Articles

Structure–Activity Relationships of the Potent Combined Endothelin-A/Endothelin-B Receptor Antagonist Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]: Development of Endothelin-B Receptor Selective Antagonists

Wayne L. Cody,* John X. He, Patricia L. DePue, Lisa A. Waite, Daniele M. Leonard, Andrea M. Sefler,† James S. Kaltenbronn, Stephen J. Haleen,‡ Donnelle M. Walker,‡ Michael A. Flynn,‡ Kathleen M. Welch,‡ Elwood E. Reynolds,‡ and Annette M. Doherty

Departments of Chemistry and Cardiovascular Therapeutics, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105

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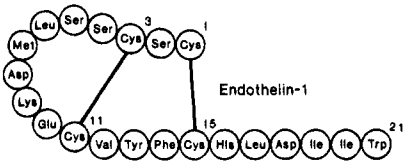
The endothelins (ETs) are a family of bicyclic 21-amino acid-containing peptides that are highly potent and prolonged vasoconstrictors. The discovery of potent ET antagonists will facilitate the understanding of the physiological and/or pathophysiological role of ET. Structure–activity studies have revealed the importance of the C-terminal hexapeptide (residues 16–21) of ET (His[16]-Leu[17]-Asp[18]-Ile[19]-Ile[20]-Trp[21]) to the development of potent antagonists at both receptor subtypes (ET_A and ET_B). In particular, it has been shown that Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21] (Dip = 3,3-diphenylalanine) has low nanomolar affinity for the two endothelin receptor subtypes and is a functional antagonist of ET activity, both *in vitro* and *in vivo* at both receptors. Herein, we will describe the structure–activity relationships of Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21] (PD 142893) with a particular emphasis on modifications that lead to enhanced receptor affinity and/or individual receptor subtype selectivity. In particular, we will demonstrate how we utilized PD 142893 to develop ET_B receptor selective ligands and the pharmacological differences that exist between species ET_B receptors with respect to their affinity for C-terminal hexapeptide antagonists.

Introduction

Endothelin-1 (ET-1, compound **1**), a bicyclic 21-amino acid peptide, is a potent constrictor of vascular smooth muscle that was first isolated and characterized from the supernatant of porcine endothelial cells.[1–3] ET-1 is one member of a family of isopeptides, including ET-2 and ET-3, that are encoded by three distinct genes.[4] In addition, the endothelins (ETs) are structurally and functionally related to mouse vasoactive intestinal contractor (VIC),[5] the cardiotoxic sarafotoxins (SRTXs),[6,7] and bibrotoxin.[8] All members of this family possess cystine bridges between positions 1 and 15 and 3 and 11, in addition to maintaining a highly conserved C-terminal hydrophobic hexapeptide culminating in a tryptophan residue with a free carboxylate (His[16]-Leu-Asp-Ile-Ile-Trp[21], $ET\text{-}1_{16-21}$).[9–11] This family of isopeptides shows potent and prolonged vasoconstricting activity and possesses a number of other important biological activities that have recently been reviewed.[9–11] Therefore, the identification of potent and specific endothelin receptor antagonists should assist in determining the physiological and/or pathophysiological roles of endothelin and its isopeptides.

Initially, two endothelin receptors were cloned, sequenced, and characterized from the bovine and rat lung, respectively.[12,13] Receptor A (ET_A) was shown to be selective for ET-1 and ET-2 over ET-3, and receptor B (ET_B) possesses equal affinity for all of the ET isopeptides. Subsequently, the corresponding human receptors have been cloned.[14,15] The endothelin receptor subtype populations (ET_A/ET_B) are widely distributed in several tissues and appear to possess different functions dependent upon species and location. For example, utilizing a highly selective ET_B receptor ligand, sarafotoxin-6c (SRTX-6c),[16] the ET_B receptor has been functionally linked to vasodilation in the rat aortic ring,[17] while it is responsible for vasoconstriction in the rabbit pulmonary artery and several other tissues.[18,19] Recent pharmacological studies suggested the existence of additional ET_B receptor subtypes and/or major species differences.[20–22] Additionally, an ET_C receptor, which is selective for ET-3, has been cloned and characterized from *Xenopus* melanophores and/or heart but remains to be fully characterized with known selective and combined ET receptor antagonists.[23] The identification of these endothelin receptors has facilitated the development of endothelin agonists and/or antagonists with high affinity for both the ET_A and ET_B receptors and with varied selectivity.



Compound 1

The first report of an endothelin receptor antagonist was the substitution of the cystine bridge between positions 1 and 15 of ET-1 with an isosteric lactam

* Author to whom correspondence should be addressed. Phone: (313) 996-7729. Fax: (313) 996-3107. e-mail: codyw@aa.wl.com.
† Current address: University of California, Department of Chemistry, Berkeley, CA 94720.
‡ Department of Cardiovascular Therapeutics.
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bridge, formed between the α-amino group of 1,3-diaminopropanoic acid (Dpr) in position 1 and the β-carboxylate of aspartic acid in position 15 of ET-1, itself.[24] This compound was shown to block ET-1-mediated vasoconstriction in the isolated perfused guinea pig lung but had no effect on ET-3-stimulated vasoconstriction in the same tissue and therefore is selective for the ET_A receptor subtype.

Several other structurally diverse peptides obtained from the screening of fermentation broths and/or compound libraries have been reported to be ET receptor antagonists. In particular, modification of a head-to-tail cyclic pentapeptide lead (BQ-18257B),[25] discovered from screening of the fermentation products of *Streptomyces misakiensis*, led to the discovery of the highly selective ET_A receptor antagonist BQ-123 (cyclo[-DTrp-DAsp-Pro-DVal-Leu-], head-to-tail cyclic).[26] Likewise, a similar screening strategy led to the discovery of a series of linear tripeptides that possessed high affinity and selectivity for the ET_A receptor as exemplified by FR 139317 ((hexahydro-1*H*-azepinyl)carbonyl-Leu-DTrp(N^{in}-Me)-D(2-pyridinyl)alanine).[27]

Several nonpeptide ET_A antagonists have also been described. The first nonpeptide endothelin antagonist was isolated from the bayberry *Myrica cerifera*. It was relatively weak and selective for the ET_A receptor.[28] Recently, several other nonpeptide ET_A receptor selective antagonists have been discovered from compound library screening strategies followed by lead optimization. ET_A selective agents include the sulfonamide BMS-182874,[29] derivatives of sulfisoazole,[30,31] the symmetrical anhydride CGS 27830,[32] and the butenolides PD 155080 and PD 156707.[33] Also, several nonpeptide combined ET_A and ET_B receptor antagonists have been recently reported, the 1,3-diarylindan-2-carboxylic acids, SB 209670,[34] Ro 46-2005,[35] Ro 47-0203 (Bosentan),[35] and L-749329.[37] (For a recent review of peptidic and nonpeptidic endothelin antagonists, see ref 37).

Conversely, the availability of ET_B receptor selective antagonists has been quite limited. For example, IRL 1038 (cyclo[Cys-Val-Tyr-Phe-Cys]-His^{16}-Leu-Asp-Ile-Ile-Trp^{21}, cyclic disulfide) was initially reported to exhibit only micromolar affinity for the ET_A receptor in a variety of species (e.g., rat aorta, guinea pig heart, pig aorta, and human umbilical vein) but low nanomolar affinity for the ET_B receptor (e.g., rat cerebellum, guinea pig cerebellum, pig lung, and human placenta, among others).[38] However, more recently, IRL 1038 has been reported to possess only low micromolar affinity at both ET_A and ET_B receptor subtypes, in similar systems/tissues.[39,40] RES-701-1 (cyclo[Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp]-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp, cyclized via a lactam bridge between the α-amine of Gly and the β-carboxylate of Asp) isolated from *Streptomyces* sp. is reported to exhibit 10 nM affinity for human ET_B receptor but greater than 5 μM affinity for the human ET_A receptor, both stably expressed in Chinese hamster ovary (CHO) cells.[41] However, a current study has shown that synthetic preparations of RES-701-1 only possess low micromolar affinity for each of the endothelin receptor subtypes.[42] Recently, it has been reported that *N-cis*-[(2,6-dimethylpiperidino)carbonyl]-L-γ-methylleucine-D-1-(methoxycarbonyl)tryptophanyl-D-norleucine (BQ-788) is highly selective for the human ET_B receptor (IC_{50} = 1300 nM for ET_A receptors in a human neuroblastoma cell line (SKN-N-MC) and IC_{50} = 1.2 nM for ET_B receptors in human Girardi heart cells).[43] Thus, BQ-788 represents the only known compound that has held up to further scrutiny and been truly demonstrated to be an ET_B receptor selective ligand.

