Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 9, pp. 967-972, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00152-2

STRUCTURE-ACTIVITY AND BIOPHYSICAL STUDIES OF THE C-TERMINAL HEXAPEPTIDE OF ENDOTHELIN

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Abstract: A series of endothelin-1 [16-21] analogs, designed to incorporate random coil and β-turn conformations, was synthesized. Compounds were compared for their endothelin receptor binding affinity and functional activity relative to the native sequence. They were studied by NMR spectroscopy in an attempt to elucidate the bioactive conformation of the C-terminal region.

Introduction: Endothelin-1 (ET-1), a potent vasoconstrictor isolated from the cultured porcine aortic endothelial cells,[1] is a bicyclic 21 amino acid peptide with two intramolecular disulfide bonds. Two subtypes of ET receptors, known as ET_A and ET_B, have been cloned and characterized in animal and mammalian systems.[2-5] A third ET receptor subtype has been cloned from *Xenopus* dermal melanophores and heart,[6,7] although this subtype has not yet been described in mammalian tissues. Structure-activity relationship studies of ET-1 related peptides have shown that the C-terminal Trp residue is important for vasoconstrictor activity in coronary artery strips (ET_A).[8] ET-1[1-15] has been shown to be inactive,[9] while the hydrophobic C-terminal hexapeptide ET-1[16-21] (His[16]-Leu[17]-Asp[18]-Ile[19]-Ile[20]-Trp[21]) showed low micromolar binding affinity in the guinea pig bronchus, the rat vas deferens and the rabbit pulmonary artery,[10,11] but was found to be devoid of functional activity in several other tissues.[10]

Structure-activity studies of the C-terminal hexapeptide of ET-1 have shown the importance of individual amino acids to receptor binding in a variety of tissue preparations (rabbit aorta, rabbit pulmonary artery and rat heart ventricle).[12-15] However, the native sequence of ET-1[16-21] was found to be devoid of functional activity in these three tissues. At position 16 (His), the D-configuration enhanced the binding affinity of the hexapeptide at both receptor subtypes[12-14,16] and N-terminal acetylation in the series with a D-amino acid at position 16 also enhanced binding at both receptor subtypes.[13] Non-selective ET_A/ET_B functional antagonists were obtained by substituting His[16] by the unnatural amino acids 3,3-diphenylalanine (D-Dip) or 10,11-dihydro-5H-dibenzo[a,d] cycloheptene glycine (D-Bhg).[13,15,17,18] Leu[17] was found to be less important for binding at both receptors,[12,13,16] while Asp[18] could be replaced by certain neutral groups without significantly altering the binding at either receptor subtype. However, substitution at position 18 with an aromatic residue (Phe) led to an increase in ET_B selectivity.[14] At Ile[19], substitutions of basic and acidic groups were not tolerated. Finally, Ile[20] and Trp[21] did not tolerate most substitutions.

NMR studies of ET-1 have been carried out in a variety of solvents (DMSO, various aqueous media).[19-23] In the aqueous media, a helical region has been reported between residues 9-15 while the remainder of the molecule has not been clearly defined. However, one report[23] suggested that the residues between Ser[2] and Ser[5] have the greatest flexibility. The C-terminal portion is undefined with most reports indicating considerable flexibility in solution. Wallace et al[24] have recently reported the X-ray structure of ET-1, showing that the 16-21 carboxy end is helical in the solid state.

In an attempt to define the bioactive conformation of the C-terminal hexapeptide in ET-1, we have incorporated specific structural constraints and examined their effects on the biological activity. One such constraint is the introduction of a turn by incorporation of the amino acid proline, well known to induce β-turns in the secondary structure of proteins.[25-27] In addition, if the amino acid side-chain adjacent to proline is capable of H-bonding, further stabilization of the turn is observed.[28] We therefore synthesized a series of ET-1[16-21] analogs where Pro was incorporated at either position 18 or 19, attempting to introduce a β-turn. The amino acid Asn was introduced at either position 18 or 19 (adjacent to Pro) in an attempt to stabilize the β-turn by H-bonding with either the -NH- of the Ile residue, or the -CO- group of the proline residue (Figure 1). Previous studies have shown that ET-1[16-21; Asn^{18}], retained the activity of the natural hexapeptide sequence in the guinea pig bronchus.[29] The monosubstitution of Ile^{19} by Pro has not been reported and was carried out prior to the incorporation of the dipeptide substitution to ensure that it retained activity. In some of these analogs, Asp was substituted for Leu^{17}, since Asp is tolerated at this position[13] and could further stabilize the turn by H-bonding.[27] The aim of these modifications was to identify whether a β-turn could define a relevant bioactive conformation of the C-terminal hexapeptide in ET-1. Several of the synthesized peptides were studied by NMR spectroscopy in an attempt to elucidate possible structural features.

