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DESIGN OF C-TERMINAL PEPTIDE ANTAGONISTS OF ENDOTHELIN: STRUCTURE-ACTIVITY RELATIONSHIPS OF ET-1[16-21,D-His^{16}]

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Abstract:

We have previously described structure-activity relationships in the hydrophobic C-terminal hexapeptide region of ET-1 known to be highly important for receptor recognition. A mono-D-amino acid scan of ET[16-21] revealed that a D-His^{16} substitution gave rise to endothelin antagonists. We will describe the discovery and development of further antagonists in this series.

Introduction:

The endothelium has been proposed to mediate vasoconstriction *via* production of endothelium derived vasoconstricting factor(s) [EDCF] in response to various chemical and physical stimuli. Endothelin-1 (ET-1), a potent vasoconstrictor peptide recently isolated from endothelial cells, has been suggested as a possible candidate for EDCF(s).[1,2] ET-1 belongs to a new class of peptides and is some ten-fold more potent than the vasoconstrictor angiotensin II, with extremely long-lasting pressor effects.[3,4] The discovery of selective endothelin (ET) receptor antagonists will facilitate identification of the physiological and pathological roles for ET-1 and its isopeptides.

The cloning and expression of two ET receptor subtypes termed ET_A and ET_B from bovine and rat lung respectively has been reported.[5,6] More recently the human receptors have also been cloned.[7,8] The ET_A receptor clearly mediates vasoconstriction and is widely distributed in vascular smooth muscle tissue of cardiovascular origin and in certain regions of the brain.[5,9] The ET_B or non-selective receptor, recognizing the ET isopeptides with equal affinity, was originally classified as the non-vascular smooth muscle receptor.[6] This receptor is localized in endothelial cells and in the brain and has been associated with vasodilator activity, possibly through the release of endothelium derived relaxing factor (EDRF).[10] However we have recently found that the rabbit pulmonary artery recognizes all three isopeptides with equal affinity, and that selective ET_B agonists are potent vasoconstrictors in this tissue.[11]

Structure-activity studies of analogs of ET-1 have indicated that the C-terminal Trp residue is important for the vasoconstrictor activity of endothelin in porcine coronary artery strips (ET_A).[12] ET[1-15] was inactive,[13] while the hydrophobic C-terminal hexapeptide ET[16-21] has been reported as a partial agonist

in guinea-pig isolated bronchus, rat vas deferans and rabbit pulmonary artery.[14,15] However, we have found that this fragment is devoid of functional activity in several tissue preparations including rat aorta and rabbit pulmonary artery.[16] It is clear that the C-terminal hexapeptide, while being important for receptor recognition, possesses very weak binding affinity in a number of animal tissues (IC_{50} = 50-70 μM).[16] We have focused our efforts on ET[16-21] by incorporating structural modifications to enhance activity and probe ET_A/ET_B specificity, in an attempt to discover smaller endothelin antagonists amenable to peptidomimetic modification.

Results and Discussion:

We have previously shown that the hexapeptide Ac-D-His^{16}-Leu-Asp-Ile-Ile-Trp^{21}, (**2**) exhibited a 5-10-fold enhancement in binding affinity compared with NAc-ET[16-21], (**1**) (see Table 1).[16,17] Compound (**2**) inhibits ET-1-induced arachidonic acid release in vascular smooth muscle cells, an ET_A functional assay.[18] Previously we reported that ET-1[Phe^{16}] was a 5-fold more potent agonist than ET-1 itself in the rat aorta (ET_A) and in rabbit pulmonary artery (ET_B).[19] This observation was key to the next modification carried out in the ET[16-21] series. Substitution of the D-His residue with D-Phe led to compound (**3**) (Table 1) which showed a further enhancement in binding affinity at both ET_A and ET_B receptors (3-fold). Comparison of the binding affinities in rabbit renal vascular smooth muscle cells (ET_A) and in rat cerebellum (ET_B) indicates that compounds (**2**) and (**3**) are non-selective ET_A/ET_B ligands.