Recently, we have reported on the rational design of a series of combined ET_A and ET_B receptor antagonists based on the C-terminal hexapeptide of ET-1 (Ac-His^{16}-Leu-Asp-Ile-Ile-Trp^{21}, Ac-ET-1_{16-21}).[44-46] Critical to our design was the recognition of the importance of the following structural features: (i) the neutralization of the N-terminal amine by acetylation, (ii) the preference for aromatic hydrophobic D-amino acids in the 16 position, (iii) the requirement for L-tryptophan in the C-terminal 21 position, and (iv) the necessity of the C-terminal free carboxylate.[44-46] (Independently, these design considerations were corroborated by the preparation of a series of C-terminal hexapeptides with low nanomolar affinity for the ET_A receptor in murine 3T3 cells, including Ac-DTrp^{16}-Orn-Asp-Ile-Ile-Trp^{21} (15 nM) and Ac-DPhe^{16}-Nphe-Asp-Ile-Ile-Trp^{21} (12 nM, Nphe = *N*-benzylglycine).)[47] Our observations have led to the preparation of compound **2** (PD 142893, Ac-DDip^{16}-Leu-Asp-Ile-Ile-Trp^{21} (Dip = 3,3-diphenylalanine)) which exhibits low nanomolar affinity for both the ET_A and ET_B receptors and was able to antagonize ET-1-stimulated vasoconstriction in the rabbit femoral artery (ET_A) and SRTX-6c-stimulated vasoconstriction in the rabbit pulmonary artery (ET_B) with pA_2 values of 6.6 and 6.3, respectively.[46,48] The complete structure–activity relationships (SAR) for this potent combined endothelin receptor antagonist and related analogues will be described. Some of these analogues exhibited ET_B receptor selectivity in different species, and these will be highlighted.

Chemistry

Peptide Synthesis, Purification, and Characterization. All of the peptide analogues were prepared by solid-phase peptide synthetic (SPPS) methodologies.[49,50] Unusual amino acids were purchased from commercial sources or prepared utilizing literature methods.[51-53] The peptide analogues were prepared utilizing either an N^{α}-*t*-Boc (*tert*-butyloxycarbonyl) protecting group strategy on a PAM[54] (phenylacetoamidomethyl) resin or an N^{α}-Fmoc protecting group strategy on a Wang[55] (HMP, [4-(hydroxymethyl)phenoxy]-methyl) resin.

For peptides prepared by the N^{α}-*t*-Boc strategy, each N^{α}-*t*-Boc group was removed with 50% trifluoroacetic acid (TFA) in dichloromethane (DCM) and neutralized with 10% diisopropylethylamine (DIEA) in DCM prior to incorporation of the next protected amino acid. For peptides prepared by the N^{α}-Fmoc strategy, each N^{α}-Fmoc group was removed with 20% piperidine in *N*-methylpyrrolidinone (NMP) prior to incorporation of the next protected amino acid. All amino acids were single coupled as either their symmetrical anhydrides or *N*-hydroxybenzotriazole (HOBt)-activated esters unless incomplete coupling was indicated by the Kaiser test.[56] If incomplete incorporation of the acylating agent was indicated, the amino acid was recoupled until a negative Kaiser test was obtained. After coupling of the last amino acid and N^{α}-amine deprotection, the peptide was acetylated with an excess of 1-acetylimidazole in DCM

or 10% acetic anhydride in DCM with a catalytic amount of 4-(dimethylamino)pyridine (DMAP). The N-terminal modifications in Table 9 were performed on the resin using the corresponding symmetrical anhydrides, HOBt-activated esters, isocyanates, or acid chlorides. The peptides prepared by the N^{α}-*t*-Boc strategy were simultaneously deprotected and cleaved from the resin by treatment with anhydrous liquid hydrogen fluoride (HF) and anisole or *p*-cresol (9:1, v/v) in the presence of 3-methylindole at 0 °C for 1 h. The resin was filtered and washed with diethyl ether, and the crude peptide was extracted into aqueous solution, concentrated under reduced pressure, resuspended in water, and lyophilized. The peptides prepared by the N^{α}-Fmoc strategy were simultaneously deprotected and cleaved from the resin by treatment with a mixture of TFA:thioanisole:phenol:1,2-ethanedithiol:water (89:5:3:2:1) at room temperature for 1–2 h. The peptide solution was separated from the resin by filtration and concentrated under reduced pressure, and the peptide was precipitated with diethyl ether, redissolved in aqueous solution, concentrated under reduced pressure, resuspended in water, and lyophilized.

All crude peptides were purified to homogeneity by preparative reversed-phase high-performance liquid chromatography (HPLC) eluting with a linear gradient of 0.1% aqueous TFA with increasing concentrations of 0.1% TFA in acetonitrile (AcCN). Peptide fractions that were determined to be homogenous by analytical reversed-phase HPLC were combined and lyophilized. For secondary *in vitro* functional evaluation, the purified peptides were converted to the corresponding disodium salt, by treating the fully protonated analogue with 5% aqueous sodium bicarbonate followed by solid-phase extraction on a C18 cartridge, elution with methanol, concentration under reduced pressure, resuspension in water, and lyophilization. All final compounds were analyzed for homogeneity by analytical HPLC and/or capillary electrophoresis (CE) and characterized for structural integrity by amino acid analysis (AAA), elemental analysis, fast atom bombardment mass spectrometry (FABMS), electrospray mass spectrometry (ESMS), and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (Table 1).

Results and Discussion

It has previously been shown that potent ET_A and ET_B receptor antagonists can be developed from the C-terminal hexapeptide of endothelin, such as Ac-DDip^{16}-Leu-Asp-Ile-Ile-Trp^{21} (PD 142893, compound **2**)[46,48] and Ac-DBhg^{16}-Leu-Asp-Ile-Ile-Trp^{21} (PD 145065, compound **3**)[57,58] (Table 2). These compounds, PD 142893 and PD 145065, showed similar binding affinity to the human cloned ET_A (IC_{50} = 60 and 7.3 nM (n = 3), respectively) and ET_B (IC_{50} = 42 and 49 nM (n = 1), respectively) receptors. *In vitro*, PD 142893 was able to block ET-1-stimulated vasoconstriction in the rabbit femoral artery (ET_A) and sarafotoxin-6c-stimulated vasoconstriction in the rabbit pulmonary artery (ET_B) with pA_2 values of 6.6 and 6.3, respectively.[46] Likewise, PD 145065 has similar effects *in vitro* with pA_2 values of 6.8 and 7.0, respectively.[57,58] In all cases, these hexapeptide antagonists were able to block ET-1-stimulated arachidonic acid release (AAR) in rabbit renal vascular smooth muscle cells which correlated well with their binding to the ET_A receptor in the same tissue. These results prompted us to perform a complete study of the SAR of these C-terminal endothelin hexapeptide antagonists to explore structural features that influence receptor affinity and selectivity.

Position 16 Modifications (Table 2). From previous studies, it is evident that N-terminal acetylation and D-stereochemistry of the amino acid in position 16 are required for high receptor affinity at both receptors. These studies have shown that both receptors possess a large hydrophobic binding pocket of finite size and depth that prefers occupation by amino acids such as DDip^{16} or DBhg^{16}.[57,58] This suggestion is further supported by the SAR observed for compounds **5**, **7**, and **8**.

All modifications other than very conservative substitutions were not tolerated.[57,58] In addition it was observed that N-terminal acetylation has a relatively small effect on the binding affinity to the rat ET_B receptor; however, significant decreases in ET_A receptor affinity were observed. Compounds **4** and **6** further support this observation.

