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N-Terminal Requirements of Small Peptide Anticoagulants Based on Hirudin$_{54-65}$

Thomas J. Owen, John L. Krstenansky,* Mark T. Yates, and Simon J. T. Mao

Merrell Dow Research Institute, 2110 E. Galbraith Road, Cincinnati, Ohio 45215. *Received October 26, 1987*

C-terminal fragment analogues of the leech anticoagulant peptide hirudin represent a unique class of thrombin inhibitors that blocks thrombin's cleavage of fibrinogen but does not block the catalytic site of thrombin. In this paper, a series of synthetic peptides were prepared by solid-phase methodology to determine the optimal N-terminal and position 56 functionalities for these C-terminal fragment analogues of hirudin. Inhibition of fibrin clot formation by thrombin in vitro was used as a measure of anticoagulant activity. In the minimal C-terminal sequence necessary for anticoagulant activity, hirudin$_{56-64}$, an L aromatic amino acid is required at position 56. Phe^{56} → Tyr substitution retained potency, whereas *p*-Cl-Phe^{56} and phenylglycine^{56} substitutions resulted in decreased potencies. Removal of the cationic amino functionality from the vicinity of Asp^{55} results in increased potency (e.g., hirudin$_{54-65}$, Ac-hirudin$_{55-65}$) and [desNH$_2$-Asp^{55}]hirudin$_{55-65}$ has a marked increase in potency over hirudin$_{55-65}$. [DesNH$_2$-Phe^{56}]hirudin$_{56-65}$ and related analogues show no detectable anticoagulant activity. The sensitivity of position 56 to modification demonstrates the significance of this residue in the interaction between the C-terminal region of hirudin and thrombin.

Bloodsucking animals produce various anticoagulant materials in order to maintain the fluidity of their meal.[1] In particular, the medicinal leech (*Hirudo medicinalis*) secretes a 65 amino acid peptide, hirudin, from its salivary glands (Figure 1). Hirudin is able to maintain the fluidity of blood for the 200 days over which the meal is digested by the symbiotic bacteria, *Pseudomonas hirudinis*.[2] Hirudin's anticoagulant activity results from its being the most potent known inhibitor of thrombin. It binds to thrombin with a reported dissociation constant of 0.8×10^{-10} M (ref 3) to 2.0×10^{-14} M (ref 4), depending on the method of determination that was used. Upon binding to thrombin the cleavage of fibrinogen and subsequent fibrin clot formation is prevented in addition to other reported functions of thrombin.[3,13] Hirudin has no known activity other than its inhibition of thrombin and is excreted from the body unmetabolized in the urine.[5,6]

Recently we reported that a C-terminal fragment of hirudin, unsulfated N^{α}-acetylhirudin$_{45-65}$, inhibited fibrin clot formation without inhibiting the amidase activity of thrombin toward the small peptide substrate D-Phe-Pip-Arg-pNA, S-2238.[7] Hirudin blocks S-2238 hydrolysis by thrombin. Thus, the inhibition of fibrin clot formation by the C-terminal fragment analogue is thought to occur by binding to a noncatalytic site on thrombin. This is similar to fibrin hydrolysates that bind thrombin but do not inhibit S-2238 hydrolysis.[8] Previously, thrombin inhibitors have been active site directed; thus peptides based on hirudin's C-terminus represent a new class of potential antithrombic drugs.

Further studies have determined the minimal C-terminal sequence for inhibition of fibrin clot formation as hirudin$_{56-64}$.[9] A series of single residue substitutions has shown that position 56 requires an L aromatic amino acid for activity and Glu^{57}, Ile^{59}, Pro^{60}, and Leu^{64} are also critical for maintaining potency.[10,11] These data led us to explore the optimal N-terminal and position 56 functionalities of these peptides. Here we report a series of synthetic analogues designed to increase anticoagulant potency of these hirudin fragment analogues.

