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STRUCTURE-ACTIVITY RELATIONSHIPS IN A SERIES OF MONOCYCLIC ENDOTHELIN ANALOGUES

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Abstract:

Monocyclic fragment analogues of endothelin-1 (ET-1) were prepared to explore the importance of the bicyclic structure of ET-1 to its binding affinity and functional activity. Most of the monocyclic analogues prepared showed low micro to high nanomolar binding affinities and were functional antagonists of ET-1 induced accumulation of inositol phosphates. However, one analogue possessed mixed antagonist/agonist activity at the two endothelin receptor subtypes.

Introduction:

The endothelins (ETs) and sarafotoxins (SRTXs) comprise a family of potent vasoconstricting peptides that contain 21-amino acids arranged in a unique bicyclic motif formed by disulfide bridges between cysteines located in positions 1-15 and 3-11 (Figure 1a).[1,2] The cloning and expression of three endothelin receptor subtypes (ET_A, ET_B and ET_C) has been reported.[3-5] All members of this family possess a hydrophobic C-terminal hexapeptide, terminating in an L-tryptophan with a free carboxylate that is important for high binding affinity to isolated endothelin receptors and for functional activity[1,2] (for recent reviews, see 6,7). The C-terminal hexapeptide has been reported to be a partial agonist in the guinea pig bronchus, rat vas deferens and rabbit pulmonary artery,[8,9] although it has also been reported that Ac-His[16]-Leu-Asp-Ile-Ile-Trp[21] (Ac-ET-1_{16-21}) only has weak affinity for either of the endothelin receptor subtypes (>50 μM) and did not mimic or block ET functional activity in either the rabbit isolated pulmonary artery or the rat isolated left atria at concentrations of up to 10 μM.[10] The C-terminal hexapeptide of ET-1 has been used to develop compounds that possess high binding affinity to both of these receptor subtypes[10-15] and that are functional antagonists of ET-1.[13-15]

Both the monocyclic analogue ET-1_{3-11} (rat aorta)[16] and the bicyclic analogue ET-1_{1-15} (porcine coronary artery strips)[17] did not possess functional activity at concentrations of up to 1 and 10 μM, respectively. In fact, the monocyclic analogue Ac-ET-1_{3-11}-NH_2 did not bind to the endothelin receptor (rabbit pulmonary artery, rabbit aorta or rat heart ventricle) at concentrations of up to 100 μM.[18] Preparation of the hybrid analogue, in which the monocyclic 3-11 fragment is attached directly to the

C-terminal hexapeptide (compound 1) led to an analogue that did not possess greater affinity for the receptor than Ac-ET-1_{16-21}, itself.[18] We have examined the importance of the bicyclic structure of ET-1 to its binding affinity and functional activity by preparing a series of monocyclic analogues with the hydrophobic C-terminal hexapeptide attached (Figure 1b).

Experimental:

Chemistry. All of the linear hexapeptides were prepared with a standard Boc solid phase synthetic techniques[19] on a N^{α}-Boc-Trp-PAM (phenylacetamidomethyl) resin on an Applied Biosystems 430A instrument. Amino acid side chain protecting groups were as follows: benzyl (Asp, Glu, Ser), 2-chlorobenzyloxycarbonyl (Lys), benzyloxymethyl or 2,4-dinitrophenyl (His), 4-methylbenzyl (Cys) and 2-bromobenzyloxycarbonyl (Tyr). Individual amino acids were coupled as their symmetrical anhydrides or their 1-hydroxybenzotriazole activated esters. After incorporation of each amino acid the peptide resin was Boc deprotected with trifluoroacetic acid (TFA) and indole (1.0 mg/mL) in dichloromethane (DCM) (1:1) and subsequently, neutralized with 10% diisopropylethylamine in DCM. For peptides utilizing the 2,4-dinitrophenyl protecting group on histidine, prior to removal of the final N-terminal Boc group, the resin was treated with thiophenol (20 mmol/N,N-dimethylformamide (DMF), 2 h) and subsequently washed with DMF, water, ethanol and DCM. The N-terminal Boc group was then removed as above and the resin was dried under reduced pressure. The peptides were cleaved from the resin and deprotected using 90% anhydrous hydrogen fluoride (HF) with 10% anisole/dimethylsulfide or p-cresol as scavengers (60 min, 0°C) and lyophilized. The linear peptides were cyclized in the presence of potassium ferricyanide at a concentration of 0.1 mg/mL of crude peptide in water (2h, pH 8). The solution was acidified to pH 4.5 with glacial HOAc and excess (10x) BioRad AG4-X4 anion resin was added (1 h). The resin was filtered and the crude peptide solution was absorbed to a disposable C18 cartridge (pre-equilibrated in water) and eluted with 70% acetonitrile, 0.1% TFA in water. After lyophilization, the peptide was purified to homogeneity by preparative reversed-phase high-performance liquid chromatography (HPLC) on a Vydac C18 column (218TP1022, 2.2 x 25.0 cm, 15 mL/min) with a mobile phase of 0.1% TFA in water and increasing concentrations of 0.1% TFA acid in acetonitrile. All synthetic peptides were analyzed for homogeneity by analytical HPLC, capillary electrophoresis (CE) and structural integrity by amino acid analysis (AAA), high field proton nuclear magnetic resonance (^{1}H-NMR) and fast atom bombardment mass spectrometry (FAB-MS).