Position 17 Modifications (Table 3). This position is tolerant of most modifications, allowing both acidic and basic substitutions (compounds **9**–**11**). In particular, compound **11** has the best binding affinity at both the ET_A and rat ET_B receptors of PD 142893 analogues. Potentially, this position could provide a handle for enhancing the aqueous solubility of this extremely hydrophobic series of molecules for further pharmacological evaluations.[60]

Interestingly, the substitution of D-phenylalanine in position 17 (compound **12**) led to a greater than 10-fold loss in binding affinity at both the ET_A and ET_B receptors. It had been previously shown in the DPhe^{16}-containing series that the substitution of Phe or DPhe for Leu in the 17 position led to a 5–20-fold loss of binding affinity at the ET_A receptor.[46] However, both substitutions (Phe^{17} and DPhe^{17}) were well tolerated at the ET_B receptor, and in fact, Ac-DPhe^{16}-DPhe-Asp-Ile-Ile-Trp^{21} (compound **13**) was a highly ET_B selective ligand (ET_A = 0.91 nM and ET_B = 0.02 nM).[45,46] This points out one of these SAR differences between the two series, such that DPhe is not well tolerated at position 17 in the DDip^{16}-containing series for binding to either receptor. This loss of binding affinity for compound **12** may be explained if the concept of hydrophobic collapse[61] is invoked via stacking of the aromatic rings of DDip^{16} and DPhe^{17}.

Position 18 Modifications (Table 4). The substitution of glutamic acid for aspartic acid had little effect on receptor binding affinity (compound **14**). However, the incorporation of basic and aromatic residues (compounds **15**–**17**) in this position maintained high affinity at the rat ET_B receptor but led to approximately a 10-fold loss of affinity for the ET_A receptor. This result suggests that selective modifications in this position could lead to ET_B receptor selective antagonists (see the discussion of binding affinities at the rat versus the cloned human ET_B receptor and Table 10). Surprisingly, neutralization of the side chain carboxylate had little effect on the binding affinity at either receptor (compound **18**), showing that this position is not involved in an important ionic interaction with either of the receptor subtypes.

Position 19 Modifications (Table 5). It has been shown in the DPhe^{16}-containing hexapeptide antagonist

Table 1. Analytical Data for the Synthetic Analogues

compd	t_R, min (purity, %) HPLC[a,b]	t_R, min (purity, %) CE[c]	FABMS or ESMS $(m/z)^+$ calcd mass	FABMS or ESMS $(m/z)^+$ obsd mass	AAA[d] (cal) obtained
2	17.6[a] (>99)	10.0 (>99)	924.12 (M)	946.6 (M + Na)	Leu(1) 1.13, Asp(1)[f] 1.00, Ile(2) 1.68[g]
3	17.5[a] (>99)	9.9 (>99)	950.15 (M)	950.3 (M)	Leu(1)[f] 1.00, Asp(1) 1.25, Ile(2) 1.68[g]
4	16.3[a] (98)	9.3 (95.2)	876.12 (M)	877.1 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.68, Ile(2) 1.81
5	18.8[a] (98)	9.4 (95.4)	918.15 (M)	940.3 (M + Na)	Leu(1)[f] 1.00, Asp(1) 2.33, Ile(2) 1.94
6	15.8[a] (99)	9.6 (94.7)	958.18 (M)	958.6 (M)	Leu(1) 0.43, Asp(1)[f] 1.00, Ile(2) 1.80
7	19.2[a] (>99)	9.2 (>99)	1000.22 (M)	1000.8 (M + H) 1022.8 (M + Na)	Leu(1)[f] 1.00, Asp(1) 0.96, Ile(2) 1.60[g]
8	18.2[a] (97)	10.1 (93.6)	892.12 (M)	914.1 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.08, Ile(2) 1.14[g]
9	17.2[b] (98)	11.7 (>99)	940.07 (M)	940.5 (M + H) 962.4 (M + Na)	Glu(1) 0.73, Asp(1)[f] 1.00, Ile(2) 1.74[g]
10	15.9[b] (>99)	8.4 (95.5)	925.10 (M)	925.1 (M) 947.1 (M + Na)	Orn(1) 1.29, Asp(1)[f] 1.00, Ile(2) 1.69[g]
11	11.6[b] (98)	6.9 (>99)	967.14 (M)	968.3 (M + H)	Arg(1)[f] 1.00, Asp(1) 1.65, Ile(2) 1.36[g]
12	16.7[b] (98)	10.5 (>99)	958.13 (M)	958.5 (M)	Leu(1)[f] 1.00, Asp(1) 1.24, Ile(2) 1.26[g]
13	17.4[a] (>99)	10.2 (98.2)	882.04 (M)	904.1 (M + Na)	Phe(2)[f] 2.00, Asp(1) 1.14, Ile(2) 1.35[g]
14	19.6[b] (>99)	9.0 (97.4)	938.14 (M)	960.5 (M + Na)	Leu(1)[f] 1.00, Glu(1) 1.31, Ile(2) 1.88
15	19.2[b] (98)	8.3 (>99)	937.20 (M)	938.6 (M + H) 959.6 (M + Na)	Leu(1)[f] 1.00, Lys(1) 1.25, Ile(2) 1.69[g]
16	17.4[a] (>99)	[e]	956.20 (M)	978.3 (M + Na)	Leu(1)[f] 1.00, Phe(1) 1.10, Ile(2) 1.38[g]
17	21.4[b] (95)	[e]	972.18 (M)	994.6 (M + Na)	Leu(1)[f] 1.00, Tyr(1) 1.02, Ile(2) 1.70[g]
18	19.5[b] (97)	8.8 (95.5)	880.11 (M)	903.1 (M + Na)	Leu(1)[f] 1.00, Ala(1) 1.47, Ile(2) 1.51[g]
19	18.8[b] (>99)	10.9 (98.5)	940.07 (M)	940.0 (M)	Leu(1) 0.83, Asp(1) 1.05, Glu(1) 0.95, Ile(1)[f] 1.00
20	19.9[b] (>99)	7.8 (>99)	939.13 (M)	940.0 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.45, Lys(1) 1.32, Ile(1) 1.11
21	18.4[a] (>99)	9.3 (99.1)	958.13 (M)	981.2 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.16, Phe(1) 1.05, Ile(1) 1.00
22	19.4[b] (95)	[e]	974.12 (M)	996.5 (M + Na)	Leu(1) 0.92, Asp(1) 1.27, Tyr(1)[f] 1.00, Ile(1) 1.03
23	19.2[b] (98)	[e]	882.02 (M)	904.3 (M + H)	Leu(1) 0.74, Asp(1) 1.02, Ala(1) 1.02, Ile(1)[f] 1.00
24	19.8[b] (98)	8.4 (>99)	910.09 (M)	910.6 (M + H) 932.3 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.13, Val(1) 0.83, Ile(1) 0.75
25	18.7[a] (97)	10.4 (93.1)	964.18 (M)	963.4 (M)	Leu(1)[f] 1.00, Asp(1) 1.15, Ile(1) 0.95
26	16.2[b] (>99)	11.8 (97.0)	940.07 (M)	962.3 (M + Na)	Leu(1) 0.70, Asp(1) 1.24, Ile(1)[f] 1.00, Glu(1) 1.10
27	18.8[b] (>99)	7.9 (>99)	939.13 (M)	939.6 (M)	Leu(1) 0.79, Asp(1) 1.33, Ile(1)[f] 1.00, Lys(1) 0.48
28	31.2[b] (>99)	6.9 (97.7)	958.12 (M)	959.2 (M + H)	Leu(1)[f] 1.00, Asp(1) 0.92, Ile(1) 1.09, Phe(1) 1.15
29	20.2[b] (>99)	[e]	910.09 (M)	910.6 (M + H) 932.3 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.13, Ile(1) 0.96, Val(1) 0.89
30	18.5[a] (94)	9.2 (93.7)	924.12 (M)	946.9 (M + Na)	Leu(2)[f] 2.00, Asp(1) 1.02, Ile(1) 0.98
31	21.1[b] (98)	9.7 (93.7[‡])	885.04 (M)	885.8 (M + H)	Leu(1)[f] 1.00, Asp(1) 1.45, Ile(2) 1.59[g], Phe(1) 1.25
32	19.0[b] (95)	[e]	901.08 (M)	901.7 (M + H) 923.4 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.17, Ile(2) 1.52,[g] Tyr(1) 0.93
33	17.9[b] (96)	9.1 (>99[‡])	886.06 (M)	886.5 (M)	Leu(1)[f] 1.00, Asp(1) 0.67, Ile(2) 1.30[g]
34	17.5[a] (98)	8.5 (98.3)	923.13 (M)	945.1 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.05, Ile(2) 1.58[g]
35	19.4[b] (97)	[e]	786.96 (M)	788.0 (M) 810.4 (M + Na)	Leu(1) 1.24, Asp(1) 0.81, Ile(1) 1.31[g], Phe(1)[f] 1.00
36	21.1[b] (96)	[e]	864.00 (M)	864.5 (M + H)	Leu(1)[f] 1.00, Asp(1) 0.87, Ile(2) 1.67[g]
37	21.8[a] (>99)	11.9 (99.0)	890.10 (M)	890.7 (M + H) 914.3 (M + Na)	Leu(1)[f] 1.00, Asp(1) 0.88, Ile(2) 1.44[g]
38	15.4[a] (>99)	9.4 (>99)	902.07 (M)	924.0 (M + Na)	Cys(2) 1.89, Asp(1) 0.95, Ile(1) 0.80
39	18.4[b] (97)	10.3 (>99)	882.08 (M)	882.1 (M)	Leu(1)[f] 1.00, Asp(1) 1.45, Ile(2) 1.61[g]
40	18.2[b] (98)	10.3 (97.6)	938.14 (M)	938.4 (M) 960.5 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.60, Ile(2) 1.57[g]
41	17.9[a] (>99)	10.4 (>99)	966.20 (M)	988.3 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.08, Ile(2) 1.20[g]
42	17.5[a] (>99)	10.3 (>99)	978.09 (M)	1001.2 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.29, Ile (2) 1.41[g]
43	16.3[a] (96)	10.7 (92.3)	960.17 (M)	982.1 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.02, Ile(2) 1.05[g]
44	17.6[a] (98)	10.7 (91.4)	939.13 (M)	961.4 (M + Na)	Leu(1)[f] 1.00, Asp(1) 0.99, Ile(2) 1.18[g]
45	17.4[a] (>99)	12.4 (>99)	982.15 (M)	1004.8 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.39, Ile(2) 1.47[g]
46	19.4[a] (>99)	10.4 (98.9)	986.19 (M)	1008.2 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.54, Ile(2) 1.67[g]
47	15.0[a] (>99)	9.8 (>99)	1001.20 (M)	1001.5 (M) 1023.4 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.16, Ile(2) 1.96
48	18.2[a] (97)	9.7 (>99)	1058.34 (M)	1080.3 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.27, Ile(2) 1.67[g]
49	17.5[a] (94)	9.7 (>99)	992.24 (M)	1014.6 (M + Na	Leu(1)[f] 1.00, Asp(1) 0.99, Ile(2) 1.19[g]
50	18.7[a] (>99)	9.6 (95.2)	1007.25 (M)	1029.5 (M + Na)	Leu(1)[f] 1.00, Asp(1) 1.54, Ile(2) 1.67[g]
51	19.5[a] (97)	9.8 (95.5)	1076.30 (M)	1098.9 (M + Na)	Leu(1) 0.44, Asp(1)[f] 1.00, Ile(2) 1.44[g]
52	22.1[a] (98)	9.5 (>99)	1116.36 (M)	1138.5 (M + H)	Leu(1)[f] 1.00, Asp(1) 0.96, Ile(2) 1.16[g]
53	21.7[a] (>99)	10.4 (>99)	880.02 (M)	880.7 (M + H) 902.4 (M + Na)	Leu(1) 0.79, Phe(2)[f] 2.00, Ile(2) 1.63[g]

