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STRUCTURE-ACTIVITY RELATIONSHIPS OF MONOCYCLIC ENDOTHELIN ANALOGS

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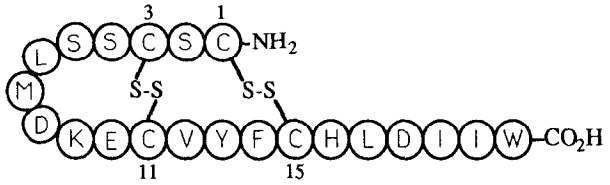
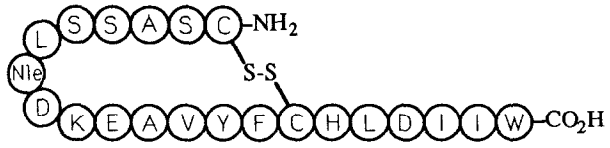
Abstract: Analogs of monocyclic ET-1 ($Ala^{3,11}$, Nle^{7}) containing alanine substitutions were prepared and assayed for vasoconstrictor and receptor binding activity. Analogs substituted at residues Glu^{10}, Phe^{14}, Leu^{17} and Asp^{18} showed significant affinity for the receptor at concentrations below those at which agonism was observed. To the extent that structure-activity relationships of monocyclic and bicyclic ETs can be compared, this study suggests that residues 10, 14, 17 and 18 are candidate sites for exploration of peptides which show ET receptor antagonism.

The endothelin (ET) family of peptides is a group of potent vasoconstrictors which may be implicated in the pathophysiology of hypertension[1], renal failure[2,3], vasospasm[4] and other disease states[5]. Despite their potential importance, few structure-activity studies of the ET family have been reported[6,7]. In addition, these studies have generally not reported the receptor affinity of ET analogs, information which is useful in the discovery of ET receptor antagonists.



Endothelin-1



Endothelin-1 ($Ala^{3,11}$, Nle^{7})

The ET family consists of 21-amino acid peptides which contain two disulfide linkages, joining cysteines 1-15 and 3-11. Among the many difficulties associated with the synthesis of ET analogs is the need to differentially protect the cysteine pairs in order to guarantee the correct disulfide structure[8]. Although the monocyclic ET-1 retaining only the 3-11 disulfide bridge is about 200-fold less active than the bicyclic ET-1, the monocyclic ET-1 retaining the 1-15 disulfide bridge maintains about 10% of the activity of ET-1[9]. The simplicity of synthesis of the monocyclic analog with the 1-15 disulfide bridge as well as its still significant

biological activity makes it an attractive framework for performing initial studies of ET structure-activity relationships. We therefore synthesized monocyclic ET-1 ($Ala^{3,11}$, Nle^{7}) analogs in which each amino acid in turn was substituted with alanine, in order to address the role of each residue of ET in receptor binding and receptor activation[10]. In order to circumvent thioether oxidation side reactions, these analogs also contain a norleucine in place of methionine[7], which has previously been shown to not be a critical residue[7].

All of the ET analogs were found to cause concentration-dependent inhibition of the specific binding of [^{125}I]-ET-1 (A10 vascular smooth muscle cell membranes[11]) and all except the Ala^{21} analog induced greater than 75 percent inhibition at 50 μM (Table 1). The Ala^{21} analog was devoid of inhibitory activity at 20 μM and was not tested at higher concentrations. Six compounds including the parent monocycle (ET-1, $Ala^{3,11}$, Nle^{7}) were associated with concentration-inhibition curves which were significantly better fitted to a two-site binding equation than to a single site equation. Of these, only one, the parent monocycle, bound with high affinity to the predominant population of binding sites, whereas two compounds, the Ala^{12} and Ala^{19} analogs, discriminated between two equally large populations of receptors. The remaining three agents with Ala at positions 2, 17 and 18, bound with low affinity to the predominant populations of receptors.

Table 1. Receptor affinity and agonist potency of ET-1 and monocyclic analogs.

