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Structure–Activity Studies Of Endothelin Leading To Novel Peptide ET_A Antagonists

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Abstract–With the goal of producing receptor antagonists, numerous monocyclic and bicyclic endothelin analogs were prepared and tested for vasoconstrictor activity, receptor affinity and functional antagonist activity. Bis-penicillamine endothelin analogs containing Ala or Asn at position 18 were functional antagonists, with K_i values of 20–40 nM but K_B values of about 1 μM (e.g., [$Pen^{1,11}$, Nle^7, Ala^{18}]-endothelin-1, K_i = 42 nM, K_B = 1.2 μM). While these peptides are antagonists at the ET_A receptor, they appear to be at least partial agonists at another receptor subtype.

Introduction

In 1988, Yanagisawa *et al.* reported the isolation of the potent vasoconstrictor peptide endothelin (ET) from the supernatant of cultured bovine aortic endothelial cells.[1] Since that time, there has been an explosion of information about this novel peptide (for a recent review, see Ref. 2). Endothelin has been shown to comprise a family of mammalian peptides as well as a family of related snake venom peptides, the sarafotoxins.[3] Although originally isolated from endothelial cells, endothelin is produced by a number of other cell types, including kidney cells.[4] Two endothelin receptor subtypes, ET_A and ET_B, have been cloned and expressed.[5,6] The ET_A subtype, which is selective for ET-1 over ET-3, appears to be the predominant vascular smooth muscle receptor while the isopeptide non-selective ET_B receptor appears to mediate either vasodilation or vasoconstriction, depending upon the tissue type.[7,8] A large body of research has attempted to define the role of endothelin in human disease. Although studies have implicated endothelin in the pathophysiology of numerous diseases, proof that endothelin is a causative agent has remained elusive. The discovery of endothelin receptor antagonists will surely remedy this situation.

Two important strategies which have been employed to aid the discovery of endothelin receptor antagonists are random screening of non-peptides and modifications of the native peptide. Random screening of natural products and synthetic compounds, though inherently serendipitous, has already uncovered several interesting antagonists. Cyclic pentapeptide antagonists selective for the ET_A receptor were discovered by two groups,[9,10] and synthetic modifications of these provided the potent antagonist BQ-123.[11] Acyltripeptide antagonists, presumably derived by synthetic modifications of the cyclic pentapeptides, were recently reported by these same groups.[12] Other endothelin antagonists which have been discovered by random screening include a family of anthraquinone derivatives,[13] myriceron caffeoyl ester,[14] asterric acid[15] and a group of cyclic depsipeptides.[16]

Although a significant amount of work has addressed the structure–activity relationships of the endothelin peptide family (reviewed in Ref. 2), there have been few reports of the production of peptide antagonists. A bicyclic endothelin-1 analog in which the Cys^1–Cys^{15} disulfide bond was replaced by an amide formed from the α-amine of a (*S*)-diaminopropionic acid residue at position 1 and the side chain carboxyl of Asp^{15} showed antagonist properties in isolated perfused guinea pig lung.[17] An analog of the hexapeptide tail of endothelin, Ac-D-diphenylalanine–Leu–Asp–Ile–Ile–Trp, was reported to be a non-selective antagonist of endothelin at both the ET_A and ET_B receptors.[18]

We have reported studies which explored the structure-activity relationships of both monocyclic and bicyclic endothelin analogs at the ET_A receptor subtype. An alanine scan of endothelin using the $Ala^{3,11}$ monocyclic framework pinpointed Glu^{10}, Phe^{14}, Leu^{17} and Asp^{18} as key residues involved in receptor activation.[19] Asp^8, Tyr^{13}, Ile^{20} and Trp^{21} were identified as residues important to receptor binding while residues Ser^2, Ser^4, Ser^5, Leu^6, Met^7, Val^{12}, His^{16} and Ile^{19} were relatively tolerant to substitution. We have also reported the preparation of bicyclic endothelin analogs containing two cysteines and two penicillamines.[20] These analogs possessed a range of activities, from potent full agonists to partial agonists, depending on the positions of the penicillamines and the disulfide patterns. In the present report, we describe extensive structure–activity studies of endothelin, using both monocyclic and bicyclic frameworks. In particular, we have discovered that residue replacement in peptides containing bis-penicillamine frameworks produced novel ET_A receptor antagonists.