Table I. Fibrin Clot Inhibition

	relative potency[a]	IC$_{50}$,[b] μM
1, N^{α}-acetylhirudin$_{54-65}$	70	5.3
2, hirudin$_{54-65}$	100	3.7[c]
3, N^{α}-acetylhirudin$_{55-65}$	72	5.2
4, hirudin$_{55-65}$	53	7.1[c]
5, [desNH$_2$-Asp^{55}]hirudin$_{55-65}$	219	1.7
6, hirudin$_{56-65}$	12	34.0
7, hirudin$_{55-64}$amide	16	23.4
8, [desNH$_2$-Phe^{56}]hirudin$_{56-64}$amide	<1.4	>250
9, N^{α}-*trans*-cinnamoylhirudin$_{57-64}$amide	<1.4	>250
10, N^{α}-benzoylhirudin$_{57-64}$amide	<1.4	>250
11, [Tyr^{56}]hirudin$_{54-65}$	112	3.3
12, [*p*-Cl-Phe^{56}]hirudin$_{54-65}$	29	12.6
13, [Pgl^{56}]hirudin$_{54-65}$	<1.4	>250

[a] Relative potency with hirudin$_{54-65}$ = 100. [b] IC$_{50}$ = molar dose of peptide that results in 50% inhibition of fibrin clot formation relative to a blank control after thrombin addition to plasma. [c] Reference 5.

Results and Discussion

All peptides in this report are unsulfated. Sulfation of Tyr^{63} in hirudin resulted in only a 2-fold increase in potency.[12] Hirudin analogues **1**, **3**, **5**–**13** (see Table I) were

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Table II. Analytical Data

	amino acid analyses									HPLC[b]	
	Asx	Glx	Pro	Gly	Ile	Leu	Tyr	Phe	calcd MW	FAB-MS[a] (M + H)	t_R gradient	k' (% CH_3CN)
1	0.99 (1)	5.07 (5)	1.00 (1)	0.96 (1)	0.97 (1)	1.02 (1)	1.00 (1)	0.98 (1)	1510	1511	16.4	4.02 (24)
3	1.01 (1)	4.97 (5)	1.00 (1)		0.96 (1)	1.02 (1)	1.00 (1)	1.03 (1)	1453	1453	16.9	4.95 (24)
5		5.08 (5)	1.05 (1)		0.95 (1)	0.99 (1)	0.96 (1)	0.97 (1)	1396	1397	18.4	3.25 (26)
6		5.01 (5)	0.99 (1)		0.97 (1)	1.03 (1)	0.99 (1)	1.01 (1)	1296	1296	12.6	2.26 (21)
7	1.00 (1)	4.02 (4)	0.99 (1)		0.96 (1)	1.02 (1)	1.02 (1)	1.00 (1)	1282	1282	14.0	3.61 (22)
8		3.98 (4)	1.01 (1)		0.97 (1)	1.02 (1)	1.01 (1)		1152	1152	19.4	5.91 (26)
9		4.08 (4)	1.01 (1)		0.94 (1)	1.00 (1)	0.97 (1)		1150	1150	20.4	8.85 (26)
10		4.09 (4)	0.99 (1)		0.95 (1)	1.02 (1)	0.95 (1)		1123	1124	18.2	7.21 (24)
11	0.99 (1)	5.10 (5)	0.98 (1)	0.96 (1)	0.95 (1)	1.05 (1)	2.04 (2)		1484	1485	12.6	3.20 (20)
12	1.02 (1)	5.06 (5)	1.01 (1)	0.98 (1)	0.95 (1)	1.01 (1)	0.99 (1)		1502	1502	16.5	4.42 (24)
13	0.99 (1)	5.07 (5)	0.97 (1)	0.97 (1)	0.95 (1)	1.03 (1)	1.01 (1)		1454	1455	13.8	2.49 (22)

[a] (M + H) ± 1. [b] Vydac 218TP54 (4.6 × 250 mm) C18 column at 2 mL/min, t_0 = 1.9 min; gradient, 15–40% acetonitrile in 0.1% trifluoroacetic acid.