[a] 80:20 to 14:86 0.1% aqueous TFA:0.1% TFA in AcCN, linear gradient over 22 min at 1.5 mL/min (λ = 214 and 280 nm) on a Vydac 218TP54 column. [b] 90:10 to 24:76 0.1% aqueous TFA:0.1% TFA in AcCN, linear gradient over 22 min at 1.5 mL/min (λ = 214 and 280 nm) on a Vydac 218TP54 column. [c] 20 mM (cyclohexylamino)propanesulfonic acid buffer (CAPS; pH 11.0), 72 cm capillary (30 °C), 10 000 Volts (140 V/cm), λ = 280 or 220[‡] nm. [d] Trp was not stable to the hydrolysis conditions and usually not determined (see the Experimental Section). Synthetic unnatural amino acids were not determined. [e] Not determined. [f] Standard amino acid for the analysis. [g] Incomplete hydrolysis of the Ile–Ile bond was routinely observed.

series that the C-terminal tripeptide (Ile-Ile-Trp[21]) is highly sensitive to even subtle modifications.[44-46] Therefore, as predicted, neither acidic (compound **19**), or basic (compound **20**) residues were tolerated in position 19. Substitution of aromatic residues, such as phenylalanine (compound **21**), maintained good binding affinity for only the rat ET_B receptor, while the tyrosine (compound **22**) analogue had good binding affinity at

Table 2. Position 16 Analogues of DDip[16]-Leu-Asp-Ile-Ile-Trp[21]

compd[a]	IC_{50}, μM ET_A[b]	ET_B[c]	AAR_A[d]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[e]	0.040[g]	0.060[g]	0.070[h]
3, Ac-DBhg-Leu-Asp-Ile-Ile-Trp·2Na[f]	0.0035[g]	0.015[g]	0.0049[g]
4, DPmp-Leu-Asp-Ile-Ile-Trp	>10	>10	*j*
5, Ac-DPmp-Leu-Asp-Ile-Ile-Trp	1.1[i]	2.4[h]	*j*
6, DTrt-Leu-Asp-Ile-Ile-Trp	11.0	10.0	*j*
7, Ac-DTrt-Leu-Asp-Ile-Ile-Trp	2.6	5.9	*j*
8, Ac-DAda-Leu-Asp-Ile-Ile-Trp	3.2	2.0	*j*

[a] Ada = 1-adamantylalanine, Bhg = 10,11-dihydro-5*H*-dibenzo[*a*,*d*]cycloheptenylglycine, Dip = 3,3-diphenylalanine, Pmp = pentamethylphenylalanine, and Trt = tritylglycine. [b] Binding data in rabbit renal vascular smooth muscle cells. [c] Binding data in rat cerebellar membranes. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [e] PD 142893; see refs 46 and 48. [f] PD 145065; see refs 57 and 58. [g] $n = 5$ IC_{50} determinations. [h] $n = 2$ IC_{50} determinations. [i] $n = 4$ IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model. [j] Not tested.

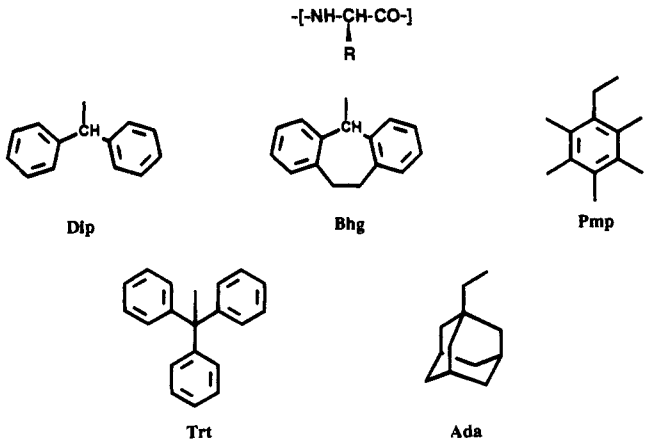

Figure 1. Structures of the unnatural amino acids incorporated in the 16 position of the C-terminal hexapeptide of endothelin-1.

