Communications to the Editor

Design of a Functional Hexapeptide Antagonist of Endothelin

Endothelin-1 (ET-1, Figure 1), a bicyclic 21-amino acid peptide, is a potent constrictor of vascular smooth muscle.[1-3] Since the isolation of ET-1 from the supernatant of cultured porcine endothelial aortic cells, human genomic analysis has identified two structurally and functionally related isopeptides (ET-2 and ET-3).[4] Previous structure–activity analyses have shown the importance of the C-terminal L-tryptophan indole ring, its carboxylate, and the two cystine bridges (1–15 and 3–11) for vasoconstrictor activity in certain tissues.[5,6] In addition, in vitro binding ($IC_{50} \approx 50$–$70\ \mu M$) to endothelin receptors in rat cardiac and rabbit pulmonary tissue preparations has been demonstrated for the C-terminal hexapeptide [His-Leu-Asp-Ile-Ile-Trp and Ac-His-Leu-Asp-Ile-Ile-Trp (compounds **2** and **3**, Table I)]. Using D-amino acids to probe the importance of the individual residues, we observed that incorporation of D-histidine in the 16 position (compound **4**) led to a 20-fold enhancement of the binding affinity in several tissue beds.[7] However, while ET-1 (16–21) and Ac-D-His-Leu-Asp-Ile-Ile-Trp did not inhibit ET-1-induced vasoconstriction in an organ bath assay, both analogues exhibited antagonist activity by inhibiting ET-1-induced inositol phosphate accumulation in rat skin fibroblasts.[8]

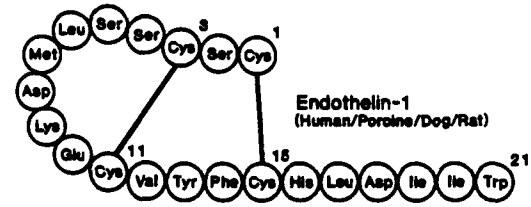

Figure 1.

D-Dip = D-Diphenylalanine =

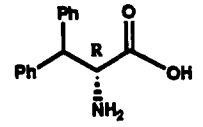

Figure 2.

Two endothelin receptor subtypes (ET_A and ET_B) have been identified, cloned, sequenced, and characterized.[9] The ET_A receptor mediates vasoconstriction and is found predominantly in peripheral tissues, such as the heart, lung, intestine, and aorta. The ET_B receptor subtype has been localized to the central nervous system (CNS) and endothelial cells. Recent studies with an ET_B receptor selective ligand [sarafotoxin-6c (SRTX-6c)] have shown that this receptor may be functionally linked to vasodilation via release of endothelium derived relaxing factor (EDRF) in the rat aortic ring.[10] In addition, we have found that [$Ala^{1,3,11,15}$]-ET-1 and other truncated linear analogues are potent and selective ET_B agonists that cause vasoconstriction in the rabbit pulmonary artery.[11,12] It is unclear whether the vascular smooth muscle ET_B-like receptor is functionally or structurally similar to the brain receptor.

Both specific and nonspecific endothelin antagonists are necessary to determine the physiological and/or pathophysiological role of endothelin and its receptor subtypes. Several peptide antagonists have recently been reported. For example, replacement of the 1–15 cystine disulfide linkage with a lactam (between aspartic acid in position 15 and 2,4-diaminobutyric acid in position 1) led to an antagonist of ET-1-stimulated vasoconstriction in the rat pulmonary artery.[13,14] Although receptor selectivity was

