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FOR THE RECORD

Incorporation of noncoded amino acids into the N-terminal domain 1–47 of hirudin yields a highly potent and selective thrombin inhibitor

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Abstract: Hirudin is an anticoagulant polypeptide isolated from a medicinal leech that inhibits thrombin with extraordinary potency (K_d = 0.2–1.0 pM) and selectivity. Hirudin is composed of a compact N-terminal region (residues 1–47, cross-linked by three disulfide bridges) that binds to the active site of thrombin, and a flexible C-terminal tail (residues 48–64) that interacts with the exosite I of the enzyme. To minimize the sequence of hirudin able to bind thrombin and also to improve its therapeutic profile, several N-terminal fragments have been prepared as potential anticoagulants. However, the practical use of these fragments has been impaired by their relatively poor affinity for the enzyme, as given by the increased value of the dissociation constant (K_d) of the corresponding thrombin complexes (K_d = 30–400 nM). The aim of the present study is to obtain a derivative of the N-terminal domain 1–47 of hirudin displaying enhanced inhibitory potency for thrombin compared to the natural product. In this view, we have synthesized an analogue of fragment 1–47 of hirudin HM2 in which Val1 has been replaced by *tert*-butylglycine, Ser2 by Arg, and Tyr3 by β-naphthylalanine, to give the BugArgNal analogue. The results of chemical and conformational characterization indicate that the synthetic peptide is able to fold efficiently with the correct disulfide topology (Cys6–Cys14, Cys16–Cys28, Cys22–Cys37), while retaining the conformational properties of the natural fragment. Thrombin inhibition data indicate that the effects of amino acid replacements are perfectly additive if compared to the singly substituted analogues (De Filippis V, Quarzago D, Vindigni A, Di Cera E, Fontana A, 1998, *Biochemistry 37*:13507–13515), yielding a molecule that inhibits the fast or slow form of thrombin by 2,670- and 6,818-fold more effectively than the natural fragment, and that binds exclusively at the active site of the enzyme with an affinity ($K_{d,\text{fast}}$ = 15.4 pM, $K_{d,\text{slow}}$ = 220 pM) comparable to that of full-length hirudin ($K_{d,\text{fast}}$ = 0.2 pM, $K_{d,\text{slow}}$ = 5.5 pM). Moreover, BugArgNal displays absolute selectivity for thrombin over the other physiologically important serine proteases trypsin, plasmin, factor Xa, and tissue plasminogen activator, up to the highest concentration of inhibitor tested (10 μM).

Keywords: anticoagulant peptides; hirudin; noncoded amino acids; peptide synthesis; thrombin

Thrombin is a serine protease that plays a pivotal role in maintaining the intricate balance between hemostasis and thrombolysis (Davie et al., 1991), and for this reason it remains the primary target of antithrombotic therapy (Fenton et al., 1998). Among natural anticoagulants, hirudin is the most potent and specific inhibitor of thrombin (Markwardt, 1994). Hirudin is a polypeptide (~7,000 Da) isolated from the salivary secretion of the medicinal leech and is composed of a compact N-terminal region (residues 1–47) cross-linked by three disulfide bridges and a flexible negatively charged C-terminal tail (48–64) (Folkers et al., 1989; Haruyama & Wüthrich, 1989; Nicastro et al., 1997). The high resolution X-ray structure of the hirudin–thrombin complex indicates that the N-terminal tripeptide binds to the active site of thrombin, while the C-terminal tail binds mainly through electrostatic interactions to exosite I (Rydel et al., 1991). Hirudin offers numerous advantages over the existing anticoagulants heparins

