensed into a microwell plate and clotted as described in the Materials and methods section. After incubation at 37 °C for 15 min the clot was overlaid with a buffered solution [0.025 M sodium phosphate/0.1 M NaCl/0.1% (w/v) poly(ethylene glycol), pH 7.4], containing u-PA or t-PA and 1 μ M inhibitor. The rate of dissolution was measured over a 10 h period at 37 °C from the decrease in absorbance at 340 nm using a Molecular Devices Thermomax reader. The results are expressed as a percentage lysis/h. The degree of inhibition is expressed with relation to rate achieved with control value (C). Results ($\pm$ S.E.M.) are means of two to four values.

Activator (nM)	Substrate	Inhibitor	Rate of dissolution (%/h)	Inhibition (%)
u-PA [30]	Plasma	C	9.2 $\pm$ 0.96	—
		PPBA	2.0 $\pm$ 1.4	78
		Compound IV	8.2 $\pm$ 1.6	11
t-PA [3]	Plasma	C	19.8 $\pm$ 3.8	—
		PPBA	0	100
		Compound IV	17.6 $\pm$ 1.94	11
u-PA [30]	Fibrinogen	C	30.4 $\pm$ 2.0	—
		PPBA	0	100
		Compound IV	30 $\pm$ 1.77	1
t-PA [30]	Fibrinogen	C	13.7 $\pm$ 0.97	—
		PPBA	0	100
		Compound IV	4.0 $\pm$ 0.25	71

inhibitor for thrombin. Owing to its selectivity, compound **IV** does not greatly inhibit tissue-type t-PA- or u-PA-mediated thrombolysis. This selectivity is probably due to the lack of a basic amino acid at the P1 position, which if present would enhance affinity for other trypsin-like serine proteinases. The absence of a basic group possibly permits the hydrophobic peptide sequence to determine the affinity for its target protein by binding to the lipophilic binding site unique to thrombin [20,21]. The boronyl group then presumably forms a tetrahedral adduct with the active-site serine or histidine of thrombin [22,23]. This compound represents the first example of a new class of thrombin inhibitor whose properties are amenable to improvement by suitable modification of the neutral side chain. (S. Elgendy, C. A. Goodwin, M. F. Scully, J. W. Deadman, V. V. Kakkar and G. Claeson, unpublished work). Since the positively charged moiety previously used in the design of thrombin inhibitor has been shown to have a pronounced lowering effect on blood pressure [24,25], the new type of inhibitor presented here may have a considerable pharmacological advantage of oral availability, high specificity and reduced toxicity.

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