Results

Receptor binding affinity and vasoconstrictor activity of monocyclic endothelin analogs

We have described the functional activity in rabbit carotid artery rings and the receptor binding affinity in rat A10 vascular smooth muscle (vsm) cell membranes of $Ala^{3,11}$ monocyclic endothelin analogs.[19] In the present study, the synthetically simple monocyclic framework was also used to study some of the other structure–activity relationships of endothelin in these assay systems which exclusively contain the ET_A receptor subtype.

Acetylation of the α-amine of endothelin has been shown to greatly diminish receptor binding.[19] While this effect could have been due to increased steric hindrance at the alpha-amine, the poor affinity shown by the mercaptopropionic (Mpr) acid analog suggests that the N-terminal ammonium ion provides an important binding interaction (Table I; for comparison purposes, several analogs reported in Ref. 19 are included). The moderate binding affinity and functional activity of the D-Cys^1 analog indicates that this binding interaction is stereospecific. This analog was also one of several partial agonists discovered in this study (see Tables 1 and 3 for other partial agonists). The monocyclic endothelin analog containing -$(Ala)_4$- in place of -Ser^4–Ser^5–Leu^6–Met^7- has been shown to be a potent agonist, suggesting that the exact nature of these turn residues is not critical.[19] The poor activity of the des-Ser^5,Leu^6 peptide indicates however that the length of the chain between the Cys^3–Cys^{11} crosslink is important.

Table 1. Agonist potency (rabbit carotid artery rings) and receptor affinity (A10 vsm cell membranes) of ET-1 and monosubstituted monocyclic analogs

Analog of [$Ala^{3,11}$,Nle^7]-ET-1	EC_{50} (nM)	% of ET-1 contraction (Conc. analog)	K_i (nM)	Slope factor
ET-1	0.32 ± 0.03		0.12 ± 0.03	0.9 ± 0.03
ET-1 ($Ala^{3,11}$,Nle^7)*	3.3 ± 0.9		6.6 ± 2.8	0.5 ± 0.01
Mpr^1 (for Cys)		15 ± 5% (10 μM)	19,200 ± 4,800	0.4 ± 0.15
D-Cys^1 (for Cys)	250 ± 50§		108 ± 42	0.7 ± 0.02
des-(Ser^5, Leu^6)		25 ± 6 % (1 μM)	1440 ± 110	1.0 ± 0.04
$chAla^{17}$ (for Leu)	14 ± 3		25 ± 11	0.7 ± 0.05
Phe^{17} (for Leu)		17 ± 9 % (10 μM)	603 ± 217	1.0 ± 0.03
Ala^{18} (for Asp)*		0 (1 μM)	61 ± 20	0.7 ± 0.06
Arg^{18} (for Asp)		0 (10 μM)	677 ± 168	0.9 ± 0.05
Asn^{18} (for Asp)		23 ± 11% (10 μM)	217 ± 72	1.1 ± 0.17
Glu^{18} (for Asp)		0 (10 μM)	806 ± 138	1.0 ± 0.17
Leu^{18} (for Asp)		0 (10 μM)	94 ± 12	0.7 ± 0.16
Orn^{18} (for Asp)		0 (10 μM)	477 ± 155	1.1 ± 0.28
Leu^{21} (for Trp)		0 (1 μM)	7,780 ± 3880	0.4 ± 0.06
His^{21} (for Trp)		0 (1 μM)	6,300 ± 1640	0.6 ± 0.13
Gly^{22}		0 (1 μM)	405 ± 45	0.6 ± 0.01
D-Glu^{10} (for Glu)	460 ± 50§		111 ± 18	0.7 ± 0.05
D-Phe^{14} (for Phe)	330 ± 48		1910 ± 600	0.7 ± 0.2
D-Cys^{15} (for Cys)	73 ± 6		94 ± 8.5	0.8 ± 0.03

* Reference 19.
§ Partial agonist.