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Abbreviations: Standard three-letter abbreviations are used for all natural amino acids. CD, circular dichroism; ChCl, choline chloride; Fmoc, 9-fluorenyl-methyloxycarbonyl; PEG, polyethylene glycol; RP-HPLC, reversed-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; UV, ultraviolet; rhir, recombinant hirudin; HM2 and HV2, hirudin variants from the leech *Hirudinaria manillensis* and *Hirudo medicinalis*, respectively; V1*t*Bug, refers to the synthetic analogue of fragment 1–47 of hirudin HM2 in which Val1 has been replaced by L-*tert*-butylglycine (*t*Bug); S2R, corresponds to the mutation of Ser2 with Arg; Y3βNal, corresponds to the analogue obtained by replacing Tyr3 with L-β-naphthylalanine (βNal); BugArgNal, refers to the triple mutant of hirudin fragment 1–47 in which the three mutations V1*t*Bug, S2R, and Y3βNal were combined.

Enzymes: chymotrypsin (EC 3.4.21.1); factor Xa (EC 3.4.21.6); pepsin (EC 3.4.23.1); plasmin (EC 3.4.21.7); thermolysin (EC 3.4.24.4); thrombin (EC 3.4.21.5); trypsin (EC 3.4.21.4).

and coumarins (for reviews, see Markwardt, 1994; Pineo & Hull, 1995), and its use in the clinical practice has been recently introduced. However, hirudin administration necessitates careful dose titration, and bleeding effects are not rare (Fenton et al., 1998).

To minimize the hirudin sequence binding to thrombin and to improve its therapeutic profile, several N-terminal fragments of hirudin have been prepared as potential anticoagulants (Chang, 1990). Their use would provide a more predictable effect in vivo compared to intact hirudin, due to their unusual stability to denaturants and proteolytic attack and lower immunogenicity. Moreover, N-terminal core fragments targeting solely the active site of thrombin are expected to have a safer therapeutic profile, in keeping with the notion that active-site reversible inhibitors of thrombin display a better antithrombotic/hemorrhagic balance than bivalent inhibitors (Callas & Fareed, 1995; Hursting et al., 1997; Verstraete, 1997). However, a major limitation of these fragments resides in their poor affinity for thrombin compared to hirudin. In fact, complexes of thrombin with N-terminal fragments of hirudin display values of dissociation constant (K_d) in the range 30–400 nM, while K_d values for hirudin–thrombin complexes are typically 0.2–1.0 pM (Chang, 1990; Betz et al., 1992; Vindigni et al., 1994). In this report we show that cumulative incorporation of amino acid substitutions at the N-terminal tripeptide of hirudin fragment 1–47 (see Fig. 1) results in a highly potent and selective thrombin inhibitor.

Results and discussion: In a recent study (De Filippis et al., 1998), we probed the S1–S3 sites of thrombin by introducing coded and noncoded amino acids at the level of the N-terminal tripeptide of hirudin fragment 1–47 (Fig. 1). In particular, the replacement of Val1 with *tert*-butylglycine (*t*Bug) led to a threefold increase in binding to the procoagulant (Na^+-bound) fast form, quantitatively interpreted as arising from a favorable reduction of the entropy of binding at the S2 site of the enzyme. Of interest, upon Ser2 → Arg exchange, the resulting analogue binds 125-fold more tightly to the anticoagulant (Na^+-free) slow form, and only 24-fold to the fast form relative to the natural fragment (see Table 1). To possibly explain this result, we proposed that Arg2 may favorably couple with Glu192, leading to a preferential stabilization of the slow form (De Filippis et al., 1998). The replacement of Tyr3, binding at the S3 site of thrombin, with phenylalanine, cyclohexylalanine, tryptophan, α-naphthylalanine (αNal), and β-naphthylalanine (βNal) improved binding by 2- to 40-fold.