Table 3. Position 17 Analogues of Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]

compd[a]	IC_{50}, μM ET_A[b]	ET_B[c]	AAR_A[d]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[e]	0.040[h]	0.060[h]	0.070[i]
9, Ac-DDip-Glu-Asp-Ile-Ile-Trp[f]	0.025	0.052	0.13[i]
10, Ac-DDip-Orn-Asp-Ile-Ile-Trp[f]	0.013[i]	0.15[i]	0.030[i]
11, Ac-DDip-Arg-Asp-Ile-Ile-Trp[f]	0.0040	0.010	0.014
12, Ac-DDip-DPhe-Asp-Ile-Ile-Trp	0.38	0.74	0.52
13, Ac-DPhe-DPhe-Asp-Ile-Ile-Trp[g]	0.91[j]	0.020[j]	2.1[i]

[a] Orn = ornithine. [b] Binding data in rabbit renal vascular smooth muscle cells. [c] Binding data in rat cerebellar membranes. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [e] PD 142893; see refs 46 and 48. [f] See ref 59. [g] See ref 46. [h] $n = 5$ IC_{50} determinations. [i] $n = 2$ IC_{50} determinations. [j] n = 3 IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model.

both receptors. Not unexpectedly, the conservative substitutions of alanine or valine led to analogues (compounds **23** and **24**) that maintained full binding affinity for both receptors. However, it was somewhat surprising to obtain a greater than 10-fold loss of binding affinity from the incorporation of cyclohexylalanine (compound **25**) in position 19. This indicates

Table 4. Position 18 Analogues of Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]

compd	IC_{50}, μM ET_A[a]	ET_B[b]	AAR_A[c]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp.2Na[d]	0.040[e]	0.060[e]	0.070[f]
14, Ac-DDip-Leu-Glu-Ile-Ile-Trp	0.021	0.019	0.43
15, Ac-DDip-Leu-Lys-Ile-Ile-Trp	0.48	0.033	0.84
16, Ac-DDip-Leu-Phe-Ile-Ile-Trp	0.58[g]	0.060[g]	1.8[f]
17, Ac-DDip-Leu-Tyr-Ile-Ile-Trp	0.50	0.080	*h*
18, Ac-DDip-Leu-Ala-Ile-Ile-Trp	0.035	0.016	0.070

[a] Binding data in rabbit renal vascular smooth muscle cells. [b] Binding data in rat cerebellar membranes. [c] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [d] PD 142893; see refs 46 and 48. [e] $n = 5$ IC_{50} determinations. [f] $n = 2$ IC_{50} determinations. [g] $n = 3$ IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model. [h] Not tested.

Table 5. Position 19 Analogues of Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]

compd[a]	IC_{50}, μM ET_A[b]	ET_B[c]	AAR_A[d]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[e]	0.040[f]	0.060[f]	0.070[g]
19, Ac-DDip-Leu-Asp-Glu-Ile-Trp	1.0	6.0	0.45
20, Ac-DDip-Leu-Asp-Lys-Ile-Trp	>10	3.7	>10[f]
21, Ac-DDip-Leu-Asp-Phe-Ile-Trp	0.20[f]	0.056[f]	0.16[f]
22, Ac-DDip-Leu-Asp-Tyr-Ile-Trp	0.068	0.012	h
23, Ac-DDip-Leu-Asp-Ala-Ile-Trp	0.10	0.33	0.42
24, Ac-DDip-Leu-Asp-Val-Ile-Trp	0.017[g]	0.051[g]	0.029[g]
25, Ac-DDip-Leu-Asp-Cha-Ile-Trp	0.65	0.21	0.20[g]

[a] Cha = cyclohexylalanine. [b] Binding data in rabbit renal vascular smooth muscle cells. [c] Binding data in rat cerebellar membranes. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [e] PD 142893, see refs 46 and 48. [f] $n = 5$ IC_{50} determinations. [g] $n = 2$ IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model. [h] Not tested.

Table 6. Position 20 Analogues of Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]

compd	IC_{50}, μM ET_A[a]	ET_B[b]	AAR_A[c]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[d]	0.040[e]	0.060[e]	0.070[f]
26, Ac-DDip-Leu-Asp-Ile-Glu-Trp	>10	>10	*g*
27, Ac-DDip-Leu-Asp-Ile-Lys-Trp	>10	4.7[f]	>10
28, Ac-DDip-Leu-Asp-Ile-Phe-Trp	4.0	7.5	>10
29, Ac-DDip-Leu-Asp-Ile-Val-Trp	0.14[f]	1.4[f]	*g*
30, Ac-DDip-Leu-Asp-Ile-Leu-Trp	0.23	0.73	0.34[f]

[a] Binding data in rabbit renal vascular smooth muscle cells. [b] Binding data in rat cerebellar membranes. [c] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [d] PD 142893; see refs 46 and 48. [e] $n = 5$ IC_{50} determinations. [f] $n = 2$ IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model. [g] Not tested.

that Ile[19] is binding in a hydrophobic pocket that has very specific and rigid steric constraints.

Position 20 Modifications (Table 6). This position is even more sensitive to modification than position 19. Similar results were obtained from the DPhe[16]-containing hexapeptide antagonist series.[46] Acidic (compound **26**), basic (compound **27**), and aromatic (compound **28** (perhaps due to the potential for hydrophobic collapse

Table 7. Position 21 Analogues of Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]

compd[a]	IC_{50}, μM ET_A[b]	ET_B[c]	AAR_A[d]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[e]	0.040[f]	0.060[f]	0.070[g]
31, Ac-DDip-Leu-Asp-Ile-Ile-Phe	5.0	6.8	>10
32, Ac-DDip-Leu-Asp-Ile-Ile-Tyr	2.2[g]	6.1[g]	4.3
33, Ac-DDip-Leu-Asp-Ile-Ile-Pal	>10	>10	*h*
34, Ac-DDip-Leu-Asp-Ile-Ile-Trp-NH_2	0.32	5.0	6.0

[a] Pal = 3-pyridinylalanine. [b] Binding data in rabbit renal vascular smooth muscle cells. [c] Binding data in rat cerebellar membranes. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [e] PD 142893; see refs 46 and 48. [f] n = 5 IC_{50} determinations. [g] n = 2 IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model. [h] Not tested.

Table 8. Cyclic/Constrained Analogues of His[16]-Leu-Asp-Ile-Ile-Trp[21] (ET_{16-21})

compd	IC_{50}, μM ET_A[a]	ET_B[b]	AAR_A[c]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[d]	0.040[g]	0.060[g]	0.070[h]
35, cyclo[-DPhe-Leu-Asp-Ile-Ile-Trp-][e]	10	>10	*i*
36, cyclo[-DDip-Leu-Asp-Ile-Ile-Trp-][e]	7.1	1.6	*i*
37, cyclo[-DBhg-Leu-Asp-Ile-Ile-Trp-][e]	>10	>10	*i*
38, Ac-DDip-cyclo[Cys-Asp-Ile-Cys]-Trp[f]	>1	>1	*i*

[a] Binding data in rabbit renal vascular smooth muscle cells. [b] binding data in rat cerebellar membranes. [c] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [d] PD 142893; see refs 46 and 48. [e] Cyclized head-to-tail. [f] Cyclic Cys–Cys disulfide. [g] n = 5 IC_{50} determinations. [h] n = 2 IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model. [i] Not tested.

between Phe[20] and Trp[21])[61] substitutions were not well tolerated in this position. Even the most conservative substitutions of valine (compound **29**) and leucine (compound **30**) for Ile[20] resulted in moderate to large losses of binding affinity at both receptor subtypes (4–20-fold).

Position 21 and C-Terminal Modifications (Table 7). The requirement of a C-terminal tryptophan with a free carboxylate in full length monocyclic, bicyclic, and linear analogues (for recent reviews, see refs 10, 11, and 37), as well as in truncated analogues, for high receptor affinity has been well documented.[44–48,57,58] Likewise, in this series the substitution of other aromatic amino acids (Phe, Tyr, or Pal, (Pal = 3-pyridinylalanine)) for Trp[21] led to significant losses (~50-fold or more) of receptor binding affinity at both receptors (compounds **31–33**). Unexpectedly, the C-terminal carboxamide of PD 142893 (compound **34**) retained reasonable affinity for the ET_A receptor (only 10-fold less than PD 142893, itself); however, this compound had almost 100-fold less affinity for the rat ET_B receptor than PD 142893. Previous studies on the interaction of the C-terminal hexapeptide analogues with the rat ET_B receptor subtype suggested that the ET_B receptor was more tolerant than the ET_A receptor to ligand modification. Therefore, compound **34** maybe useful for designing ET_A receptor selective ligands from the C-terminal hexapeptide template.

Cyclic/Constrained Analogues (Table 8). The use of constrained small peptides that lead to analogues with either enhanced receptor affinity, selectivity, and/or metabolic stability has been well documented.[25,26,62] In this regard, analysis of potent constrained peptides by high-field proton NMR spectroscopy and molecular modeling can provide insights into the three-dimensional binding pharmacophore and assist in the design of peptidomimetics.[62] As expected, the linear hexapeptide PD 142893 and related analogues proved to be quite flexible in solution without defined secondary structure (unpublished observations). In order to gain insights into the bioactive conformation of PD 142893 and related analogues, several attempts have been made to incorporate specific structural constraints while maintaining high receptor affinity. These included incorporation of β-turn mimics[63] and cyclizations head-to-tail via lactams (compounds **35–37**) or through individual amino acid side chains via disulfides (compound **38**). Unfortunately, all of the cyclic analogues prepared possessed only minimal receptor binding affinity, and the biophysical structural evaluation was not pursued.