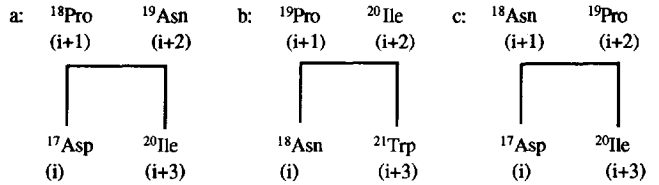

Figure 1: Differences in sequences designed to incorporate a β-turn: a: The β-turn induced by ^{18}Pro at position i + 1; ^{19}Asn (i + 2) can stabilize the turn by H-bonding to the backbone; b: ^{19}Pro can either induce a β-turn (position i +1), as in a:, or c: it can complete the turn if at position i + 2.

Experimental-Chemistry: The peptides were synthesized by solid phase peptide synthesis techniques on an Applied Biosystems Model 430A peptide synthesizer. The analogs were prepared via Fmoc strategies, using an HMP resin (hydroxymethylphenoxymethyl). The amino acid side-chains were protected as follows: O-t-butyl (Asp) and trityl (His). The peptide was Fmoc-deprotected with 20% piperidine in N-methylpyrrolidone (NMP). Acetylation of the peptide was carried out while the peptide was still on the resin: an excess of 1-acetylimidazole (20 equivalents) was added to the peptide-resin in methylene chloride, and the suspension was stirred at room temperature for 1-3 hours. Completion of the reaction was verified by the Kaiser test.[30] The peptide was then cleaved with trifluoroacetic acid (TFA) /thioanisole /anisole /ethanedithiol /water (86: 5: 2: 3: 4) (two hours, room temperature). The peptides were purified by RP-HPLC C_{18} preparative scale Vydac column (2.2 x 25 cm) eluting with a linear gradient of 0.1% aqueous TFA with increasing concentrations of CH_3CN (containing 0.1% TFA), at 13 ml/min. The purified products were isolated by lyophilization. All peptides were assessed for homogeneity by analytical HPLC, TLC and characterized by amino acid analysis, FAB mass spectrometry, proton NMR spectroscopy and elemental analysis. HPLC and MS data are shown in Table 1.

Experimental-Pharmacology: The endothelin receptor binding assays and functional assays (inhibition of ET-1 and ET-3 induced arachidonic acid release, AAR) were carried out using previously reported procedures.[13,14,18]

Table 1: Data (HPLC and MS) for the synthesized peptides

Compound	HPLC t_R[a](min)	HPLC purity (%)	MS (m/z)+ cald mass	obsd mass
6 Ac-His-Leu-Asn-Ile-Ile-Trp	13.07	99	837.0(M)	838.3[b](M+1)
7 Ac-His-Leu-Asp-Pro-Ile-Trp	13.97	96	821.94(M)	822.1[c](M+1)
8 Ac-His-Asp-Asp-ILe-Ile-Trp	14.27	96	839.91(M)	840.4[b](M+1)
9 Ac-His-Leu-Asn-Pro-Ile-Trp	11.70	98	820.95(M)	821.3[c](M+1)
10 Ac-D-His-Leu-Asn-Pro-Ile-Trp	11.36	94	820.95(M)	821.4[c](M+1)
11 Ac-Phe-Leu-Asn-Pro-Ile-Trp	16.28	99	831.00(M)	831.8[c](M+1)
12 Ac-D-Phe-Leu-Asn-Pro-Ile-Trp	16.76	94	853.00(M + Na)	853.6[c](M+Na)
13 Ac-D-Dip-Leu-Asp-Ile-Ile-Trp	17.15	95	929.09(M + Na)	929.4[c](M+Na)
14 Ac-D-Dip-Leu-Asn-Pro-Ile-Trp	15.28	98	822.88(M)	823.1[c](M+1)

a: 10:90-76:24 CH_3CN with 0.1% TFA-0.1%aqueous TFA; linear gradient over 22 min. Flow rate 1.5 ml/min (column conditions in Experimental-Chemistry); b: Analyzed by Electrospray-MS (Finnigan TSQ70 mass spectrometer); c: Analyzed by FAB-MS (VG Analytical 7070E/HF mass spectrometer)