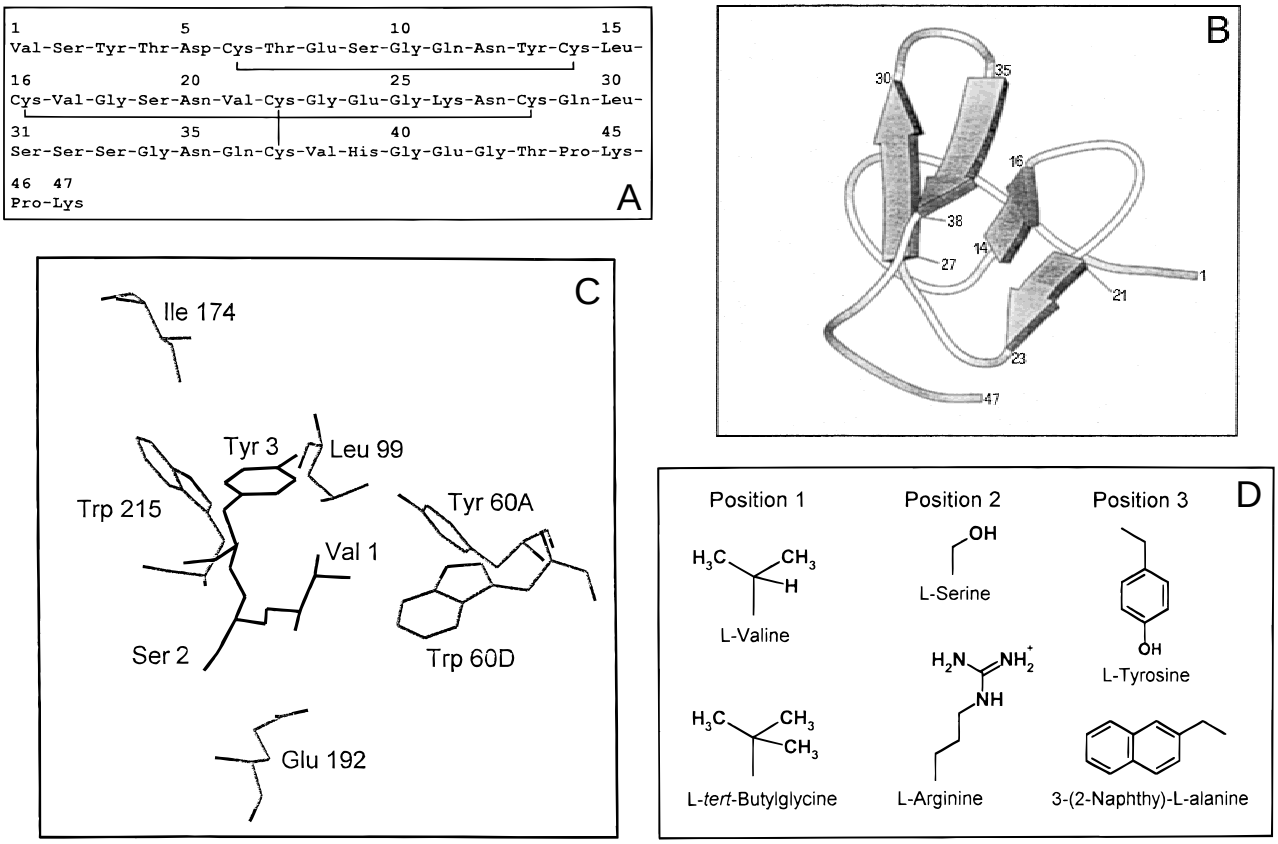

Fig. 1. A: Amino acid sequence of the N-terminal fragment 1–47 of hirudin HM2 from *Hirudinaria manillensis* (Scacheri et al., 1993). The three disulfide bridges between residues 6–14, 16–28, and 22–37 are indicated by plain lines. **B:** Ribbon drawing of the solution structure of hirudin fragment 1–47 from *H. manillensis* (Nicastro et al., 1997). Arrows indicate regions of the β-structure. **C:** Schematic representation of the interaction of the N-terminal tripeptide of hirudin HM2 with the active site of thrombin. The model structure of hirudin HM2 bound to thrombin was based on the coordinates of the complex between human α-thrombin and hirudin HV2 variant, possessing an Ile at position 1 and a Val at position 2 (Rydel et al., 1991; PDB code 4HTC). Amino acid residues at the S1–S3 sites of thrombin are indicated. The ribbon drawing was generated using the program MOLSCRIPT (Kraulis, 1991). **D:** Chemical structure of the amino acid substitutions at positions 1–3 of hirudin fragment 1–47.