Extensive SAR around this series has been carried out and Table 1 lists a selection of compounds with differing selectivity for ET_A vs ET_B receptors. In an attempt to discover smaller peptides with antagonist activity some deletion analogs of compound (**5**) were also synthesized. Comparison of compounds (**1**) and (**2**) and (**3**) and (**4**) indicates that D-stereochemistry at position 16 affords a large increase in binding affinity at both ET_A and ET_B receptors. This was found to be a fairly general phenomenum throughout the series. Many different substituents were tolerated at the Leu^{17} that maintained similar ET_A/ET_B binding, illustrated by compounds (**5**) and (**6**). Independent replacement of the 17 and 18 positions with aromatic residues led to an increase in ET_B selectivity (rat cerebellum ET_B receptor)(compounds (**7**),(**8**), (**9**)). Few changes are well tolerated in the Ile-Ile-Trp tripeptide without marginal losses in antagonist activity[20] at the ET_A receptor. Interestingly some modifications in the C-terminal tripeptide have led to an increase in ET_B binding (compare compounds (**3**) and (**10**)).

Clearly, shortening the peptide (**3**) generally leads to some loss in activity at both receptor subtypes (compounds **12-14**). A low energy overlay of compound (**3**) and the recently discovered ET_A selective antagonist BQ-123 (cyclo[Leu-D-Trp-D-Asp-Pro-D-Val])[21] is shown in Figure 1. This overlay illustrates a potential conformer of compound (**3**) that could be consistent with the solution conformation of BQ-123.[22] In the fitting process, the Asp of compound (**3**) was not placed with the D-Asp of BQ-123, consistent with our observed SAR (compound (**9**)). Further SAR around this series of ET antagonists will be reported at a later date.

Table 1: Analogs of ET-1[16-21]

	Compound	Binding ($IC_{50}/\mu M$)		Functional
		ET_A	ET_B	AAR
1	Ac-His[16]-Leu-Asp-Ile-Ile-Trp[21]	>50	>50	c
2	Ac-D-His-Leu-Asp-Ile-Ile-Trp	9.5	10	3.2
3	Ac-D-Phe-Leu-Asp-Ile-Ile-Trp	2.8	3.3	3.1
4	Ac-Phe-Leu-Asp-Ile-Ile-Trp	18.4	18.5	c
5	Ac-D-Phe-Glu-Asp-Ile-Ile-Trp	0.65	1.3	0.60
6	Ac-D-Phe-Orn-Asp-Ile-Ile-Trp	0.75	4.0	2.0
7	Ac-D-Phe-Phe-Asp-Ile-Ile-Trp	0.20	0.05	2.6
8	Ac-D-Phe-D-Phe-Asp-Ile-Ile-Trp	0.93	0.03	1.6
9	Ac-D-Phe-Leu-Phe-Ile-Ile-Trp	1.18	0.035	4.5
10	Ac-D-Phe-Leu-Asp-Ile-Ile-Tyr	>10	0.14	c
11	Ac-D-Phe-Leu-Asp-Ile-Trp	>10	>10	c
12	Ac-D-Phe-Asp-Ile-Ile-Trp	9.1	9.3	c
13	Ac-D-Phe-Ile-Ile-Trp	>10	>10	c
14	Ac-D-Phe-Ile-Trp	8.0	>10	c

ET_A = rabbit renal artery vascular smooth muscle cells ET_B = rat cerebellum
AAR= Inhibition of ET-1 induced arachidonic acid accumulation in rabbit renal artery vascular smooth muscle cells
c= not tested

Conclusions:

Ac-D-Phe-Leu-Asp-Ile-Ile-Trp (3) is a non-selective ET_A/ET_B ligand antagonizing endothelin-induced arachidonic acid release in vascular smooth muscle cells. SAR studies of the D-Phe[16] series have shown that D-stereochemistry at position 16 affords a large increase in binding affinity and antagonist activity at ET receptors. Several different residues are tolerated at position 17 without a loss in binding affinity and antagonist activity. Aromatic residues at either of positions 17 and 18 maintain ET_A receptor binding affinity while substantially increasing ET_B receptor binding affinity. Substitution of the C-terminal Trp for Tyr leads to a substantial decrease in ET_A binding (rabbit renal artery smooth muscle cells), while ET_B binding affinity (rat cerebellum) is increased.