ET-1 ($Ala^{3,11}$,Nle^{7})	EC_{50} (nM)*	k_d (nM)	B_{max1}/B_{max2}
ET-1	0.94± 0.31	0.15± 0.07	
ET-1 ($Ala^{3,11}$,Nle^{7})	3.3± 0.9	0.35± 0.07/16.6± 6.3	57/43
α-Ac	36± 5 % @ 1 μM	4,778± 508	
Ala^{2} (for Ser)	38± 7	9.6± 7.4/409± 345	38/62
$Ala^{4,5,6,7}$ (for SSLM)	36± 5	130± 1.4	
Ala^{8} (for Asp)	60 ± 16% @ 1 μM	3,074± 131	
Ala^{10} (for Glu)	7 ± 5% @ 1 μM	188± 67	
Ala^{12} (for Val)	130± 15	31± 40/1390± 1412	49/51
Ala^{13} (for Tyr)	21 ± 3% @ 1 μM	3920± 2330	
Ala^{14} (for Phe)	6 ± 4% @ 1 μM	646± 93	
Ala^{16} (for His)	31± 11	51.9± 3.5	
Ala^{17} (for Leu)	0 @ 1 μM	6.6± 4.2/1907± 62	30/70
Ala^{18} (for Asp)	0 @ 1 μM	0.7± 0.4/80± 21	28/72
Ala^{19} (for Ile)	182 ± 35	96± 92/3122± 1875	50/50
Ala^{20} (for Ile)	37 ± 22% @ 1 μM	2,109± 723	
$Ala^{19,20}$ (for Ile-Ile)	50 ± 22% @ 1 μM	929± 84	
Ala^{21} (for Trp)	0 @ 1 μM	>20,000	

* For analogs which produced less than 60% stimulation of vascular contraction, the percent of the ET-1 induced contraction at 1 μM analog is reported.

Similar to published reports, ET-1 ($Ala^{3,11}$, Nle^{7}) was found to retain about 10% of the activity of ET-1 in both our functional assay[12] (rabbit carotid rings) and in our receptor binding assay. Substitution of individual amino acids by alanine produced analogs with a range of activities. Based on the biological activity

of the substituted monocyclic ETs, we roughly grouped residue sites into three classes. The 'tolerant sites' (see Ref. 13) are defined as those sites which tolerate substitution in that replacement with alanine produced agonists with $k_d \leq 100$ nM. The 'binding sites' are those sites which are important to receptor binding in that replacement with alanine produced analogs with $k_d \geq 1{,}000$ nM. Finally, the 'functional sites' are those sites for which substitution leads to little change in receptor binding affinity but large changes in the functional consequences of binding.

Substitution of Ser^2, Val^{12}, His^{16}, Ile^{19} as well as a multiple substitution of residues Ser^4-Ser^5-Leu^6-Met^7- with -$(Ala)_4$- produced agonists of reasonable potency. These residues are thus identified as tolerant sites, although it should be noted that each of these substitutions led to a 10-30 fold loss of potency compared to the parent monocycle. Lys^9 is presumed to also be a tolerant site, since a previous study indicated that the functional activity of ET-1 was unaffected by a leucine replacement at this position[7]. Considering that there is little conservation of residue type in the region of residues 4-7 among the ETs and sarafotoxins, it is not surprising that these positions are tolerant. Because the sequence differences between ET-1 and ET-3 occur primarily at residues 4-7, it will be interesting to compare the effects of ET-1 ($Ala^{4,5,6,7}$) in tissues which show a preference for ET-3.

Substitution of Asp^8, Tyr^{13}, Ile^{20} and Trp^{21} afforded analogs which showed greater than a thousand-fold poorer affinity for the receptor than the parent monocycle, identifying these sites as being important to receptor binding. This suggests that the poor vasoconstrictor activity of the previously reported Asn^8 analog is due to poor receptor binding[7]. It has been shown that the Phe^{13} analog is a potent vasoconstrictor peptide, indicating that the tyrosine hydroxyl is not critical for this activity[7]. The poor receptor affinity of the Ala^{13} monocyclic analog indicates that an aromatic residue is important at this site. A previous study showed that other aryl amino acids (Phe, Tyr) at position 21 led to vasoconstrictor peptides while a des-Trp^{21} peptide was inactive[7]. Reports of the poor binding affinity[14] of ET-1 (1-20) or its poor vasoconstrictor activity[6,7] could previously be ascribed to an inappropriate location for the important C-terminal carboxylate. Our studies show that the aromatic side chain of Trp^{21} is absolutely critical to binding, because the peptide containing Ala^{21} had dramatically poorer receptor affinity, showing no displacement of [^{125}I]-ET-1 when tested at concentrations as high as 20 μM. Because the affinity of all of these analogs is so poor, the role of these amino acids in receptor activation is difficult to discern.