Table 1. *Thermodynamic data for the binding of synthetic analogues 1–47 to the fast and slow form of thrombin*[a]

Synthetic analogues 1–47	Fast form K_d	Fast form $\Delta\Delta G_b$[b] (kcal/mol)	Slow form K_d	Slow form $\Delta\Delta G_b$[b] (kcal/mol)	ΔG_c[c] (kcal/mol)
Natural[d]	41 ± 2 nM	—	1,500 ± 100 nM	—	−2.1
V1*t*Bug[d]	14 ± 0.4 nM	−0.63	1,000 ± 80 nM	−0.24	−2.5
S2R[d]	1.7 ± 0.07	−1.88	12 ± 2 nM	−2.86	−1.1
Y3βNal[d]	1.1 ± 0.1 nM	−2.14	94 ± 4 nM	−1.64	−2.6
BugArgNal	15.4 ± 3 pM	−4.67	220 ± 50 pM	−5.23	−1.6
*r*hir HM2	0.20 ± 0.03 pM	−7.25	5.5 ± 0.6 pM	−7.36	−1.9

[a] K_d values for *r*hir HM2 and BugArgNal were determined from the analysis of the competitive inhibition of the hydrolysis of the substrate D-Phe-Pro-Arg-*p*-nitroanilide (20 μM), and calculated according to the slow tight-binding model of thrombin inhibition (Ayala & Di Cera, 1994). All measurements were carried out at 25 °C in 5 mM Tris, pH 8.0 containing 0.1% PEG-6,000 in the presence of 200 mM NaCl for the fast form or 200 mM ChCl when the slow form was being studied.

[b] $\Delta\Delta G_b$ is the difference in binding free energy between the synthetic analog (ΔG_b^*) and the natural fragment (ΔG_b^{wt}): $\Delta\Delta G_b = \Delta G_b^* - \Delta G_b^{wt}$. The values of ΔG_b were calculated by the equation $\Delta G_b = RT \cdot \ln K_d$. A negative value of $\Delta\Delta G_b$ indicates that the mutated species binds more tightly to thrombin than the natural fragment. Errors are ±0.1 kcal/mol.

[c] ΔG_c is the coupling free energy, measured as $\Delta G_c = \Delta G_{b,fast} - \Delta G_{b,slow}$ (Ayala & Di Cera, 1994). The value of ΔG_c is negative if the inhibitor binds preferentially to the fast form.

[d] Data taken from De Filippis et al. (1998).

The introduction of sufficiently large structural and chemical variability at position 3 allowed us to conclude that both hydrophobicity and side-chain packing effects strongly influence hirudin binding, while indicating that the presence of an aromatic moiety preferentially stabilizes the fast form.

At this point, the possibility to enhance thrombin inhibitory potency of hirudin fragment 1–47 by multiple amino acid replacements seemed attractive. Hence, we selected the best-performing amino acid exchanges tested in our previous work (i.e., Val1*t*Bug, Ser2Arg, and Tyr3βNal; De Filippis et al., 1998) and combined them in the same analogue, hereafter denoted as BugArgNal. The BugArgNal analogue was synthesized by combining automated and manual solid-phase Fmoc chemistry and allowed to fold under air-oxidizing conditions, as previously described (De Filippis et al., 1995, 1998). Overall, the final yield of synthesis, including peptide assembly, resin cleavage, oxidative folding, and purification, was higher than 30%. The reaction of disulfide reconstitution of BugArgNal proceeded with an efficiency comparable to that observed for the other synthetic analogs (Fig. 2), and the correctness of the disulfide pairing was established by the peptide-mapping strategy previously detailed (De Filippis et al., 1995). The homogeneity of the disulfide oxidized BugArgNal was accessed by RP-HPLC and capillary zone electrophoresis, while its chemical identity was established by quantitative amino acid analysis and electrospray mass spectrometry, giving an experimental molecular weight of 4,988.2 Da, in agreement with the theoretical value (4,988.7 Da).