Experimental-Chemistry:

Peptides were prepared by solid phase synthesis techniques on an Applied Biosystems Model 430A peptide synthesizer. The analogs were prepared using either (a) an N-α-BOC protection scheme with a PAM resin; amino acid side chain protection: OBzl (Asp, Glu), 2-Cl-Z (Lys), Tos (His); deprotected and cleaved from the resin using anhydrous liquid HF/anisole or HF/ p-cresol (9:1 v/v) or (b) an N-α-FMOC protection scheme on an HMP resin (hydroxymethylphenoxymethyl); amino acid side-chain protection: O^{t}Bu (Asp, Glu, Tyr), trityl (His); these peptides were FMOC-deprotected with 20% piperidine in N-methylpyrrolidinone: deprotection and cleavage from the resin with TFA:thioanisole:ethanedithiol:H_2O (89:5:2:3:1). Acetylation was carried out on the resin (for both BOC and FMOC methods) in methylene chloride with an excess of 1-acetylimidazole (20 equivalents).

Purification was carried out by RP-HPLC (C18 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 at 13-15 ml/min. Peptides were isolated by lyophilization and assessed for homogeneity by analytical HPLC, TLC and characterized by amino acid analysis, FAB mass spectroscopy, proton NMR spectroscopy and elemental analysis.

Experimental-Pharmacology:

Endothelin Receptor Binding Assay Protocol

Incubations were performed in 12 x 75 mm polypropylene tubes containing 20 mM Tris, 2 mM EDTA, 100 μM PMSF, 100 μM bacitracin, 30pM [^{125}I]-ET-1 (2,000Ci/mmol) and 5 μg rabbit renal artery vascular smooth muscle membranes (ET_A) or 5 μg rat cerebellar membranes from adult blue laurie rats (ET_B) (total volume of 250 μL) (pH 7.4 at 37°C). The order of the additions (tubes on ice) were i) test compound, ii) [^{125}I]-ET-1 and iii) membranes. Test compounds were diluted in a buffer (20 mM Tris, 2 mM EDTA, 1 mg/mL BSA and 1% DMSO), to five times the final incubation concentration. [^{125}I]-ET-1 was diluted in the same buffer without DMSO. Membranes were diluted in buffer containing 100 μM PMSF and 100 μM bacitracin, without BSA or DMSO. Immediately following the last addition, the incubation was agitated by hand. Tubes were then incubated at 37°C for 2 hours. Incubations were terminated by filtration through Whatman GF/B filters which were presoaked with 50 mM Tris, containing 0.2% BSA and 100 μM bacitracin (pH 7.3 at 5°C). Nonspecific binding is defined as binding in the presence of 100 nM ET-1 and specific binding is defined as total binding minus nonspecific binding. IC_{50} values were derived from single competition experiments in which all data points were measured in triplicate. Binding data was computer analyzed by non-linear least squares analysis giving the best fit for a one-site model, from which the IC_{50} value was estimated.