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Table I. Relative Activities and Mass Spectral Data for the C-Terminal Hexapeptide Analogues

no.	analogue	binding assay[a] ET_A	binding assay[a] ET_B	biochemical assay[b] IP_3	biochemical assay[b] AAR	mass spec [M + 1]
1	ET-1	0.0002	0.0016	0.0012[c]	0.0003[c]	
2	His-Leu-Asp-Ile-Ile-Trp	>50	>50	>50	*d*	796.3
3	Ac-His-Leu-Asp-Ile-Ile-Trp	>50	43	>50	*d*	838.1
4	Ac-D-His-Leu-Asp-Ile-Ile-Trp	9.5	10.0	1.4	3.2	838.6
5	Ac-D-Phe-Leu-Asp-Ile-Ile-Trp	2.8	3.3	0.86	3.1	848.4
6	Ac-D-Tyr-Leu-Asp-Ile-Ile-Trp	0.40	7.0	0.43	0.25	864.0
7	Ac-D-Trp-Leu-Asp-Ile-Ile-Trp	0.13	1.8	*d*	0.45	887.0
8	Ac-D-Dip-Leu-Asp-Ile-Ile-Trp	0.015	0.15	0.014	0.070	924.6
9	Ac-D-Nal-Leu-Asp-Ile-Ile-Trp	1.0	4.0	0.63	1.9	898.5
10	Ac-D-Bip-Leu-Asp-Ile-Ile-Trp	4.4	3.5	6.0	*d*	925.3

[a] All data is expressed as micromolar IC_{50} values. Competitive binding versus ET-1 was determined in cultured rabbit renal artery vascular smooth muscle cells and the rat cerebellar membranes for ET_A and ET_B, respectively.[23,24] [b] Antagonism of the endothelin stimulated accumulation of inositol phosphates (IP_3) and arachidonic acid release (AAR) was determined in cultured rat skin fibroblasts and rabbit renal artery vascular smooth muscle cells, respectively.[24,25] [c] EC_{50} value. [d] Not measured.

not determined, this compound did not inhibit ET-3-induced vasoconstriction, which suggests that it may be an ET_A-selective antagonist.[13] Another antagonist was designed from a cyclic pentapeptide lead isolated from *Streptomyces misakiensis* fermentation products.[15] This antagonist, cyclo-[D-Trp-D-Asp-Pro-D-Val-Leu] (BQ-123), is selective for the ET_A receptor subtype and is a functional antagonist with a pA_2 value of 7.4 in the isolated porcine coronary artery.[16] Recently, 2(*R*)-[(*R*)-2-[2(*S*)-[[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino]-4-methylpentanoyl]amino]-3-[[3-(1-methyl-1*H*-indolyl)propionyl]-amino]-3-(2-pyridyl)propionic acid (FR139317) was also disclosed as an ET_A selective antagonist.[17] We wish to report the first functional antagonist of endothelin-stimulated vasoconstriction (Ac-D-Dip-Leu-Asp-Ile-Ile-Trp; compound 8, PD 142893, [D-Dip = D-diphenylalanine,[18-20] Figure 2)] which exhibits high affinity for both the ET_A and ET_B receptor subtypes.

Experimental[21] Summary. Chemistry. All the linear hexapeptides were prepared using standard Boc or Fmoc solid-phase synthetic techniques[22] on an Applied Biosystems 430A instrument. The peptides were cleaved from the resin and deprotected using anhydrous hydrogen fluoride for Boc syntheses and trifluoroacetic acid for Fmoc syntheses with the appropriate scavengers.[6,7,22] All peptides were purified to homogeneity by preparative HPLC on a C18 column with a mobile phase of 0.1% trifluoroacetic acid in water and increasing concentrations of 0.1% trifluoroacetic acid in acetonitrile. The peptides were analyzed for homogeneity and structural integrity by analytical HPLC, capillary zone electrophoresis, amino acid analysis (AAA), high-field proton nuclear magnetic resonance (^1H NMR), and fast atom bombardment mass spectrometry (FAB-MS).