Endothelin Receptor Binding Assay Protocols. The binding protocol using the rat heart ventricle from adult male Sprague-Dawley rats (RaH) has been previously described.[10] Likewise, the binding

protocols using rabbit renal artery vascular smooth muscle membranes (ET_A) and rat cerebellar membranes (ET_B) from adult blue laurie rats has been previously described.[14]

Inositol Phosphate Accumulation (IP_3). The protocol utilized for this functional assay in rat skin fibroblasts has been previously described.[20]

Figure 1a:

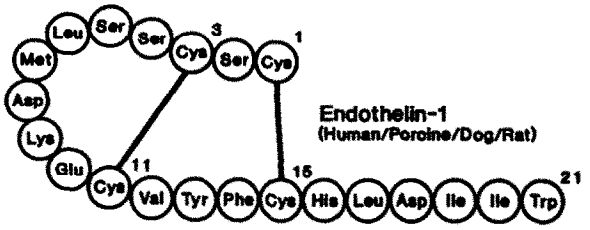


Figure 1b:

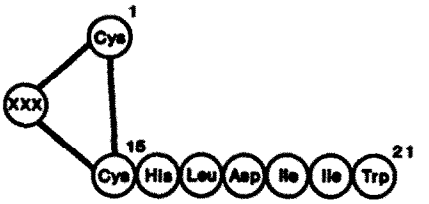


Table 1: Pharmacological Profiles of Monocyclic Analogues of ET-1.

	Compound	Binding[a] ($IC_{50}/\mu M$)			Functional[b]
	XXX =[c]	RaH	ET_A	ET_B	IP_3
1	-Ser-Ser-Leu-Met-Asp-Lys-Glu	49	>10	>10	d
2	-Ser-Apa-Val-Tyr-Phe-[e]	1.8	2.5	0.35	>10
3	-Ser-Aha-Val-Tyr-Phe-[e]	2.5	d	d	8.0
4	-Ser-Aoa-Val-Tyr-Phe-[e]	1.6	1.8	0.24	3.0
5	-Val-Tyr-Phe-	6.5	2.6	1.7	2.2
6	-Ser-Val-Tyr-Phe-	2.8	3.8	1.7	d
7	-Ser-Ser-Val-Tyr-Phe-	1.8	2.0	1.4	d
8	-Ser-Ser-Ser-Val-Tyr-Phe-	1.2	2.5	0.06	5.0
9	-Ser-Asp-Lys-Glu-Val-Tyr-Phe-	3.5	7.1	1.1	>10

[a]Binding data: Rat heart ventricle (RaH); Rabbit renal artery vascular smooth muscle cells (ET_A); Rat cerebellar membranes (ET_B). [b]Functional data: Inhibition of ET-1 induced accumulation of inositol phosphates in rat skin fibroblasts. [c]See Figure 1b. [d]Not determined. [e]Apa = 5-aminopentanoic acid; Aha = 7-aminoheptanoic acid; Aoa = 8-aminooctanoic acid.