Functional Assay Protocols: Arachidonic Acid Release (AAR)[18]

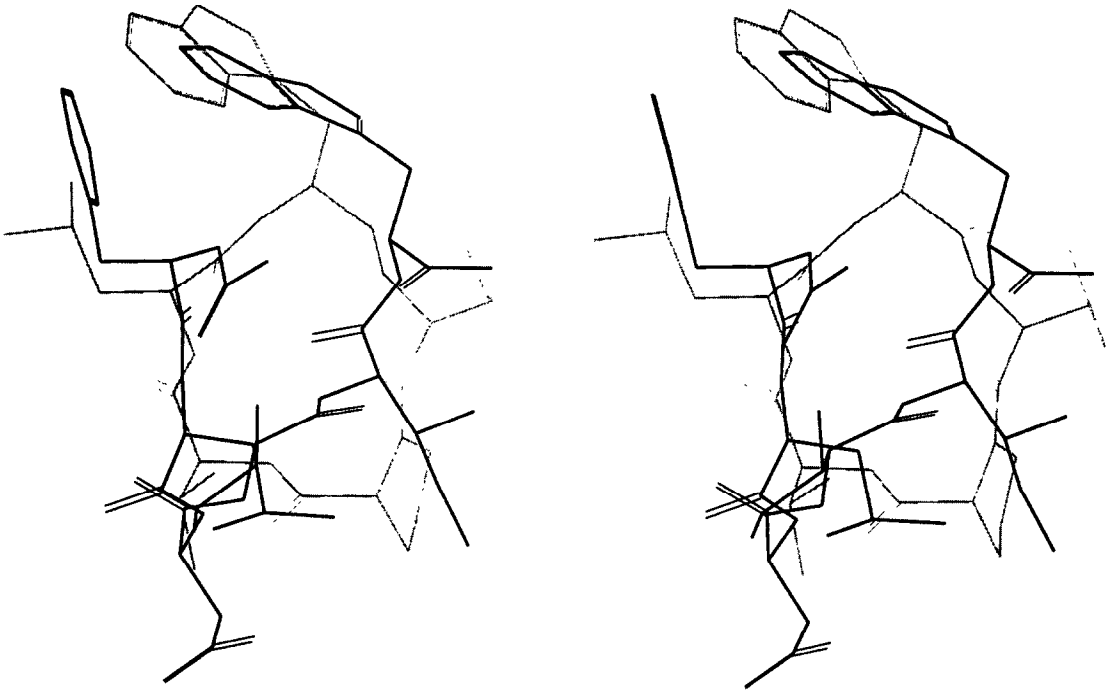
[^{3}H] Arachidonic Acid Loading Media (LM): Dulbecco's Eagle Medium and Ham's Nutrient Mixture F12 (1:1) with 0.5 % FCS (fetal calf serum) and 0.25 μCi/mL [^{3}H] arachidonic acid (218 Ci/mmol) (Amersham).

Confluent monolayers of cultured rabbit renal artery vascular smooth muscle cells were incubated in 0.5 mL of the LM over 18 hours, at 37°C, in 5% CO_2. The LM was aspirated and the cells were washed once with the assay buffer (Hank's BSS + 10 mM HEPES + fatty acid-free BSA (1 mg/mL)), and incubated for 5 minutes with 1 mL of the prewarmed assay buffer. This solution was aspirated, followed by the addition of 1 ml of prewarmed assay buffer and further incubated for another 5 minutes. A final 5 minute incubation was carried out in a similar manner. The same procedure was repeated with the inclusion of 10 mL of the test compound (1 nM to 1 μM)) and 10 mL ET-1 (0.3 nM) and the incubation was extended for 30 minutes. This solution was then collected, 10 mL of scintillation cocktail was added and the amount of [^{3}H] arachidonic acid was determined in a liquid scintillation counter.