The conformational characterization of BugArgNal was conducted in comparison with the natural fragment 1–47 by far- and near-ultraviolet (UV) circular dichroism (CD). The far-UV CD spectra indicate the presence of β-structure, as given by the negative band in the 210–220 nm region and by the positive absorption centered at 195 nm (Brahms & Brahms, 1980) (Fig. 3A). However, the CD spectrum of BugArgNal is strongly influenced by the intense positive contribution of βNal around 230 nm, which is absent in the spectrum of the Tyr3αNal (De Filippis et al., 1998), thus reflecting the different chiroptical properties of the α- and β-isomer (Sisido et al., 1983a, 1983b). The CD spectrum of BugArgNal in the near-UV region is similar to that of the natural fragment 1–47 (Fig. 3B), dominated by a positive band centered at ~255 nm, due to the positive contributions of βNal (Sisido et al., 1983a) and disulfide bonds in right-handed conformation (Kahn, 1979). As previously pointed out

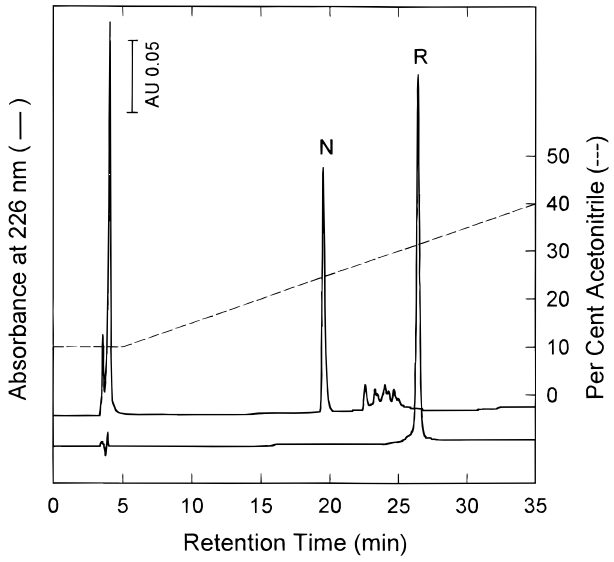

Fig. 2. RP-HPLC analysis of the oxidative folding reaction of BugArgNal. Reduced, purified BugArgNal was allowed to fold (1 mg/mL) for 24 h at 25 °C under air-oxidizing conditions in 0.1 M $NaHCO_3$ buffer, pH 8.3, in the presence of 100 μM β-mercaptoethanol (De Filippis et al., 1995, 1998). Aliquots of the refolding mixture (20 μL) were blocked with a double volume of 4% (v/v) aqueous TFA and loaded onto a Vydac C18 column (4.6 × 250 mm, 5-μm particle size) eluted with a linear gradient of acetonitrile containing 0.05% TFA. R indicates the fully reduced peptide BugArgNal and N the disulfide oxidized species. The correctness of disulfide pairing was established by the peptide mapping strategy previously detailed (De Filippis et al., 1995).