Leu^{17} has been identified as a residue which contributes to receptor activation.[19] This site appears to require an aliphatic residue, as shown by the poor activity of the Phe^{17} analog and the high vasoconstrictor activity of the cyclohexylalanine^{17} analog.

Of the alanine substituted monocycles which were previously prepared, the Ala^{18} analog displayed the highest receptor affinity with minimal agonist activity.[19] In order to further explore this high affinity analog, we prepared analogs with alternate residues at this position as well as several disubstituted monocyclic endothelin analogs containing Ala^{18} (Table 2). Surprisingly, the Glu^{18} analog showed poor affinity and no vasoconstrictor activity. The Arg^{18} and Orn^{18} analogs, which carry an opposite charge to the natural Asp residue, displayed moderate affinity and no vasoconstrictor activity. The isosteric Asn^{18} analog displayed moderate affinity and was a weak agonist while the analog containing the isosteric hydrophobic Leu^{18} substitution displayed moderate affinity but no vasoconstrictor activity. With the aim of improving binding affinity and eliminating the partial agonism of the bicyclic Ala^{18} analog (*vide infra*) we used simultaneous multiple peptide synthesis[21] to prepare disubstituted monocyclic peptides. However, substitution of Asn, Leu, Lys or Phe at position 8, substitution of Arg, Gln, His, Leu, Lys, Phe or Ser at position 10, substitution of Asp, Leu, Lys or Ser at position 14, and substitution of cyclohexylalanine at position 17 of the Ala^{18} monocycle all produced peptides with only moderate affinity and no vasoconstrictor activity. We have shown that in monocyclic endothelin analogs, the helix-inducing residue aminoisobutyric acid (Aib) at position 11 provides high affinity agonists.[22] The Aib^{11}, Ala^{18} analog had the highest affinity among monocyclic analogs which displayed no vasoconstrictor activity.

Table 2. Receptor affinity (A10 vsm cell membranes) of disubstituted monocyclic analogs

Analog of [$Ala^{3,11}$,Nle^7]-ET-1	K_i (nM)	Slope factor
Asn^8, Ala^{18}	171 ± 10	0.8 ± 0.06
Leu^8, Ala^{18}	400 ± 124	0.7 ± 0.02
Lys^8, Ala^{18}	341 ± 206	0.7 ± 0.07
Phe^8, Ala^{18}	629 ± 47	0.5 ± 0.05
Arg^{10}, Ala^{18}	668 ± 280	0.7 ± 0.03
Gln^{10}, Ala^{18}	85 ± 13	0.8 ± 0.09
His^{10}, Ala^{18}	662 ± 125	0.8 ± 0.16
Leu^{10}, Ala^{18}	1630 ± 430	0.8 ± 0.17
Lys^{10}, Ala^{18}	350 ± 96	0.8 ± 0.04
Phe^{10}, Ala^{18}	855 ± 247	0.5 ± 0.13
Ser^{10}, Ala^{18}	195 ± 41	1.0 ± 0.01
Aib^{11}, Ala^{18}	12.7 ± 2	0.8 ± 0.06
Asp^{14}, Ala^{18}	676 ± 285	1.1 ± 0.3
Leu^{14}, Ala^{18}	84 ± 43	0.9 ± 0.3
Lys^{14}, Ala^{18}	730 ± 91	0.8 ± 0.03
Ser^{14}, Ala^{18}	553 ± 71	1.0 ± 0.03
chA^{17}, Ala^{18}	308 ± 18	0.7 ± 0.03

None of these compounds at 10μM caused contraction of rabbit carotid artery rings