References:

1. Hickey, K. A.; Rubanyi, G.; Paul, R. J.; Highsmith, R. F. *Am. J. Physiol.* **1985**, *248*, C550.
2. Yanagisawa, M.; Kurihara, H.; Kimura, S.; Tomobe, Y.; Kobayashi, M.; Mitsui, Y.; Yazaki, Y.; Goto, K.; Masaki, T. *Nature (Lond.)* **1988**, *332*, 411.
3. Masaki, T.; Yanagisawa, M. *Cardiovascular Drug Rev.*, **1990**, *8*, 373.
4. Doherty, A.M. *Annu. Rep. Med. Chem.* **1991**, *26*, 83.
5. Arai, H.; Hori, S.; Aramori, I.; Ohkubo, H.; Nakanishi, S. *Nature (London)*, **1990**, *348*, 730.
6. Sakurai, T.; Yanagisawa, M.; Takuwa, Y.; Miyazaki, H.; Kimura, S.; Goto, K.; Masaki, T. *Nature (London)* **1990**, *348*, 732.
7. Sakamoto, A.; Yanagisawa, M.; Sakurai, T.; Takuwa, Y.; Yanagisawa, H.; Masaki, T. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 656.
8. Hosoda, K.; Nakao, K.; Arai, H.; Suga, S.; Ogawa, Y.; Mukoyama, M.; Shirakami, G.; Saito, Y.; Nakanishi, S.; Imura, H. *FEBS Lett.* **1991**, *287*, 23.
9. Lin, H.Y.; Kaji, E.H.; Winkel, G.K.; Ives, H.E.; Lodish, H.F. *Proc. Natl. Acad. Sci. USA*, **1991**, *88*, 3185.
10. Saeki, T.; Ihara, M.; Fukuroda, T.; Yamagiwa, M.; Yano, M. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 286.
11. Panek, R.L.; Major, T.C.; Hingorani, G.P.; Doherty, A.M.; Taylor, D.G.; Rapundalo, S.T. *Biochem. Biophys. Res. Commun.*, **1992**, *183*, 566.
12. Kimura, S.; Kasuya, Y.; Sawamura, T.; Shinmi, O.; Sugita, Y.; Yanagisawa, M.; Goto, K.; Masaki, T. *Biochem. Biophys. Res. Commun.*, **1988**, *156*, 1182.
13. Takayanagi, R.; Hashiguchi, T.; Ohashi, M.; Nawata, H. *Regul. Pept.* **1990**, *27*, 247.
14. Maggi, C.A.; Giuliani, S.; Patacchini, R. Santicioli, P.; Rovero, P.; Giachetti, A.; Meli, A. *Eur. J. Pharmacol.*, **1989**, *166*, 121.
15. Maggi, C.A.; Giuliani, S.; Patacchini, R.; Rovero, P.; Giachetti, A.; Meli, A. *Eur. J. Pharmacol.*, **1989**, *174*, 23.
16. Doherty, A.M.; Cody, W.L.; Leitz, N.L.; DePue, P.L.; Taylor, M.D.; Rapundalo, S.T.; Hingorani, G.P.; Major, T.C.; Panek, R.L.; Taylor, D.G. *J. Cardiovasc. Pharmacol.*, **1991**, *17* (Suppl.7), S59.
17. Doherty, A.M. *J. Med. Chem.* **1992**, *35*, 1493.
18. Reynolds, E.; Mok, L. *FASEB J.* **1991**, *5*, A1066.
19. Panek, R.L.; Major, T.C.; Taylor, D.G.; Hingorani, G.P.; Dunbar, J.B.; Doherty, A.M.; Rapundalo, S.T. *Biochem. Biophys. Res. Commun.* **1992**, *183*, 572.
20. Doherty, A.M.; Cody, W.L. Unpublished results.
21. Ihara, M.; Fukuroda, T.; Saeki, T.; Nishikibe, M.; Kojiri, K.; Suda, H.; Yano, M. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 132.
22. Reily, M.D.; Thanabal, V.; Omecinsky, D.O.; Dunbar, J.B.; Doherty, A.M.; DePue, P.L. *FEBS Lett.*; **1992**, *300*, 136.

Figure 1:



Ac-D-Phe-Leu-Asp-Ile-Ile-Trp (black) overlayed on BQ123(gray)