Experimental-NMR Studies: All NMR data was acquired and processed on a Bruker AMX500 spectrometer on 1-3 mg samples dissolved in 30% CD_3CN / 70% H_2O at pH between 2 and 3. In certain cases two dimensional TOCSY[31] spectra were recorded as a function of temperature when amide proton resonances were overlapped. In one case, a ROESY[32] spectrum (200ms, 4kHZ cw field strength) was acquired to try to extract NOE information. Two dimensional data were acquired as 512 blocks of 1024 data points and were processed using cosine-squared weighting functions with a final matrix size of 1k x 1k data points. Amide proton temperature coefficents, $\Delta\delta/\Delta TNH$, were measured by determining the chemical shift of amide protons (relative to internal TSP) at two, at least, different temperatures (Table 2).

Table 2: Temperature coefficients for amide protons in selected ET-1[16-21] analogs[a].

	$\Delta\delta/\Delta T$ (ppb)					
Compound	16	17	18	19	20	21
1 His-Leu-Asp-Ile-Ile-Trp	-5.8	-6.0	-5.6	-5.6	-5.6	
2 Ac-His-Leu-Asp-Ile-Ile-Trp	-6.0	-5.0	-4.4	-4.2	-5.6	-6.9
6 Ac-His-Leu-Asn-Ile-Ile-Trp	-6.0	-5.4	-5.1	-4.8	-6.0	-7.8
7 Ac-His-Leu-Asp-Pro-Ile-Trp	-5.8	-5.6	-5.8	b	-5.6	-5.2
10 Ac-D-His-Leu-Asn-Pro-Ile-Trp	-7.0	-7.4	-4.9	b	-6.1	-5.7
4 Ac-Phe-Leu-Asp-Ile-Ile-Trp	-7.4	-6.3	-3.6	-3.4	-6.3	-6.2
11 Ac-Phe-Leu-Asn-Pro-Ile-Trp	-5.5	-6.7	-5.1	b	-5.7	-4.1
5 Ac-D-Phe-Leu-Asp-Ile-Ile-Trp	-8.5	-6.9	-3.8	-2.7	-5.9	-5.9
12 Ac-D-Phe-Leu-Asn-Pro-Ile-Trp	-8.4	-7.4	-4.6	b	-6.2	-4.0
13 Ac-D-Dip-Leu-Asp-Ile-Ile-Trp	-6.5	-7.1	-3.6	-3.2	-5.2	-5.4
14 Ac-D-Dip-Leu-Asn-Pro-Ile-Trp	-6.9	-8.4	-4.5	b	-6.3	-3.4

a: Temperature coefficients were determined by measuring chemical shifts at 3 temperatures between 15°-30°C.
b: Sequences where Proline is at position 19

Results and Discussion: The ET-1[16-21] analogs were studied for their binding affinity in rabbit renal artery vascular smooth muscle cells (ET_A) and rat cerebellum (ET_B).[13,14] All peptides were acetylated at the N-terminus.

Table 3 shows selected analogs incorporating one amino acid substitution and their respective binding affinities. Compounds **1-5** have been reported previously,[13-15] and are important references for this study. The D-configuration at the 16-position was shown to be favored for both His^{16} and Phe^{16} analogs (**3** and **5**), with the D-Phe^{16} analog being more potent than the D-His^{16} analog. Monosubstitutions of Asn^{18} and Pro^{19} respectively,

were carried out to determine their effects on binding affinity at either receptor subtype (cf. **2**). At position 18, Asn substitution (**6**) gave rise to a better ligand at both receptors. Proline substitution for Ile^{19} (**7**) was tolerated, giving similar results as the parent peptide. Substitution of the Asp for Leu^{17} (**8**) indicated that this modification could also be tolerated at both receptor subtypes. These monosubstitutions indicated that incorporation of the turn-inducing amino acids was feasible.