Table 3. Receptor affinity and agonist and antagonist potency of bicyclic analogs

Analog of [Nle^7]-ET-1	EC_{50} (nM)	% of ET-1 contraction (Conc. Analog)	K_i (nM)	Slope factor	K_{Bapp} or K_B (nM)*
Ala^{18}	4.4 ± 0.4§		2.1 ± 2.0	0.54 ± 0.03	
Pen^{1,11} ¶	7.5 ± 0.5§		4.5 ± 0.5	0.7 ± 0.09	
Pen^{3,15} ¶	0.89 ± 0.22		0.7 ± 0.1	0.6 ± 0.1	
Pen^{1,11}, D-Asp^8		0 (10 μM)	619 ± 136	0.8 ± 0.1	7000 ± 600
Pen^{1,11}, Ala^{10}		0 (10 μM)	63 ± 13	0.8 ± 0.2	9700 ± 7900
Pen^{1,11}, Gln^{10}		0 (10 μM)	150 ± 27	0.9 ± 0.02	>10000
Pen^{1,11}, D-Glu^{10}		0 (10 μM)	114 ± 17	0.7 ± 0.01	8500 ± 6400
Pen^{1,11}, Phe^{10}		0 (10 μM)	93 ± 1	1.1 ± 0.05	
Pen^{1,11}, Ala^{18} (BMS-180403)		0 (10 μM)	42 ± 8	0.8 ± 0.02	1200 ± 300*
Pen^{1,11}, Asn^{18} (BMS-180402)		0 (10 μM)	18 ± 5	0.9 ± 0.1	930 ± 220*
Pen^{1,11}, Leu^{18}		0 (10 μM)	80 ± 1	0.8 ± 0.1	5400 ± 1600
Pen^{3,15}, Asn^8		9 ± 3% (10 μM)	52 ± 20	0.7 ± 0.05	>10000
Pen^{3,15}, Lys^8		19 ± 18% (10 μM)	482 ± 14	0.9 ± 0.15	>10000
Pen^{3,15}, Phe^8		7 ± 3% (10 μM)	244 ± 114	0.8 ± 0.09	>10000
Pen^{3,15}, Gln^{10}	600 ±60		47 ± 6	0.9 ± 0.06	
Pen^{3,15}, D-Glu^{10}		0 (1 μM)	5 ± 4	0.5 ± 0.10	
Pen^{3,15}, Ala^{18}		28 ± 11% (10 μM)	0.9 ± 0.1	0.7 ± 0.01	2000 ± 700
Pen^{3,15}, Asn^{18}		2 ± 1% (10 μM)	0.7 ± 0.1	0.7 ± 0.02	2000 ± 700*
Pen^{3,15}, Leu^{18}		0 (10 μM)	1.8 ± 0.4	0.7 ± 0.03	2000 ± 200

§ Partial agonist.
¶ Reference 20.

Although replacement of Trp^{21} with the aromatic residues Phe or Tyr has been shown to produce a reasonably potent vasoconstrictor peptide,[23] we previously showed that the Ala^{21} monocycle displayed extremely poor receptor affinity.[19] The requirement for an aromatic, hydrophobic residue appears to be quite stringent, in that neither a peptide with the aliphatic hydrophobic Leu^{21} nor the polar aromatic His^{21} displayed significant receptor affinity (Table 1). These results are consistent with a similar study performed with bicyclic endothelin analogs.[24] While maintaining a polar aromatic residue at position 21, a C-terminal extended monocyclic peptide containing Gly at position 22 was prepared and found to be poorly active. This is in agreement with reports that ET-22 is 1000-fold less potent than ET-21.[25,26] Interestingly, these same reports indicated that further elongation to ET-23 improved both binding and vasoconstrictor potency.