Substitution of Glu^{10}, Phe^{14}, Leu^{17} and Asp^{18} produced analogs which showed appreciable affinity for the receptor at concentrations below those at which agonism was observed. The Ala^{14} monocyclic peptide corroborates the lack of vasoconstrictor activity of the bicyclic Ala^{14} peptide[7] but also shows that this substitution does not completely abolish receptor affinity. The still significant receptor affinity of the Ala^{10} analog suggests that the bicyclic peptide with the more isosteric Gln residue at position 10, reported to have no vasoconstrictor activity[7], may also bind to the endothelin receptor. All of these 'functional sites' appear to play a more significant role in receptor activation than in receptor binding, making them candidate sites for future exploration of receptor antagonism.

It has been noted that the C-terminal tail of ET is very important to ET binding and receptor activation[6] and this is corroborated by our determination of Leu^{17}, Asp^{18}, Ile^{20} and Trp^{21} as important residues. In order to further explore the importance of the aliphatic amino acids in the extremely hydrophobic tail, we prepared the -Ala^{19}-Ala^{20}- analog. Interestingly, this peptide showed functional and binding activity which was

intermediate to the two monosubstituted analogs rather than showing an additive effect. This suggesting that these residues may play an important role in biasing the tail conformation as well as providing hydrophobic binding interactions.

To determine whether a correlation exists between the functional data (ability to induce concentration-dependent smooth muscle contraction) and the binding data (ability to bind to the vascular smooth muscle cell ET receptor), several comparisons were made. Nine agents induced less than 60% stimulation of vascular contraction at a concentration of 1 μM, and therefore EC_{50} values were not determined. Since four of the remaining eight compounds were able to discriminate two coexisting binding sites, correlation studies were performed between the agonistic potency data (EC_{50}) and either of four corresponding receptor dissociation constants: the high affinity k_d, the low affinity k_d, the k_d value characteristic of binding to the high density receptor population, or that characteristic of binding to the low density receptor population. The four correlation studies also included the k_d values for the four analogs which bound to a single receptor population. Table 2 shows the results from this correlation analysis.

Table 2. Correlation analysis: Stimulatory potency vs. receptor affinity

Correlation of log EC_{50} to:	r	r^2	slope
log k_d (high affinity)	0.95	0.89	1.37
log k_d (low affinity)	0.93	0.87	1.39
log k_d (high B_{max})	0.94	0.89	1.53
log k_d (low B_{max})	0.94	0.88	1.65

The correlation analysis for the alternative yielding the highest correlation coefficient and the slope least different from unity (log EC_{50} vs. log k_d high affinity) is shown in Figure 1. These data suggest that functional stimulation of a receptor by any of the compounds studied is primarily the result of its interaction with the high-affinity binding site. However, the differences between the correlation coefficients and slopes derived from the four alternative analyses were not statistically significant.

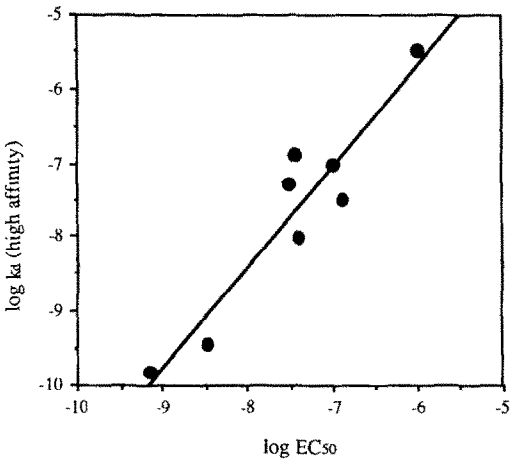


Figure 1. Correlation between agonist potency in isolated rabbit carotid artery rings and affinity for the high affinity population of ET-1 receptors in A10 vascular smooth muscle cell membranes.

A natural question arises about the validity of extrapolating to bicyclic ETs the structure-activity conclusions gained from studies of monocyclic ETs. Although we are currently investigating this comparison with selected peptides, correlation of our data with published structure-activity studies of bicyclic analogs supports a favorable comparison. For example, both ET-1 (Ala^4) and ET-1 (Ala^5) were reported to be full agonists with 43% and 24% of the potency of ET-1[7] while we have shown that the monocyclic analog with alanines replacing residues 4-7 is a full agonist with about 10% of the potency of the monocyclic parent peptide. The bicyclic analog with an α-acetyl group had 0.5% of the potency of ET-1[7] while the similarly substituted monocyclic analog showed significant vasoconstrictor activity at 10 μM, or ~0.03% of the activity of the parent monocycle. The bicyclic analog with Ala^{14} was reported to have an ED_{25} of ~1 μM and at this concentration the corresponding monocyclic analog showed no vasoconstrictor activity. Thus, rough correlations in activity are apparent, although some differences between the structure-activity relationships of monocyclic and bicyclic ETs are to be expected, considering the increased conformational flexibility of a peptide constrained by only one disulfide bridge.