Table 3: Structure-Activity Relationships of monosubstituted ET-1[16-21] hexapeptide analogs

Compound		ET_A Binding (IC_{50} μM)	ET_B
1	His^{16}-Leu-Asp-Ile-Ile-Trp^{21}	>50	>50
2	Ac-His-Leu-Asp-Ile-Ile-Trp	>50	43
3	Ac-D-His-Leu-Asp-Ile-Ile-Trp	8.9[a]	9.1[a]
4	Ac-Phe-Leu-Asp-Ile-Ile-Trp	30	>50
5	Ac-D-Phe-Leu-Asp-Ile-Ile-Trp	1.8[a]	2.0[a]
6	Ac-His-Leu-Asn-Ile-Ile-Trp	13[a]	8.3[a]
7	Ac-His-Leu-Asp-Pro-Ile-Trp	>50	34
8	Ac-His-Asp-Asp-Ile-Ile-Trp	>25	14

ET_A: inhibition of ^{125}I-[ET-1] binding to rabbit renal artery vascular smooth muscle cells

ET_B: inhibition of ^{125}I-[ET-3] binding to rat cerebellum

a: n=2 IC_{50} determinations, all other values represent one determination.

IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data was computer-analyzed by non-linear least square analysis giving the best fit for a one-site model.

Table 4 shows the ET_A/ET_B binding affinities when the dipeptide Asn^{18}-Pro^{19} was substituted in the ET-1[16-21] hexapeptide. This substitution lead to an increase in binding affinity at the ET_B receptor (**2, 9**). However, the introduction of the dipeptide in the DHis analog (**3**) increased the binding affinity at both receptor subtypes. Since previous results[13,14] had shown that substitution of His^{16} by Phe or D-Phe increased binding affinity at both receptors, these same substitutions for the Asn^{18}-Pro^{19} analog series were carried out (**11, 12**). The Phe^{16} substitution increased the affinity at the rabbit renal artery (ET_A) (**9, 11**), but when compared to **4**, compound **11** showed an increase in binding affinity only at the ET_B receptor. The D-Phe^{16} analog showed dramatically increased binding affinity at both receptors (**9, 12**), as also seen in Table 3 (**2, 5**). Comparison of **5** and **12** showed that the dipeptide substitution in the D-Phe^{16} analog (**5**) lead to an increase in affinity at both receptor subtypes: this increase being more significant at the ET_A receptor. Substitution of the unnatural amino acid D-Dip for His^{16} was also carried out (**14**) but the compound was less active than **12** and **13**, compound **14**, however, was more active at both ET_A and ET_B receptors when compared to **9**, **10** and **11**.

Table 4: Hexapeptide analogs: Asn^{18}-Pro^{19} Derivatives.

Compound		ET_A Binding (IC_{50} μM)	ET_B
9	Ac-His-Leu-Asn-Pro-Ile-Trp	>50	6.6[a]
10	Ac-D-His-Leu-Asn-Pro-Ile-Trp	>50[a]	>50[b]
11	Ac-Phe-Leu-Asn-Pro-Ile-Trp	24[a]	13[b]
12	Ac-D-Phe-Leu-Asn-Pro-Ile-Trp	0.04[b]	0.27[b]
13	Ac-D-Dip-Leu-Asp-Ile-Ile-Trp(ref.[9,11])	0.014[a]	0.06[a]
14	Ac-D-Dip-Leu-Asn-Pro-Ile-Trp	3.2[b]	0.78[c]

a: n=2 IC_{50} determinations; b: n=3 IC_{50} determinations; c: n=4 IC_{50} determinations, all other values represent one determination.

IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data was computer-analyzed by non-linear least square analysis giving the best fit for a one-site model.

From the results in Table 4, the substitution of the dipeptide Asn^{18}-Pro^{19} in the ET-1[16-21] hexapeptide is tolerated and indicates that introduction of Asn-Pro at positions 18-19 is beneficial in increasing binding affinity at the ET_B receptor, when His^{16} is of the L-configuration. If the D-configuration is at position 16, this substitution is favorable for binding at both receptor subtypes only in the Phe^{16} series.

Introduction of Asp^{17} in the Asn^{18}-Pro^{19} derivatives was not tolerated leading to a loss in binding affinity at both receptor subtypes (compounds not shown).

In an attempt to examine the effect of changing the position of turn-inducing amino acids, Asn^{18}-Pro^{19} to Pro^{18}-Asn^{19}, the synthesis of peptide analogs with Pro^{18}-Asn^{19} substitution was carried out. The derivatives were found to be devoid of activity up to 50 μM at both ET_A and ET_B receptors, indicating that the possible conformation (Figure 1a) represented by this substitution is not favoured or that Pro^{18} or Asn^{19} individually are not tolerated.