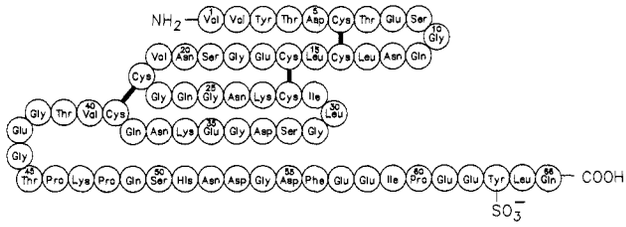

Figure 1. Sequence and structure of hirudin as reported by Dodt et al.

synthesized by solid-phase methods with an Applied Biosystems 430A peptide synthesizer. Cleavage and deprotection were accomplished with anhydrous HF at 0 °C containing 5% anisole. Purification by reversed-phase high-performance liquid chromatography (RP-HPLC), yielded the desired peptides as determined by FAB mass spectrometry and amino acid analysis (Table II). Homogeneity was assessed by analytical RP-HPLC (Table II). Inhibition of fibrin clot formation (anticoagulant activity) was measured as the inhibition of turbidity of a human plasma solution to which human thrombin has been added (Table I).[7]

Previously, it has been shown that hirudin$_{54-65}$ (compound **2**), containing Gly at the N-terminus, was almost 2-fold more potent than hirudin$_{55-65}$ (**4**).[10] It was thought that the positively charged N-terminus in hirudin$_{55-65}$ was less favorable for the interaction with a cationic site on thrombin and was responsible for its lower potency. The binding of hirudin's C-terminus to such a region on thrombin has been postulated.[3,4,12-14] Thus, we synthesized the N-terminally blocked N^{α}-acetylhirudin$_{54-65}$ (**1**) and N^{α}-acetylhirudin$_{55-65}$ (**3**). Both were found to be more potent than hirudin$_{55-65}$ (1.3- and 1.4-fold, respectively; Table I). These data suggest that removal of the N-terminal amino group from Asp^{55} of the hirudin$_{55-65}$ sequence may enhance potency, and indeed [desNH$_2$-Asp^{55}]hirudin$_{55-65}$ (**5**) was 4-fold more potent than hirudin$_{55-65}$ (**4**).

Since [desNH$_2$-Asp^{55}]hirudin$_{55-65}$ (**5**) had improved potency, we investigated the desamino modification at position 56. The analogues [desNH$_2$-Phe^{56}]hirudin$_{55-64}$amide (**8**), N^{α}-*trans*-cinnamoylhirudin$_{57-64}$amide (**9**), and N^{α}-benzoylhirudin$_{57-64}$amide (**10**) all had no detectable anticoagulant activity. This loss of activity was not due to the removal of the C-terminal Gln^{65}, since hirudin$_{55-64}$amide (**7**) was still active. Thus, it appears that a positively charged N-terminus decreases the potency of the thrombin–peptide interaction; position 56 requires a complete L aromatic amino acid; and the carboxylate side chain of Asp^{55} contributes significantly to the overall potency.

We further investigated the aromatic requirement at position 56 by substituting Tyr, *p*-Cl-Phe, and Pgl (L-phenylglycine) for Phe, resulting in compounds which were 1.1, 0.3, and <0.01 times as active as hirudin$_{54-65}$, respectively. The addition of the hydroxyl then was well-tolerated, but addition of the chloro group decreased potency.