In addition to position 1, the effects of side chain stereochemistry at several other sites were probed by the synthesis of D-amino acid containing analogs (Table 1). The D-Glu^{10} monocycle showed moderate affinity and was a partial agonist, producing only 20% of the force of a maximal endothelin induced contraction. The analog with D-Phe^{14} was a moderately potent full agonist. Incorporation of D-Cys for Cys^{15}, the anchor residue for attachment of the C-terminal tail to the bicyclic core, afforded a surprisingly potent agonist.

Receptor binding affinity and vasoconstrictor activity of bicyclic endothelin analogs

Because the Ala^{18} monocycle possessed high receptor affinity but did not cause vasoconstriction, we prepared the bicyclic Ala^{18} analog (Table 3). This peptide was a potent partial agonist, producing about 50% of an endothelin-1 induced contraction with an EC_{50} value of 4.4 nM. We have previously reported the synthesis of [Nle^7]-endothelin analogs which contain 2 cysteine residues and 2 penicillamine residues in the 1–15, 3–11 disulfide pattern (Fig. 1).[20] The $Pen^{1,11}$ analog was a moderately potent partial agonist while the $Pen^{3,15}$ analog was an agonist approximately equipotent to endothelin-1. Combining the features of these two partial agonists, we prepared [$Pen^{1,11}$, Nle^7, Ala^{18}]-endothelin-1, BMS-180403. This analog exhibited moderately high affinity but no vasoconstrictor activity. The [$Pen^{1,11}$, Nle^7, Asn^{18}]-endothelin-1 analog BMS-180402 and the Leu^{18} analog with the $Pen^{1,11}$ framework exhibited similar affinity and also showed no vasoconstrictor activity. The effects of substitution at positions 8 and 10 were also examined in this framework. The D-Asp^8 analog showed no vasoconstrictor activity but only moderate receptor affinity. At position 10, each of the substitutions Ala, Gln, D-Glu and Phe provided analogs with reasonably high affinity and no functional activity.

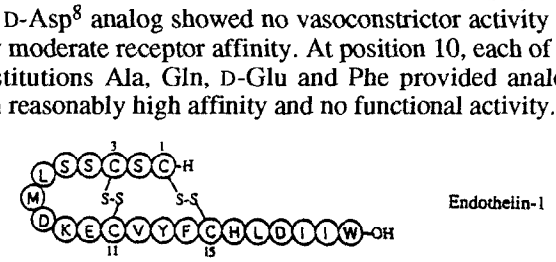

Figure 1 Amino acid sequence and disulfide structure of endothelin-1.

Although the $Pen^{3,15}$ analog of endothelin was a full agonist, the effect of substitution was also investigated in this framework. At position 8, substitution with Asn, Lys or Phe provided compounds with weak to moderate affinity and weak vasoconstrictor activity. At position 10, substitution with Gln provided a moderately potent agonist while substitution with D-Glu produced a high affinity analog which displayed no vasoconstrictor activity. Substitution at position 18 with Ala, Asn or Leu provided analogs of high affinity. Of these compounds, the Ala^{18} and Asn^{18} analogs showed some vasoconstrictor activity at 10 μM while the Leu^{18} analog was devoid of such activity.

Testing of endothelin analogs for functional antagonism

A number of monocyclic and bicyclic endothelin analogs which showed high affinity and little or no vasoconstrictor activity were tested for their ability to antagonize endothelin-induced contractions in rabbit carotid artery rings *in vitro*. Apparent K_B values (K_{Bapp}) were calculated for those analogs which were tested at a single concentration and full K_B values were determined for analogs which were tested at multiple concentrations.

Of the monocyclic analogs from the alanine scan reported earlier,[19] those containing Ala^{10}, Ala^{17}, Ala^{19}, Ala^{20} or Ala^{21} produced non-reproducible shifts in the endothelin concentration–response curve when tested at a single concentration. Similar results were obtained with the monocyclic analogs containing His^{21}, Leu^{21} and Gly^{22}. The Ala^{14}, Ala^{18}, Arg^{18}, Asn^{18}, Leu^{18} and Orn^{18} monocycles produced rightward shifts in the endothelin concentration–response curve when tested at a single concentration. In further studies, however, it was determined that increasing concentrations of the Ala^{14} and Ala^{18} analogs did not cause progressive, parallel rightward shifts in the endothelin concentration–response curve, suggesting that these compounds are not competitive with endothelin. Because of these results, experiments aimed at determining full K_B values for the other monocyclic analogs were not performed.