In a related study, selective truncations of the C-terminal hexapeptide were performed in order to generate smaller potent analogues for peptidomimetic design strategies. In particular, individual amino acids or groups of amino acids were deleted, all of which led to compounds with minimal receptor affinity (data not shown). These results were similar to those previously reported for the DPhe[16]-containing series.[48]

Modifications of the N-Terminus (Table 9). It has been previously shown that the N-terminal acetyl group is critical for the high binding affinity of several of the C-terminal hexapeptide analogues.[44,46,48,57,58] This trend is also observed in the PD 142893 series (compound **39**). Replacement of the acetyl group with a free carboxylate via condensation of the free amine with succinic anhydride led to an analogue (compound **45**) that was devoid of binding affinity at either receptor subtype. Several substitutions for the acetyl group were carried out, all of which led to a loss of binding affinity for the ET_A receptor. Even the subtle addition of a methylene group (compound **40**) or the replacement of the acetyl group with a *tert*-butyl (compound **41**) or trifluoroacetyl (compound **42**) group led to a significant loss of binding affinity at the ET_A subtype. The effects on binding to the rat ET_B receptor with most of the compounds were not as substantial, providing further evidence that the rat ET_B receptor is not as sensitive as the ET_A receptor to ligand modification. In fact, compounds **46** and **50** maintained significant binding affinity at the rat ET_B receptor suggesting that these peptides may provide starting points for the development of ET_B receptor selective C-terminal hexapeptide antagonists. However, it has been previously reported that the rat and human ET_B receptors can have dramatically differing binding selectivities for the C-terminal hexapeptide antagonists.[21,22] In fact, compound **40** (PD 147452) had almost 150-fold less affinity for the human cloned ET_B receptor (Table 10).[22] All of the N-terminally modified analogues were evaluated for their ability to block SRTX-6c-stimulated arachidonic acid release (AAR_B) from confluent monolayers of cultured CHO cells expressing recombinant rat ET_B receptors. An exact correlation between the ET_B binding and functional activity was not always observed, and this may be related to the different tissues used.

Table 9. N-Terminally Modified Analogues of Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]

compd[a]	IC_{50}, μM				
	ET_A[b]	ET_B[c]	AAR_A[d]	AAR_B[e]	ET_B/ET_A[f]
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[g]	0.040[h]	0.060[h]	0.070[i]	0.020	0.67
39, DDip-Leu-Asp-Ile-Ile-Trp	2.1	1.9	1.9	9.2	0.90
40, Prl-DDip-Leu-Asp-Ile-Ile-Trp	1.0[j]	0.26[j]	0.23	0.22	3.8
41, Tbu-DDip-Leu-Asp-Ile-Ile-Trp	10	0.40[i]	*l*	0.89	25
42, Tfa-DDip-Leu-Asp-Ile-Ile-Trp	0.25	0.60[i]	1.7	0.29	0.42
43, Msl-DDip-Leu-Asp-Ile-Ile-Trp	0.54	0.26	2.1	1.3	2.1
44, Mal-DDip-Leu-Asp-Ile-Ile-Trp	0.85[k]	0.14[k]	0.28[i]	0.020	6.1
45, Suc-DDip-Leu-Asp-Ile-Ile-Trp	>10	>10	*l*	*l*	*m*
46, Bzl-DDip-Leu-Asp-Ile-Ile-Trp	6.3[i]	0.030[i]	*l*	0.29	210
47, Pyl-DDip-Leu-Asp-Ile-Ile-Trp	5.0	0.19	*l*	2.5	26.3
48, Aal-DDip-Leu-Asp-Ile-Ile-Trp	3.5[i]	0.63[i]	*l*	4.0	5.6
49, Chl-DDip-Leu-Asp-Ile-Ile-Trp	6.2[i]	0.35[i]	*l*	0.56	17.8
50, Cal-DDip-Leu-Asp-Ile-Ile-Trp	2.0[i]	0.068[i]	*l*	0.21	29.4
51, Dpl-DDip-Leu-Asp-Ile-Ile-Trp	2.6[i]	1.3[i]	*l*	9.3	2.0
52, Dsl-DDip-Leu-Asp-Ile-Ile-Trp	3.6	0.39	*l*	3.6	9.2

[a] Aal = 1-adamantylacetyl, Bzl = benzoyl, Cal = cyclohexylacetamidyl, Chl = cyclohexylcarbonyl, Dpl = diphenylacetyl, Dsl = dibenzylsuberylacetyl, Mal = methylacetamidyl, Msl = methylsulfonyl, Prl = propionyl, Pyl = 3-pyridinylacetyl, Suc = succinyl, Tbu = *tert*-butylacetyl, and Tfa = trifluoroacetyl. [b] Binding data in rabbit renal vascular smooth muscle cells. [c] Binding data in rat cerebellar membranes. [d] Inhibition of ET-1-stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells. [e] Inhibition of ET-1-stimulated arachidonic acid release in CHO cells expressing recombinant rat ET_B receptors. [f] Relative ratio of binding affinities (IC_{50}s) to the ET_A and ET_B receptors. [g] PD 142893; see refs 46 and 48. [h] $n = 5$ IC_{50} determinations. [i] $n = 2$ IC_{50} determinations. [j] $n = 4$ IC_{50} determinations. [k] $n = 3$ IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model. [l] Not tested. [m] Not determined.

Table 10. Binding Affinities of Selected Analogues to the Cloned Human ET_B Receptor

compd	IC_{50}, μM		ET_B vs hET_B[c]
	ET_B[a]	hET_B[b]	
2, Ac-DDip-Leu-Asp-Ile-Ile-Trp·2Na[d]	0.060[h]	0.96[i]	16
3, Ac-DBhg-Leu-Asp-Ile-Ile-Trp·2Na[e]	0.015[h]	0.21	14
11, Ac-DDip-Arg-Asp-Ile-Ile-Trp	0.010	0.59	59
14, Ac-DDip-Leu-Glu-Ile-Ile-Trp	0.019	1.8	95
15, Ac-DDip-Leu-Lys-Ile-Ile-Trp	0.033	>10	>300
16, Ac-DDip-Leu-Phe-Ile-Ile-Trp	0.060[j]	>1.6	17
21, Ac-DDip-Leu-Asp-Phe-Ile-Trp	0.056[h]	0.90[i]	16
46, Bzl-DDip-Leu-Asp-Ile-Ile-Trp[f]	0.030[i]	4.4[k]	147
52, Dsl-DDip-Leu-Asp-Ile-Ile-Trp	0.39	5.9	15
53, Ac-DPhe-Leu-Phe-Ile-Ile-Trp[g]	0.036[j]	0.037[i]	1.0

[a] Binding data in rat cerebellar membranes. [b] Binding data in CHO cells stably transfected with the human ET_B receptor. [c] Relative ratio of the binding affinities (IC_{50}s) for the rat and human ET_B receptors. [d] PD 142893; see refs 46 and 48. [e] PD 145065; see refs 57 and 58. [f] PD 147452; see refs 21 and 22. [g] See ref 46. [h] $n = 5$ IC_{50} determinations. [i] $n = 2$ IC_{50} determinations. [j] n = 3 IC_{50} determinations. [k] $n = 4$ IC_{50} determinations. All other values represent one IC_{50} determination. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data were computer-analyzed by nonlinear least squares analysis giving the best fit for a one-site model.

Binding Affinity of Selected Analogues for the Cloned Human ET_B Receptor (Table 10). As previously discussed, recent pharmacological evaluations have suggested the existence of multiple, species dependent ET_B receptor subtypes.[20-22] Therefore, we compared a selected number of the most potent and/or selective C-terminal hexapeptide antagonist analogues at the rat ET_B cerebellar receptor and the cloned human ET_B receptor stably expressed in CHO cells (hET_B). In Table 10, it can be clearly seen that PD 142893 (compound **2**) and PD 145065 (compound **3**) have distinctly less affinity for the human ET_B receptor than for the rat ET_B receptor (16- and 14-fold, respectively). Likewise, all the other analogues of this series examined at the cloned human ET_B receptor (compounds **11**, **14**–**16**, **21**, **46**, and **52**) displayed a loss of binding affinity (15-fold to greater than 300-fold).[22] Interestingly, the DPhe[16]-containing analogue of compound **16** (compound **53**)[46] was shown to maintain high affinity for the human cloned receptor as well as for the rat receptor. In fact, compound **78** is the only C-terminal hexapeptide that has been identified to maintain equal, nanomolar affinity for both the rat and human cloned ET_B receptors. These results further support the proposal for species-differentiated ET_B receptor subtypes.