In summary, optimal N-terminal modifications of the minimal C-terminal functional domain of the anticoagulant hirudin are desNH$_2$-Asp^{55} and Phe^{56} or Tyr^{56}. The inability of the desNH$_2$-Phe^{56} analogue to inhibit fibrin clot formation demonstrates the specificity of the interaction between thrombin and this particular residue. Glu, Leu, and D-Phe substitutions at position 56 similarly led to inactive analogues.[9] The Asp^{55} side chain and Gln^{65} residue, although not essential for activity, contribute significantly to the potency of these peptides. The acidic residue Asp^{55} may contribute to the interaction with a cationic site on thrombin. The location of this site has been proposed as lying in the 65–83 region of thrombin (α-chymotrypsin numbering).[13,14] The fibrin portion of fibrinogen binding to thrombin may also utilize this binding region.[8,13] In addition, CD studies have shown that the binding of hirudin$_{54-65}$ to thrombin induces a conformational change in thrombin.[9] Thus, the inhibition of thrombin's cleavage of fibrinogen by the C-terminal functional domain of hirudin may exert its effect by direct competition with fibrinogen, by a conformational change in thrombin that decreases its affinity for fibrinogen, or by a combination of these mechanisms.

Experimental Section

Synthesis, Purification, and Characterization. The peptides were synthesized by solid-phase methodology with an Applied Biosystems 430A peptide synthesizer as previously described.[7,10] Boc-Gln-(OCH_2)-Pam resin (Applied Biosystems, 0.64 mmol/g) or *p*-methylbenzhydrylamine resin (Applied Biosystems, 0.48 mmol/g), were used with double symmetrical anhydride couplings. N_{α}-*t*-Boc amino acids were used with the following side chain protection: Asp(Chx), Glu(Bzl), Tyr(2-Br-Z). *trans*-Cinnamoyl, dihydrocinnamoyl, and benzoyl N-terminal modifications were made by coupling the symmetrical anhydrides of *trans*-cinnamic, dihydrocinnamic, and benzoic acids, respectively, to the resin-bound peptides. Succinylation and acetylation of the N-terminus of the peptide resins were accomplished with succinic anhydride and N-acetylimidazole, respectively.

Peptides were treated with anhydrous HF (containing 5% anisole) at 0 °C for 30 min to remove side chain protecting groups and cleave the peptides from the resins. Peptides were precipitated with ether and extracted with 30% acetic acid, dilute sodium bicarbonate, water, and a small amount of DMF. The extracts were lyophilized and purified by reversed-phase HPLC

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on a Rainin Dynamax C-18 (21.4 × 250 mm) column at 10 mL/min with various gradients of acetonitrile/0.1% trifluoroacetic acid. The resulting peptides were analyzed for homogeneity by RP-HPLC and for identity by amino acid analysis with a Beckman 6300 amino acid analyzer and by FAB-MS with a Finnigan TSQ-4C instrument (Table II).

Anticoagulant Assay. Inhibition of plasma clot formation was determined as previously described.[7] Human plasma from a healthy female (fasting for 12 h) was collected in a final EDTA concentration of 0.1%. The plasma was immediately sterilized by filtration through a 0.2-μM filter disk (Gelman). It was then aliquoted as 1 mL/vial and stored at −20 °C. All peptide samples were assessed by using the same plasma preparation. $Hirudin_{45-65}$ was always included[7] as a standard for quality control. Intra- and interassay coefficients of variation were less than 5% and 10%, respectively. Briefly, 50 μL (0.2 pmol) of bovine thrombin (Sigma) was added to the wells of a 96-well microtiter plate (Falcon) containing 50 μL of a solution of the synthetic peptide to be tested (0.25 nmol). After 1 min of agitation and additional incubation for 10 min at 24 °C, 100 μL of diluted human plasma (1:10) in 0.1 M NaCl and 0.012 M sodium phosphate (PBS) was added and vortexed for 20 s. The turbidity of the solution was monitored by an autoreader (EL 309, Bio-Tek Instruments) at 405 nm at 5-min intervals. Typically turbidity at 30 min for various doses of peptide was used to construct dose–response curves for the IC_{50} values. All of the above reagents were diluted in an assay buffer containing 0.12 M sodium chloride, 0.01 M sodium phosphate, 0.01% sodium azide, and 0.1% bovine serum albumin, pH 7.4.