Results and Discussion:

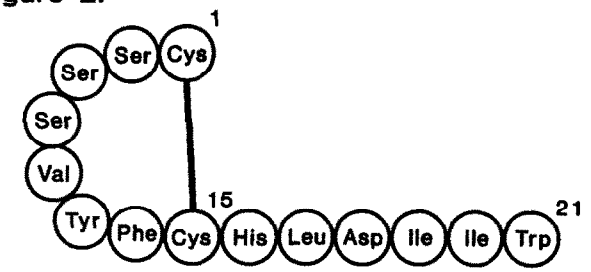
Initially, we replaced the 3-11 disulfide bridge of ET-1 with various hydrocarbon spacers (compounds **2**, **3** and **4**) to determine the preferred ring size for ET receptor binding. In all of these cases, we observed a greater than 20-fold enhancement in binding affinity for endothelin receptors in the rat heart ventricle and a corresponding enhancement in binding affinity to the isolated ET_A and ET_B receptors. Likewise, these compounds were also micromolar functional antagonists of ET-1 induced accumulation of inositol phosphates (IP_3) in rat skin fibroblasts. The accumulation of inositol phosphates in rat skin fibroblasts is mediated through the ET_A receptor[20] and the inhibition observed correlates well with the binding observed to the ET_A receptor.

In an effort to further explore the structural requirements for high affinity binding to the endothelin receptors and in an attempt to increase aqueous solubility we have prepared a series of analogues in which the size of the monocyclic ring was modified by the inclusion of serine residues (these residues are also present in ET-1, compounds **5-8**). Interestingly, all compounds exhibited low micromolar affinity for both receptors with the exception of compound **8**, which exhibited 60 nM affinity for the ET_B receptor subtype (Figure 2). It appears that both of the receptor subtypes are relatively tolerant of modifications in the size of the second monocyclic ring, but that the enhancement in binding affinity over that of Ac-ET-$1_{16\text{-}21}$ and compound **1** may be related to the hydrophobic tripeptide -Val^{12}-Tyr-Phe^{14}-. This suggests that an auxiliary hydrophobic binding pocket is present in both receptor subtypes. Interestingly, compound **8** is an antagonist of ET-1 induced accumulation of inositol phosphates in rat skin fibroblasts (IC_{50} = 5.0 μM) and ET-1 stimulated arachidonic acid (AAR)[14,20] release in rabbit renal vascular smooth muscle cells (IC_{50} = 7.0 μM) (both ET_A), but is an agonist of AAR in CHO-K1 cells stably transfected with the recombinant rat ET_B receptor[21] at 10 μM. This is the first report of a compound with mixed antagonist/agonist activity at the two endothelin receptor subtypes.

It has been reported that compound **5** (IRL 1038)[22,23] is a low nanomolar ET_B receptor-selective antagonist (6 nM in the rat cerebellum) inhibiting ET_B receptor mediated contractions of the guinea pig ileal and tracheal smooth muscle. In our hands, compound **5**, at least in the rat cerebellum, is best described as a nonselective ligand for both ET_A and ET_B receptors being a weak antagonist of ET-1 induced accumulation of inositol phosphates mediated through the ET_A receptor (IC_{50} = 2.2 μM).[20] In addition, compound **5** did not have any functional antagonistic activity in the ET_B AAR assay at concentrations of up to 10 μM, providing further evidence that this compound is a nonselective ET_A/ET_B antagonist.

Finally, in an effort to determine if the highly conserved tripeptide -Asp^8-Lys-Glu^{10}- between the

Figure 2:



Compound 8

RaH	ET_A	ET_B	AAR_A	AAR_B
1.2	2.5	0.06	antagonist	agonist

All values are micromolar.

endothelins has a significant role in ET-1 binding or function, this tripeptide was incorporated into compound **6**. No enhancement in binding affinity was observed for compound **9** over that of compound **6**; suggesting that these residues are not critical to the binding affinity. However, it is possible that in this monocyclic series, these residues cannot adopt a three-dimensional orientation that fits into an additional hydrophilic binding pocket.

Conclusions:

Amino acid residues in the 1-3 and 11-15 monocyclic loop are more critical for binding to the endothelin receptor than residues in the 3-11 monocyclic loop. In fact, weak functional antagonists of ET-1 induced accumulation of inositol phosphates in rat skin fibroblasts can be obtained from this monocyclic series. It can be inferred that an auxiliary hydrophobic binding pocket exists in both endothelin receptor subtypes that accounts for the enhanced binding of analogues containing the residues -Val^{12}-Tyr-Phe^{14}-. In addition, compound **8** is the first reported mixed antagonist/agonist in tissues containing the ET_A and ET_B receptors, respectively. Finally, in our hands, compound **5**, (IRL 1038) is a nonselective low micromolar ligand for both of the endothelin receptor subtypes.

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