***In Vitro* Contractility Studies.** *In vitro* contractility data for several of these analogues have been previously reported.[46,48,57-59] A close correlation was observed between the ability of these antagonists to inhibit ET-1-stimulated arachidonic acid release and the ability to block ET-1-induced vasoconstriction in the rabbit pulmonary artery (ET_A). In general, a slightly enhanced pA_2 value has been reported for the same compound in its ability to block SRTX-6c-stimulated vasoconstriction (ET_B). For the new compounds reported in this manuscript and tested for activity in *in vitro* contractility, a similar trend has been observed (data not shown).

Conclusions

Compound **2** (PD 142893) is a potent combined ET_A/ET_B receptor antagonist (40 and 60 nM, respectively) with limited tolerance for modification, particularly for ET_A receptor binding. For example, position 17 (Leu) can incorporate acidic and basic residues while maintaining high affinity for both receptor subtypes. Position 18 only tolerates acidic and aliphatic substitutions without loss of ET_A receptor affinity; however, the rat ET_B receptor allowed all modifications (acidic, basic, aromatic, and aliphatic). Thus compounds **15** (Ac-DDip[16]-Leu-Lys-Ile-Ile-Trp[21]), **16** (Ac-DDip[16]-Leu-Phe-Ile-Ile-Trp[21]), and **17** (Ac-DDip[16]-Leu-Tyr-Ile-Ile-Trp[21]) are rat ET_B selective ligands; however, compound **15** did not bind to the human ET_B receptor at concentrations of up to 10 μM. Only conservative (aliphatic and some aromatic) substitutions are allowed in positions 19 and 20, while the C-terminal tryptophan could not be modified. Surprisingly, converting the C-terminal carboxylate to a carboxamide maintained significant

ET_A receptor affinity (Ac-DDip[16]-Leu-Asp-Ile-Ile-Trp[21]-NH_2, compound **34**). The N-terminal acetyl group is critical for ET_A receptor recognition, such that even subtle modifications (compounds **40** and **42**) led to losses of receptor affinity. However, some of the N-terminal-modified peptides (compounds **46** and **50**) maintained high-affinity binding for the rat ET_B receptor.

Thus, position 17 may provide a handle for modification of the physicochemical properties of PD 142893, such as solubility and transport, while maintaining high receptor affinity. In fact, compound **11** (Ac-DDip[16]-Arg-Asp-Ile-Ile-Trp[21]) is the most potent analogue in the PD 142893 series at both receptor subtypes (ET_A = 4.0 nM; ET_B = 10 nM). Position 18 (compounds **15**–**17**) and the N-terminus (compounds **41, 46, 47**, and **50**) are sites where selective modifications can lead to ET_B selective antagonists. However, compound **53**[46] (Ac-DPhe[16]-Leu-Phe-Ile-Ile-Trp[21]) is the first report of a C-terminal hexapeptide that has been identified that maintains high affinity for both the rat and human cloned ET_B receptors (36 and 37 nM, respectively). Interestingly, the corresponding analogue (compound **16**) in the PD 142893 series did not maintain this binding profile to the species differentiated receptors. Overall, the SAR in the N-terminal portion of the molecule displayed a larger structural and functional diversity than the very tight SAR at the C-terminus. In addition the ET_A receptor appears to be much more sensitive to modifications than the ET_B receptor, although this will require further evaluation with the continued evaluation of species and tissue ET_B receptor subtypes.[20-22]

Experimental Section

Materials and Methods. Orthogonally protected N^{α}-*t*-Boc amino acids, N^{α}-Fmoc amino acids, N^{α}-*t*-Boc-Trp-PAM resin, and Wang (HMP) resin were purchased from either Advanced Chemtech, Applied Biosystems Inc., Bachem California, Bachem Bioscience, Novabiochem, Peninsula Laboratories, Inc., or Synthetech Inc. The unnatural N^{α}-*t*-Boc and N^{α}-Fmoc amino acids, Ada (1-adamantylalanine), Bhg (10,11-dihydro-5*H*-dibenzo[*a*,*d*]cycloheptenylglycine), Cha (cyclohexylalanine), Dip (3,3-diphenylalanine), Pal (3-pyridinylalanine), Pmp (pentamethylphenylalanine), and Trt (tritylglycine) were purchased from commercial sources or prepared by previously reported synthetic methods.[51-53] All amino acids were of the L-configuration unless otherwise noted.

TFA was purchased from Halocarbon. *N*,*N*'-Dicyclohexylcarbodiimide (DCC), DIEA, and HOBt were purchased from Applied Biosystems Inc. (ABI). *N*,*N*-Dimethylformamide (DMF), DCM, and NMP were purchased from Burdick & Jackson and were of reagent grade or better. HPLC grade solvents (AcCN and water) were obtained from Burdick and Jackson, EM Science, or Mallinckrondt. HF was purchased from Matheson Gas Products. Indole, 3-methylindole, *p*-cresol, anisole, 1,2-ethanedithiol, thiophenol, and DMAP were purchased from Aldrich Chemical Co., Inc. Tris(hydroxymethyl)aminomethane (Trizma), ethylenediaminetetraacetate (EDTA) and *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma Chemical Co. Phenylmethanesulfonyl fluoride (PMSF) and bacitracin were purchased from Boehringer Mannheim Biochemicals. Bovine serum albumin (BSA) was purchased from Miles Inc., Diagnostics Division. [^{125}I]ET-1 (2000 Ci/mmol) was purchased from New England Nuclear, DuPont, and [^{3}H]arachidonic acid (218 Ci/mmol) was purchased from Amersham.

The peptides were prepared on an ABI Model 430A or 431A peptide synthesizer using software versions 1.40 (Boc) or 1.00 (Fmoc). For Boc syntheses, the HF cleavages were performed on an Immuno-Dynamics Inc. Model 2A/2B HF apparatus. High-pressure liquid chromatographs were obtained on a Waters HPLC system from Millipore Corp. equipped with a Model 600E system controller, a Model 600 solvent delivery system, a Model 490 variable wavelength detector operating at 214 and 280 nm, and a Bio-Rad Laboratories Model AS-100 autosampler. Vydac analytical and preparative C18 HPLC columns were purchased from The Nest Group. Preparative reversed-phase HPLC was performed using a C18 preparative scale Vydac column (218TP1022) (2.2 × 25.0 cm, 10–20 μM particle size) eluting with a linear gradient of 0.1% aqueous TFA with increasing concentrations of AcCN at 13–15 mL/min. Analytical reversed-phase HPLC analysis was carried out on a Vydac column (218TP54) (0.46 × 25.0 cm, 5 μM particle size). The analytical HPLC system used was the same as that described in detail above for peptide purification. Two different conditions were utilized for analytical HPLC analysis: (a) 80:20 to 14:86 0.1% aqueous TFA:0.1% TFA in AcCN, linear gradient over 22 min at 1.5 mL/min (λ = 214 and 280 nm) on a Vydac 218TP54 column, or (b) 90:10 to 24:76 0.1% aqueous TFA:0.1% TFA in AcCN, linear gradient over 22 min at 1.5 mL/min (λ = 214 and 280 nm) on a Vydac 218TP54 column.

Capillary electrophoreses were performed on an ABI Model 270A instrument using a 20 mM (cyclohexylamino)propanesulfonic acid buffer (CAPS, pH 11.0) operating at 10 000 V (140 V/cm) on a 72 cm silica capillary observing at 220 or 280 nm. Amino acid analyses were performed using an ABI automated derivatizer/analyzer (Model 420A) with a two-component aqueous TFA/AcCN buffer system. Automated hydrolysis was carried out with 6 N hydrochloric acid at 155–160 °C for 75 min. Phenyl isothiocyanate derivatization in the presence of DIEA was carried out followed by separation of the derivatized amino acids using an ABI Model 130A separation system. Data analysis was performed on an ABI Model 610A system (data analysis program version 1.2).