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[1,2,4]Triazolo[4,3-*a*]quinoxalin-4-amines: A New Class of A_1 Receptor Selective Adenosine Antagonists

Bharat K. Trivedi*† and Robert F. Bruns‡

Departments of Chemistry and Pharmacology, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, Michigan 48105. *Received October 30, 1987*

Several [1,2,4]triazolo[4,3-*a*]quinoxalines that were reported as antidepressants in the patent literature were found to possess moderate affinity for the adenosine A_1 and A_2 receptors. On the basis of structural parallels with adenine and adenosine, the *N*-cyclopentyl derivative was synthesized and found to have improved affinity and selectivity for the A_1 receptor. In the *N*-cyclopentyl series, affinity was optimal with trifluoromethyl substitution at the 1-position, resulting in a compound (**9**) with 7.3 nM A_1 affinity and 138-fold selectivity for the A_1 receptor.

Adenosine elicits a wide variety of physiological actions via membrane-bound receptors, which have been divided into A_1 and A_2 subtypes.[1] Xanthine derivatives such as caffeine and theophylline have long been known to block adenosine receptors.[2] However, their low potency and nonspecific actions such as phosphodiesterase inhibition and calcium modulation have limited their utility as pharmacological tools.[3] Attempts to improve upon the potency of theophylline have led to the discovery of potent xanthine antagonists such as 8-cyclopentyltheophylline[4] and the xanthine amine congener *N*-(2-aminoethyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purine-8-yl)phenoxy]acetamide (XAC).[5] Although the search for more potent and receptor-selective xanthine antagonists continues,[6] there are key features of these molecules (for instance, poor aqueous solubility) that impede their utility as pharmacological tools for in vivo studies and tend to limit their development as potential therapeutic agents. Because of these limitations of the xanthines, there is considerable interest in identifying nonxanthine adenosine antagonists that might not share these drawbacks.

Chronic administration of caffeine and theophylline has been shown to result in down-regulation of β-adrenergic receptors,[7-9] an activity that is considered a useful preclinical predictor of antidepressant activity. In light of these reports, our attention was drawn to two accounts[10,11] describing [1,2,4]triazolo[4,3-*a*]quinoxalin-4-amines with antidepressant-like activity of unknown mechanism. An examination of these molecules (Figure 1) revealed several striking features that suggest similarity to the adenine moeity, including (1) a 6:5 fused ring system, (2) an exocyclic amino function, and (3) the presence of four ring nitrogen atoms arranged in a manner similar to those of

Table I. Physical and Chemical Properties of Novel [1,2,4]Triazolo[4,3-*a*]quinoxalin-4-amines

example	method of preparation[a]	mp, °C[b]	(formula) anal.
4	A	257–260	($C_{14}H_{15}N_5$) C, H, N
5	B	196–198	($C_{15}H_{17}N_5$) C, H, N
6	B	166–168	($C_{16}H_{19}N_5$) C, H, N
7	A	186–188	($C_{17}H_{21}N_5$) C, H, N
8	A	195–197	($C_{18}H_{23}N_5$) C, H, N
9	C	183–184	($C_{15}H_{14}N_5F_3$) C, H, N, F
10	A	190–192	($C_{14}H_{15}N_5$) C, H, N
11	B	215–217	($C_{15}H_{17}N_5$) C, H, N
12	A	217–220	($C_{17}H_{21}N_5$) C, H, N
13	B	170–172	($C_{18}H_{21}N_5$) C, H, N
14	B	172–174	($C_{17}H_{15}N_5$) C, H, N
15	A	236–238	($C_{14}H_{17}N_5O$) C, H, N

[a] Methods of preparation A–C are described in the Experimental Section. [b] Melting points are uncorrected.

† Department of Chemistry.
‡ Department of Pharmacology.

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