Pharmacology. Test compounds were dissolved in DMSO and brought to a final DMSO concentration of 0.1–0.5% in the assay buffer.[21] Inhibition of the binding of [^{125}I]ET-1 to the ET_A and ET_B receptor subtypes was determined in cultured rabbit renal artery vascular smooth cells and rat cerebellar membranes.[23,24] Antagonism of ET-1 stimulated accumulation of inositol phosphates and arachidonic acid release was measured in cultured rat skin fibroblasts and rabbit renal artery smooth muscle cells, respectively.[24,25] Antagonism of ET-1 stimulated vasoconstriction was determined in the rabbit femoral and pulmonary arteries.[12] The pA_2 values were calculated by the method of Arunlakshana and Schild.[26]

Results and Discussion. We have designed an antagonist of ET-1-stimulated vasoconstriction for tissues containing either the ET_A or the ET_B receptor subtype. The enhanced receptor binding affinity observed for Ac-D-His-Leu-Asp-Ile-Ile-Trp (4) and structure–activity relationships developed from various D-amino acid substitutions in position 16 were critical to our design strategy. It has been previously reported that the substitution of a D-amino acid in the 16 position of ET-1 itself led to a

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300-fold loss in binding affinity to human vascular smooth muscle cells, suggesting that the structure–activity relationships of the full molecule are quite different than those of the C-terminal hexapeptide.[27]

Previously, it was shown that D-aromatic amino acids in the 16 position of the C-terminal hexapeptide enhances receptor affinity.[7,8] The D-phenylalanine substitution (5) led to approximately a 3-fold enhancement in binding affinity to both receptor subtypes (cf. 4). An enhancement in binding affinity to the ET_A receptor over the ET_B receptor was realized from the D-Tyr[16] and D-Trp[16] (6 and 7) substitutions, (approximately 15-fold). A further 10-fold increase in binding was obtained by incorporation of the hydrophobic D-diphenylalanine[18-20] (D-Dip) residue in position 16.

Although, Ac-D-Dip-Leu-Asp-Ile-Ile-Trp (8) displayed high affinity for both the ET_A and ET_B receptors, it showed some selectivity for the ET_A receptor (IC_{50} = 15 nM and 150 nM, respectively, Table I). The enhanced binding of 8 was not simply a function of the hydrophobicity of Dip, since both the naphthyl (Nal) and biphenyl (Bip) substituted analogues (9 and 10) exhibited approximately 100-fold less receptor affinity.

The ability of these linear hexapeptides (2–10) to inhibit endothelin-stimulated arachidonic acid release (rabbit renal artery vascular smooth muscle cells (ET_A)) correlates well with binding to the ET_A receptor. Only 8 was a functional antagonist of ET-1-stimulated vasoconstriction in both the rabbit femoral and pulmonary artery with pA_2 values of 7.19 and 7.27, respectively. The rabbit femoral artery expresses only the ET_A receptor since SRTX-6c has no activity at concentrations up to 1.0 μM, while the rabbit pulmonary artery has predominantly an ET_B-like receptor.[12] None of the other analogues tested showed antagonism of ET-1 induced vasoconstriction at concentrations up to 10 μM.

This analogue (8) represents the first known functional antagonist of endothelin at both the ET_A and ET_B receptor subtypes. This compound may provide a critical tool for determining the physiological and/or pathophysiological role of endothelin.

Supplementary Material Available: Physical (proton NMR and mass spectral) data for all the peptides and a detailed description of the pharmacological assays (binding, IP_3, AAR, and vasoconstriction) is provided (23 pages). Ordering information is given on any current masthead page.

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Time-Dependent Inhibition of Human Placental Aromatase with a 2,19-Methyleneoxy-Bridged Androstenedione

Aromatase is the rate-limiting enzyme in the conversion of androgens to estrogens.[1] Inhibitors of aromatase have demonstrated therapeutic utility in estrogen-dependent metastatic breast cancer[2a,b] and have potential for use in the management of other estrogen-dependent processes and diseases.[2c] Several categories of steroidal aromatase inhibitors have been designed.[3,4] We recently described hydroxylated 2,19-methylene-bridged androstenediones[5]

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