Fast atom bombardment mass spectra were measured with a VG analytical 7070E/HF mass spectrometer in either a thioglycerol or 3-nitrobenzyl alcohol matrix using xenon as the target gas. Electrospray mass spectra were obtained on either a Finnigan TSQ70 or Fisons VG Trio 2000 quadrupole mass spectrometer using 50:50 water:methanol made 1% in acetic acid as the solvent. Proton NMR spectra were measured with a Varian XL 200 or Brüker AM 250 instrument using tetramethylsilane as an external standard in dimethyl sulfoxide (DMSO-d_6; Cambridge Isotope Laboratories).

Peptide Synthesis. All of the peptides were synthesized by solid-phase peptide synthetic techniques[49,50] on an ABI Model 430A or 431A peptide synthesizer. The peptide analogues were prepared using one of the following two methods. (a) An N^{α}-*t*-Boc protection scheme with N^{α}-*t*-Boc-Trp-PAM resin—the amino acid side chains were protected as follows: benzyl (Asp, Glu), 4-methylbenzyl (Cys), [(2-bromobenzyl)oxy]carbonyl (Tyr), [(2-chlorobenzyl)oxy]carbonyl (Lys), and tosyl (Arg); N^{α}-*t*-Boc amino acids were coupled via DCC in DMF; N-terminal acetylation was carried out on the resin in DCM with an excess of 1-acetylimidazole (20-fold) or 10% acetic anhydride in DCM with a catalytic amount of DMAP. The N-terminal modifications (Table 9) were performed on the resin using the corresponding symmetrical anhydride, HOBt-activated ester, isocyanate, or acid chloride. (All N-terminal modifications were synthesized using N^{α}-*t*-Boc chemistry.) The peptides were then deprotected and cleaved from the resin using anhydrous liquid HF/anisole or HF/p-cresol (9:1, v/v) in the presence of 3-methylindole (100 mg/g of resin). (b) An N^{α}-Fmoc protection scheme on a Wang resin—the amino acid side chains were protected as follows: *tert*-butyl (Asp, Glu, Tyr), *t*-Boc (Lys, Orn), and pentamethylchroman-6-sulfonyl (Pmc) (Arg); N^{α}-Fmoc amino acids were coupled via DCC in NMP; N-terminal acetylation was carried out on the resin in DCM with an excess of 1-acetylimidazole (20-fold) or 10% acetic anhydride in DCM with a catalytic amount of DMAP, and the peptides were then deprotected and cleaved from the resin using TFA:thioanisole:phenol:1,2-ethanedithiol:water (89:5:2:3:1). The crude peptides were precipitated with anhydrous diethyl ether and separated from ether-soluble non-

peptide material by filtration. (See ref 46 for the solid-phase synthesis of compound **2**, which is representative of all syntheses.)

Peptide Purification. Crude peptides were dissolved in a mixture of aqueous TFA and acetonitrile (exact ratio depended on the solubility of the peptide) and then purified by preparative reversed-phase HPLC (see above). Peptide fractions determined to be pure by analytical HPLC were combined, concentrated under reduced pressure, and lyophilized.

Peptide Homogeneity and Characterization. Peptides were assessed for homogeneity by analytical reversed-phase HPLC and CE. The peptides were characterized by AAA, FABMS, or ESMS and proton NMR spectroscopy (Table 1).

Endothelin Receptor Binding Assay Protocol. The binding assay protocol using rat heart ventricle has been reported previously.[44] The ET_A and ET_B binding assays were described previously.[45,46,58,59] Briefly, incubations were performed in 12 × 75 mm poly(propylene) tubes containing 20 mM Trizma·HCl buffer, 2 mM EDTA, 100 μM PMSF, 100 μM bacitracin, 30 pM [^{125}I]ET-1 (2000 Ci/mmol), and 5 μg of rabbit renal artery vascular smooth muscle membranes (ET_A) or 5 μg of rat cerebellar membranes (ET_B) from adult blue laurie rats (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 Trizma, 2 mM EDTA, 1 mg/mL BSA, and 1% DMSO) to 5 btimes 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 initiated by hand agitation. Tubes were then incubated at 37 °C for 2 h. Incubations were terminated by filtration through Whatman GF/B filters which were presoaked with 50 mM Trizma, 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 calculated by weighted nonlinear regression curve fitting to the mass action equation (giving the best fit for a one-site model).[64]

Endothelin Receptor Binding Assay Protocol (Cloned human ET_B receptor).[22] Rat brain ET_B receptor cDNA was obtained as previously described.[65] A human placenta cDNA library was constructed in bacteriophage, and plaque hybridization was carried out for 16 h at 42 °C. The membranes were then washed twice for 30 min each and a final wash was done at 55 °C. The positive clones were purified and subcloned. DNA sequencing was performed by the dideoxynucleotide chain termination method and identified as human ET_B receptor (hETBR) by reading both DNA strands. The restriction fragment of clone 11 of the hETBR was inserted into the eucaryotic expression vector pRcCMV (pRcCMV-hETBR). CHO-K1 cells were transfected by electroporation at 300 V, 800 μF, low ohms for 1 s. Cell populations expressing hETBR were selected with G418 (0.5 mg/mL), and from these selected cell populations, clonal cell lines were isolated by single-cell cloning. Expression levels of hETBR were determined by the receptor binding assay described below using [^{125}I]ET-1 as the radioligand. CHO-K1 cells were grown in DME/F12 (1:1) supplemented with 10% FCS and G418 (0.5 mg/mL). Membranes were prepared from confluent transfected CHO-K1 cells by lysing cells and homogenizing and centrifuging the homogenate at 30000*g* for 20 min at 4 °C. Cell pellets were resuspended in cold binding buffer and frozen at −80 °C until use. Membranes were thawed and homogenized and then diluted in tissue buffer. Radioligand and competing ligands were prepared in drug buffer containing 0.1% BSA. Competition binding assays were initiated by combining membranes, [^{125}I]ET-1 (30 000 cpm), and competing ligand in a final volume of 250 μL and incubating for 2 h at 37 °C. The assay was terminated by filtration over Whatman GF/B filters, which were presoaked to prevent unbound [^{125}I]ET-1 from binding to the filter. Nonspecific binding is defined as binding in the presence of 100 nM unlabeled ET-3, and specific binding is defined as total binding minus nonspecific binding. Specific binding was analyzed by nonlinear least squares curve fitting.

Endothelin Receptor Binding Assay Protocol (Cloned human ET_A receptor).[66] Human ET_A receptors expressed in mouse Ltk cells were a gift of Dr. M. Yanagisawa and prepared as in ref 66. The binding assay was performed as outlined for the cloned human ET_B receptor, above.

Arachidonic Acid Release (AAR_A and AAR_B) Assay.[45,46,58,59,67] The loading media (LM) used for the [^{3}H]-arachidonic acid assay consisted of Dulbecco's modified Eagles/Ham's nutrient mixture F12 (DME/F12) (1:1) which contained 0.5% fetal calf serum (FCS) and 0.25 μCi/mL [^{3}H]arachidonic acid (218 Ci/mmol). Confluent monolayers of cultured rabbit renal artery vascular smooth muscle cells (AAR_A) or CHO cells expressing recombinant rat ET_B receptors (AAR_B) were incubated in 0.5 mL of the LM over 18 h, at 37 °C, in 5% CO_2. The LM was aspirated, and the cells were washed once with the assay buffer (Hank's balanced salt solution, 10 mM HEPES buffer, and fatty acid-free BSA (1 mg/mL)) and incubated for 5 min with 1 mL of the prewarmed assay buffer. This solution was aspirated followed by addition of 1 mL of prewarmed assay buffer and further incubated for another 5 min. A final 5 min 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−1 μM) and 10 mL of ET-1 (0.3 nM) or SRTX-6c (1 nM), and the incubation was extended for 30 min. 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.

***In Vitro* Contractility Studies.**[19,46,58,59] Vascular rings of rat femoral artery (ET_A) or rabbit pulmonary artery (ET_B) were placed in 37 °C maintained organ baths containing oxygenated Krebs bicarbonate solution and continuously gassed with 5% CO_2 in oxygen. Peptides were added to the endothelium-intact preparations. Vascular contractile responses were measured isometrically. The ET-1 (ET_A) or SRTX-6c (ET_B) response in the presence of novel compounds was expressed as a percent of the ET-1 or SRTX-6c response in the absence of compounds.

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