A number of the bicyclic analogs were also tested at a single concentration as functional antagonists. Analogs with either the $Pen^{1,11}$ pattern or the $Pen^{3,15}$ pattern containing substitutions at position 8 or at position 10 were poor antagonists. However, analogs in either series which contained substitutions at position 18 were moderately potent functional antagonists. Full K_B values were then determined for those analogs which exhibited K_{Bapp} values less than 1 μM. BMS-180402, BMS-180403 and the $Pen^{3,15}$, Asn^{18} analog were competitive antagonists, with K_B values of approximately 1 μM, values significantly higher than the K_i values for these compounds (20–40 nM).

Additional antagonist testing of BMS-180402 and BMS-180403

One of the two most potent functional antagonists in the rabbit carotid artery preparation, BMS-180403, was evaluated as an antagonist in several model systems. The ability of endothelin to stimulate phosphoinositide hydrolysis and to increase intracellular calcium concentrations provides cell based assay systems to study endothelin antagonists. BMS-180403 was a potent antagonist of endothelin stimulated phosphoinositide hydrolysis in cultured A10 rat vsm cells (K_B = 44.5 ± 16.3 nM, n=2). The maximal response to endothelin was not suppressed by the presence of the peptide, consistent with a competitive mechanism of action. This peptide also depressed the calcium mobilization in fura-2 loaded A10 cells induced by 3 nM endothelin-1 (IC_{50} = 43 ± 13 nM, n=3). Therefore, BMS-180403 was a potent competitive antagonist of endothelin-1.

Because the discrepancy between binding and functional potency of BMS-180403 (and by inference the other peptides) arose between A10 rat vsm cell and rabbit carotid artery tissue assays, we investigated the receptor binding characteristics of our rabbit carotid artery vasoconstrictor assay system. Rabbit carotid artery membranes were prepared and used for radioligand binding under the same conditions as those for A10 vsm cell membranes. Preliminary competition binding experiments demonstrated that BMS-180402 inhibited [^{125}I]–ET-1 binding with an IC_{50} value of 26 nM and a slope of 0.8, numbers quite similar to those obtained from the A10 cell membranes. However, while competition binding experiments using endothelin-1 indicated a single class of binding sites (K_i = 0.05 ± 0.01 nM, slope = 0.8 ± 0.02), data from endothelin-3 competition binding was best fit to a two site model (high affinity K_i = 3.9 ± 0.1 nM, low affinity K_i = 171 ± 81 nM; B_{max1}/B_{max2} = 77%/23%), suggesting the presence in the rabbit carotid of a receptor subtype in addition to ET_A.

BMS-180403 was tested for vasoconstrictor activity and the ability to antagonize endothelin-1 induced vasoconstriction in guinea pig trachea and rabbit saphenous vein (Fig. 2). BMS-180403 at 10 μM produced 14 ± 5% and 90 ± 4% of the maximal force of an endothelin-induced contraction in guinea pig trachea and rabbit saphenous vein respectively. The contractions in the saphenous vein were transient while those in the guinea pig trachea were biphasic in that they reached a peak and then fell to suprabasal levels which were maintained. After the tissues relaxed to the pre-drug level (vein) or to the maintenance level (trachea), cumulative concentration–response curves were generated for endothelin. The K_{Bapp} value for BMS-180403 in the saphenous vein was 5.6 ± 1.6 μM, compared to the K_B value of 1.2 μM in the rabbit carotid artery.