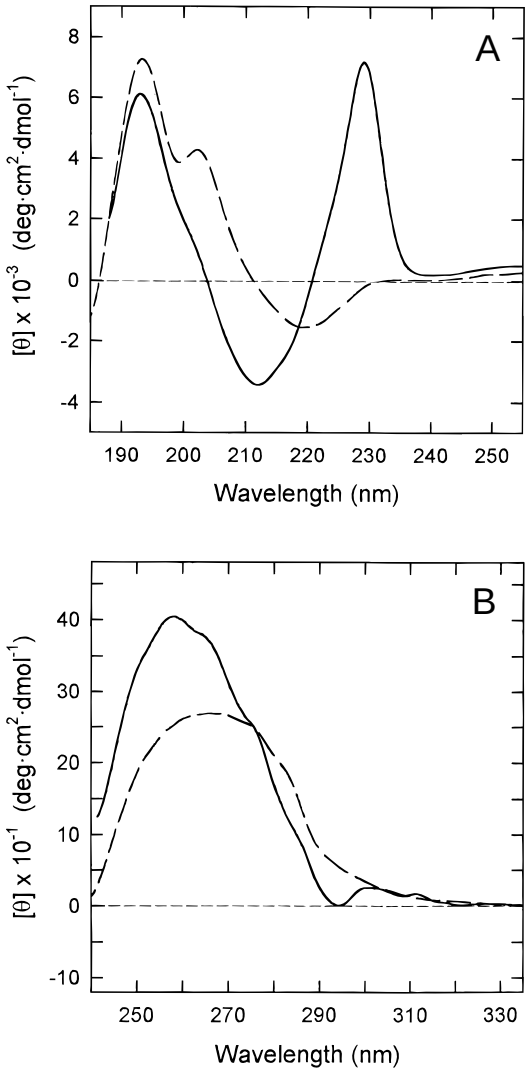

Fig. 3. (**A**) Far- and (**B**) near-UV CD spectra of BugArgNal (—) and natural fragment 1–47 (---). The spectra were recorded on a Jasco J-710 spectropolarimeter at 20 °C in 10 mM sodium phosphate buffer, pH 7.0, at a peptide concentration of 20 μM in the far-UV and 100 μM in the near-UV region, using a 1-mm and 1-cm pathlength cuvette, respectively. Peptide concentration was determined by UV absorption at 280 nm, using extinction coefficients of 0.67 and 1.48 mg^{-1} cm^2 for the natural fragment 1–47 and for the synthetic analogue BugArgNal, respectively.

(De Filippis et al., 1995, 1998), the spectral differences observed between the natural fragment 1–47 and its synthetic analogue(s) do not reflect global structural changes, but only their different content in aromatic residues. To further investigate the consequences of amino acid substitutions on the conformation and dynamics of fragment 1–47, we used the approach of limited proteolysis (Fontana et al., 1997). The synthetic analogue BugArgNal and the natural fragment were incubated at 37 °C under identical experimental conditions with pepsin, trypsin, chymotrypsin, and thermolysin, using an enzyme:substrate ratio of 1:50 (w/w). RP-HPLC analysis of the reaction mixtures indicates that natural and synthetic species are both resistant to the hydrolytic action of all the proteases tested. The combination of these results with those obtained from the conformational and peptide mapping analysis, establishing the correctness of disulfides topology, indicates that amino acid exchanges at positions 1–3 do not alter the overall fold and dynamics of the natural fragment 1–47.

Thrombin inhibition: The inhibitory potency of BugArgNal for the slow (Na^+-free) and fast (Na^+-bound) forms of thrombin (Di Cera, 1998a) was determined from the analysis of the competitive inhibition of the hydrolysis of the substrate D-Phe-Pro-Arg-*p*-nitroanilide and compared to that of the singly substituted analogs (see Table 1). The introduction of only three mutations at the N-terminal end of hirudin fragment 1–47 yields a molecule that inhibits the fast or slow form by 2,670- and 6,818-fold more effectively than the natural counterpart and that binds exclusively at the active site of thrombin with an affinity ($K_{d,fast}$ = 15.4 pM, $K_{d,slow}$ = 220 pM) comparable to that of full-length hirudin ($K_{d,fast}$ = 0.2 pM, $K_{d,slow}$ = 5.5 pM). As shown in Table 1, the effects of the amino acid replacements are additive in both the fast and slow forms of thrombin, indicating that S1, S2, and S3 sites behave independently, that is perturbation of a given site of the enzyme does not affect the binding properties of the other two sites. This result is of particular relevance, because it allows the properties of a multiple mutant to be inferred directly from those of the singly mutated species and to engineer incremental increases in binding strength and selectivity for either allosteric form of thrombin. For instance, combination of Tyr3 → Phe and Val1 → *t*Bug exchanges (De Filippis et al., 1998) should yield an analogue binding 180-fold more tightly to the fast form.