The most potent peptides in these series included incorporation of the Asn^{18}-Pro^{19} dipeptide as shown in Table 4, in particular, **12**: Ac-D-Phe-Leu-Asn-Pro-Ile-Trp. Compound **12** was found to be an antagonist of ET-1 stimulated arachidonic acid release (AAR)[13,18] in rabbit renal vascular smooth muscle cells (ET_A) (IC_{50} = 0.21 μM) and of ET-3 stimulated arachidonic acid release in rat cerebellum (ET_B) (IC_{50} = 0.64 μM).

NMR studies indicated that $^3J_{NH,H\alpha}$ coupling constants tended to be in "motionally averaged" range of 6-8 Hz for all of the peptides studied and that the observed chemical shifts are close to those observed for random coil peptides.[33] For compound **12**, which was subjected to a more detailed investigation, including 2-D NMR, no long range NOE's were observed using rotating frame Overhauser spectroscopy (ROESY). These observations are consistent with a high degree of flexibility for these peptides, and adequate conformational constraints could not be obtained for a detailed structural analysis. Therefore, in order to access the structural impact of a Pro^{19} substitution, amide proton temperature coefficients were measured (Table 2). In water, temperature coefficients for the chemical shifts of amide protons are affected by exchange with bulk solvent and by conformational equilibria.[34] In the case of exchange with bulk solvent, a negative coefficient is observed, the magnitude of which is inversely related to the degree of sequestration from solvent. In H_2O, a fully exposed NH should shift about -10 ppb/K with a more positive number indicating reduced exchange with solvent and possible involvement in hydrogen bonding.[34] In the case of confomational equilibria, the temperature coefficient need not be negative and non-exchangeable protons (e.g. Hα) should also be affected.[34] In N-terminal acetylated non-proline containing peptides $\Delta\delta/\Delta T_{NH}$ is invariably smallest for the two internal residues: Asn^{18} and Ile^{19}. Introduction of a proline at position 19 resulted in a significant increase (by ca. 2 ppb/K) of the $\Delta\delta/\Delta T_{NH}$ for Trp^{21}and a decrease (ca. 1 ppb/K) in $\Delta\delta/\Delta T_{NH}$ for Asn^{18} in all of the peptides. All of the Hα proton chemical shifts were found to be independent of temperature (I $\Delta\delta/\Delta T_{H\alpha}$ I≤0.6ppb/K), suggesting that the observed $\Delta\delta/\Delta T_{NH}$ arises primarily from exchange with solvent and not conformational equilibria. Although it is clear that all of the analogs, including those containing Pro, are highly flexible, these results suggest that the proline-containing peptides have a different distribution of conformers from the parent (non-proline containing) peptides. Furthermore, at least some of the conformations include a solvent-shielded Trp^{21} NH in the Pro^{19} analogs, consistent with the expected H-bond between the Trp^{21} NH and the Asn backbone CO in the predicted turn (Figure 1b).

Although the structural impact of the Pro^{19} substitution is consistent across the entire series of peptides, it is less clear how this substitution affects biological activity. Neglecting Asn^{18} substitutions, 4 pairs of peptides which differ only by the presence of Pro or Ile at position 19 display variable biological activity. Analogs **2** and **7**

are both inactive at ET_A, and both have similar (weak) activity at ET_B. The Pro^{19} analogs of the Phe^{16} and $\underline{D}Phe^{16}$ peptides **(11, 12)** show improved activity at one or both of the receptors, compared to their parent analogs **(4, 5)**. In contrast, a significant loss in activity is observed in the $\underline{D}$-Dip^{16} peptides on going from Ile^{19} to Pro^{19} **(13, 14)**.

Our results indicate that insertion of proline at position 19 in analogs of the C-terminal peptide of ET-1 might favor a β-turn structure, since reduced amide proton temperature coefficients at Trp^{21}, the i+3 residue, were observed. The possible presence of a β-turn in the hexapeptide may position the N- and C-termini within close proximity leading to unfavorable interactions and decreased biological activity for certain analogs.

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(*Received in USA* 24 *January* 1995; *accepted* 23 *March* 1995)