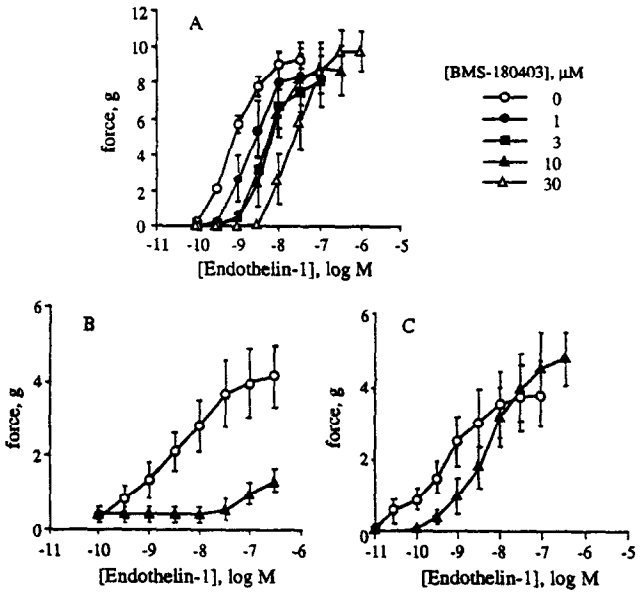

Figure 2 Antagonist potency of BMS-180403 in rabbit carotid artery (A), guinea pig trachea (B) and rabbit saphenous vein (C).

Discussion

Our previous alanine scan of monocyclic Ala^{3,11} endothelin pinpointed those residues which are important to binding and vasoconstrictor potency at the ET_A receptor.[19] This study expands on that work in several ways. An N-terminal ammonium ion and a turn of the correct length at residues 5–8 were found to be important. For high affinity binding, an aliphatic residue at position 17 and a hydrophobic aromatic residue at position 21 were important. D-Amino acid substitution at positions 1, 10, 14 and 15 led to agonists or partial agonists of moderate potency. At the important position 18, replacement of Asp with Ala, Asn or Leu provided analogs with moderate affinity and no vasoconstrictor activity. Nevertheless, even the most potent of the monocyclic peptides failed to provide a competitive antagonist of endothelin in our vasoconstrictor assay.

Using the synthetically simple and regiochemically defined bispenicillamine endothelin frameworks, we further studied the pharmacology of monosubstituted endothelin analogs. [Pen^{1,11} Nle^7, Ala^{18}]–endothelin-1 (BMS-180403) and [Pen^{1,11}, Nle^7, Asn^{18}]–endothelin-1 (BMS-180402) were identified as antagonists of endothelin in the rabbit carotid artery, with K_B values of about 1 μM. The reason for the discrepancy between the micromolar K_B values and the double digit nanomolar K_i values for these compounds is unclear. In order to gain some insight into this puzzling phenomenon, K_B values were determined in two A10 vsm cell based assays, namely phosphoinositide hydrolysis and calcium mobilization. In each of these systems, BMS-180403 exhibited K_B or IC_{50} values (40 nM) in line with its K_i value. Therefore the discrepancy in potency was observed between a cell and a tissue based assay. While

preliminary binding studies with BMS-180403 in membranes prepared from rabbit carotid artery (our tissue assay system) suggested the presence of a single receptor subtype, similar studies using endothelin-3 did provide evidence that rabbit carotid arteries are composed of multiple and/or different endothelin receptor subtypes. This difference in receptor composition may provide part of the explanation for the difference between the K_i and K_B values for these peptide antagonists.

While neither BMS-180402 nor BMS-180403 was an agonist in the rabbit cartoid artery, each was a modest agonist in the guinea pig trachea and a more potent agonist in the rabbit saphenous vein. In each of the latter tissues, there is evidence for the presence of non-ET_A receptor subtypes.[8,27] It is therefore probable that these bis-penicillamine, 18-substituted endothelin analogs, while clearly antagonists at the ET_A receptor, are at least partial agonists at this alternate receptor subtype.