The additivity of mutational effects observed with hirudin derivatives is in contrast with the nonadditivity observed with small tripeptide substrates binding in the transition state (Vindigni et al., 1997). The coupling (or lack of it) of mutational effects in protein–ligand interactions is a property of both the free and bound states of the various components participating in the reaction and is expected to change when ligands of very different structures are considered (Di Cera, 1998b). Thrombin makes 126 contacts shorter than 4 Å with the hirudin fragment 1–47 and buries approximately 240 $Å^2$ upon inhibitor binding (Rydel et al., 1991; Protein Data Bank (PDB) code 4HTC), whereas it makes 60 contacts with D-Phe-Pro-Arg (~60% of these contacts are at the S1 site) and buries only ~100 $Å^2$ (Bode et al., 1992; PDB code 1PPB). Binding of the bulky and rigid fragment 1–47 may impose steric and conformational constraints on thrombin that are not seen upon binding of small and flexible substrates.

Protease selectivity: The issue of protease selectivity is of crucial importance in the design of thrombin inhibitors, because inhibition of other physiologically relevant serine-proteases, also including those of the fibrinolytic pathway (*t*PA and plasmin), can compromise their clinical use (Tapparelli et al., 1993; Cleason, 1994; Das & Kimball, 1995; Fenton et al., 1998; Sanderson & Naylor-Olsen, 1998) and has been recognized as the major determinant of severe hypotension and respiratory depression observed in vivo (Hauptmann & Markwardt, 1992). The molecular basis underlying this lack of selectivity resides in the fact that the positively charged residue generally present at the P1 position of the inhibitor, interacts electrostatically at the S1 site of thrombin with Asp189, which is highly conserved among the endogenous enzymes prevalent in the vascular system.

To investigate the selectivity of BugArgNal for thrombin over other serine proteases, we incubated at 25 °C in separate experi-

ments trypsin (4 nM), plasmin (240 nM), factor Xa (0.8 nM), and tissue plasminogen activator (8 nM) with increasing concentrations (from 0.25 to 10 μM) of hirudin HM2, natural fragment 1–47, and BugArgNal in 5 mM Tris-HCl, pH 8.0, containing 0.1% (w/w) PEG-6,000 and 200 mM NaCl. The reaction was started by addition of 20 μM substrate D-Phe-Pro-Arg-*p*NA, except for factor Xa, where Arg-Gly-Arg-*p*-nitroanilide was used, and monitored by recording the absorbance at 405 nm. Similarly to the natural fragment 1–47 and full-length hirudin, BugArgNal does not inhibit any other protease up to the highest concentration of inhibitor tested (10 μM). Only a slight inhibition was observed for factor Xa, with an estimated K_d value higher than 8 μM.

The results reported in this study demonstrate that the presence of an Arg residue at the N-terminal end of the hirudin fragment 1–47 strongly improves binding, while retaining the extraordinary selectivity of the natural product. This is in contrast with the results obtained for low molecular weight inhibitors, where it is paradigmatic that the introduction of a positive charge at P1 position improves binding, but strongly reduces selectivity (Cleason, 1994; Das & Kimball, 1995; Sanderson & Naylor-Olsen, 1998). Taken together, these results are in keeping with our proposal (De Filippis et al., 1998) that Arg2 may couple electrostatically with Glu192, that is peculiar to thrombin.

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