In conclusion, these findings indicate that many residues from position 8 to position 21 as well as a free N-terminal amino group contribute to ET receptor binding and receptor activation. It appears that the production of smaller peptide agonists or antagonists acting at the ET receptor will be a challenging undertaking.

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10. ET-1 was purchased from Peninsula Laboratories or Peptides International. All other peptides were prepared by automated solid phase synthesis using standard t-Boc protocols. Peptides were deprotected using anhydrous HF/anisole, air oxidized in 8M urea, desalted on an HP-20 column (water, then 50% aq acetonitrile), sized on Sephadex G-25 (2M aq acetic acid) and purified by gradient preparative HPLC (acetonitrile:water:0.1% TFA). Peptides were purified to ≥95% homogeneity and characterized by amino acid analysis and fast atom bombardment mass spectrometry.
11. Receptor binding assay: A10 rat thoracic aorta smooth muscle cells (American Type Culture Collection, Rockville, MD) at passage 14 were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 1% penicillin-streptomycin in a 95% air, 5% CO_2 humidified atmosphere at 37°C. Confluent cells were detached with trypsin (0.05%) and collected in DMEM containing 0.1 mM phenyl methylsulfonyl fluoride, 10 μg/ml soy bean trypsin inhibitor and 20 mM Hepes, at a cell concentration of 300-400 x 10^3/ml. The cell suspension was stored at -70°C, thawed in a 37°C water bath, homogenized and centrifuged at 100,000 x g for one hr at 4°C. The supernatant was discarded, the pellet was resuspended in DMEM (10 ml/per flask of initial cell culture) and aliquots were frozen and stored at -70°C. Aliquots of membranes (100 μl; 10-15 μg protein) were incubated with 0.05 nmol of [^{125}I]-ET-1 (NEN DuPont de Nemours: NEX-259; 2,200 Ci/mmol) in the absence or presence of competing compound in a final volume of 0.25 ml assay buffer. Specific binding was determined as the difference in

binding attained in the absence and presence of 100nM unlabelled ET-1. The binding reaction was carried out at 35°C for 1 hr and was interrupted by 40-fold dilution with ice-cold Tris-isosaline (50 mM Tris, 0.154 M NaCl). The assay samples were filtered through BSA-treated glass fiber filters (Schleicher & Schuell # 30), the filters were washed with 10 ml of Tris-isosaline, dried under vacuum and collected in polystyrene tubes. The radioactivity retained on the filter discs was measured in a multi-well gamma counter (Squibb Ria-Stat).

Graphic analysis of the binding characteristics of nonlabelled test compounds was performed by a computer-aided non-linear regression least-square curve fitting procedure, which allows for evaluation of the affinity of the competing drug for one or more subtypes of receptors which the radioligand nonselectively recognizes as specific binding sites. Calculation of the dissociation constant (K_d) from observed IC_{50}-value (concentration of test compound which causes 50% inhibition of specific binding of the radioligand) was performed using the formula:

$$K_d = IC_{50}/(1+[RL]/K_{RL}),$$

where [RL] is the concentration of radioligand and K_{RL} is the affinity of the radioligand for its specific receptors.

12. Functional assay: Male New Zealand white rabbits were sacrificed by an overdose of Brevital in the marginal ear vein. Both external carotid arteries were removed and placed in ice cold physiological salt solution (PSS) of the following composition (in mM): 140.0 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.2 Na_2HPO_4, 1.2 $MgSO_4$, 0.02 Na_2-EDTA, 5.6 D-glucose, and 2.0 MOPS. Rings, approximately 3 mm wide, were cut and mounted for isometric force recording. Experiments were conducted at 37° C and pH 7.4 with aeration by 100% O_2. Following equilibration, the rings were gradually stretched to a maintained 4 g preload and stimulated with K+PSS (substitution of KCl for NaCl to bring [KCl] to 110 mM) several times to assess viability. Cumulative concentration response curves were obtained to each of the test peptides; only one peptide was tested in each ring. Concentrated stock solutions of the peptides were prepared daily in water or dilute acetic acid as necessary. Results are reported as the mean EC_{50} (concentration producing half-maximal force) ± SE; n = at least 4 rings from different rabbits. The EC_{50} values were determined by linear regression analysis of the concentration response data near 50%.
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