Experimental

Materials

Amino acids were obtained from Bachem California, [^{125}I]ET-1 (2,200 Ci/mmol) and [^{125}I]ET-3 (2,200 Ci/mmol) from New England Nuclear, [^{3}H]myoinositol from Amersham, ET-1 and ET-3 from Peninsula Labs or Peptides International, cell culture reagents from Gibco and rat VSM-A10 cells from the American Type Culture Collection.

Solid phase peptide synthesis

Peptides were prepared by automated solid phase synthesis as previously described.[19] Sets of peptides differing only in the nature of the residue at one site were prepared as mixtures by multiple peptide synthesis using a single support and separated by preparative HPLC.[21] Nle was incorporated at position 7 in place of Met, a substitution known to have minimal effect on endothelin function.[23] Preliminary purification of some peptides was accomplished by chromatography on Sephadex LH-20 or LH-60 (aq. CH_3CN, dilute (0.2–2%) NH_4OH). All peptides were purified by gradient preparative HPLC (acetonitrile:water:0.1% TFA) to >95% homogeneity and characterized by amino acid analysis and fast atom bombardment or ionspray mass spectrometry. The disulfide structures of most of the bis-Cys, bis-Pen analogs, including BMS-180402 and 180403, were determined by a combination of trypsin/mass spectrometry (to rule out the possibility of the 1–3, 11–15 isomer) and CH_3SO_3H-catalyzed hydrolysis followed by LC/MS.[20]

Smooth muscle testing

New Zealand white rabbits and Hartely guinea pigs were anesthetized and tissues of interest were removed and mounted as rings for isometric force recording. Tissues were placed in individual water jacketed muscle chambers in a physiological salt solution (PSS) composed of (in mM): 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.9 $CaCl_2$, 25 $NaHCO_3$, 10.1 D-glucose, and 0.01 Na_2EDTA. The solution was warmed to 37°C and a pH of 7.4 was maintained by aeration with 95% O_2/5% CO_2. During the equilibration period, preparations were stretched to a maintained preload of 4 g (rabbit carotid artery), 2 g (guinea pig trachea) or 0.5 g (rabbit saphenous vein). The preparations were contracted twice with 110 mM K^+PSS, prepared by equimolar substitution of KCl for NaCl, then returned to PSS. Cumulative concentration response curves were obtained for the peptides in individual preparations and the EC_{50} value, the concentration of agonist causing half-maximal force, was determined. EC_{50} values are reported as mean ± S.E.; $n \geq 3$ tissues from different animals. For antagonist testing, the tissues were exposed to various concentrations of peptide for 20 min prior to and during the concentration–response curves to endothelin-1. Stock solutions were prepared immediately before use by dissolving the peptides in a small amount of 1 M acetic acid which was adjusted to the appropriate volume with water containing 0.05% bovine serum albumin.

Radioligand binding

Rat A10 vascular smooth muscle cell membranes were prepared as previously described.[28] Carotid arteries from New Zealand White rabbits were homogenized twice for 10 sec with a Brinkman Polytron (setting 8) in ice cold 50 mM Tris-HCl pH 7.4 with 0.24 units/ml aprotinin and 1 mM EDTA. The crude particulate matter was removed by centrifugation at 750 g for 10 min at 4°C. The membranes were sedimented from the supernatant fraction by centrifugation at 48,000 g for 30 min. Membrane pellets were resuspended in the above buffer and stored in aliquots at -80° C until use. Radioligand binding was conducted as previously described.[28] Data were analyzed by iterative curve fitting to a one or two binding site model, and inhibition constants (K_i) calculated from IC_{50} values.[29] K_i values and slope factors are means ± S.D. derived from 10-concentration point curves with each concentration run in duplicate. $K_i = IC_{50}/1 + [RL \text{ concentration} / K_D \text{ of RL}]$ where RL denotes radioligand.

Phosphoinositide metabolism

Assays were performed as previously described.[28] Calculation of apparent K_B values was made from Schild plots on the assumption that slopes were not significantly different from unity.[30]

Free intracellular calcium ion determinations.

Assays were performed as previously described.[28]

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