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Development of agonists of endothelin-1 exhibiting selectivity towards ET_A receptors

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1 Endothelin-1 (ET-1) is a bicyclic 21-amino-acid peptide causing a potent and sustained vasoconstriction, mainly through the ET_A receptor subtype. So far, no selective ET_A agonists are described in the literature.

2 A series of truncated and chemically modified ET-1 analogues were obtained through solid-phase peptide synthesis and their biological activity was assessed on rat thoracic aorta rings (ET_A receptors) and guinea-pig lung parenchyma strips (ET_B receptors).

3 Structure–activity studies led to the identification of ET-1 fragments exhibiting an ET_A selective agonistic activity.

4 In particular, [D-Lys^9]cyclo$^{11-15}$ ET-1(9–21) was the most potent peptide. It appeared as a full agonist of ET_A receptors, being under two orders of magnitude less potent than ET-1 (EC_{50}: 2.3×10^{-7} vs 6.8×10^{-9} M). Interestingly, even a linear formylated analogue, [Ala^{11,15}, Trp(For)^{21}]ET-1(9–21), showed a selective ET_A activity (EC_{50}: 3.0×10^{-6} M). None of the numerous analogues of the series exhibited substantial effects in the guinea-pig lung parenchyma bioassay.

5 Thus, this study describes the first compounds showing a significant bioactivity in an ET_A pharmacological preparation while being inactive in an ET_B paradigm. They show that the ET-1 pharmacophores, responsible for the ET_A-mediated actions, are located within the 9–21 segment of the molecule. Moreover, the bicyclic structure of ET-1 does not appear as essential for the ET_A-related vasoconstriction. Results also suggest that the positive charge of the Lys^9 side chain participates in an intramolecular ionic bond with the carboxylate function of Asp^{18}.
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Abbreviations: Ac, acetyl; Acm, acetamidomethyl; Ahx, aminohexanoic acid; Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate; DMF, dimethylformamide; ET, endothelin; eq, equivalent; For, formyl; HF, hydrofluoric acid; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; NMR, nuclear magnetic resonance; STX_{6c}, sarafotoxin 6c; Suc, succinoyl; TFA, trifluoroacetic acid

Introduction

Endothelin (ET) was first isolated from the supernatant of cultured porcine aortic endothelial cells by Yanagisawa *et al.* (1988). This peptide exhibits potent vasoconstrictor properties along with long-lasting pressor effects. These biological features suggest a key role for this peptide in the cardiovascular system and, likely, a relation with pathophysiologies such as hypertension, renal failure, asthma and atherosclerosis (Huggins *et al.*, 1993; Vanhoutte, 1996; Boulanger, 1999; De Artiñano & Gonzalez, 1999; Boss *et al.*, 2002; Dasgupta *et al.*, 2002).

ET is a member of a highly conserved 21-amino-acid peptide family, sharing about 60% of sequence homology. Two disulfide bridges, located at positions 1–15 and 3–11, a charged region involving residues 8–10 and a hydrophobic C-terminal segment (residues 16–21) characterize this molecule. Human ET peptide isoforms, described so far and identified as ET-1, ET-2 and ET-3, mediate their biological activities through two receptor subtypes: ET_A exhibiting a clear-cut preference for the ET-1 and ET-2 ligands, and ET_B showing an identical affinity towards all three related isoform molecules. Activation of the ET_A receptors found on vascular smooth muscle cells generates a sustained vasoconstriction. On the other hand, ET_B receptors localized on endothelial cells can mediate a vasoconstriction as well as a vasodilatation (Opgenorth, 1995; Ortega Mateo & De Artiñano, 1997).

Various techniques have been used to elucidate the conformational characteristics of ET-1 or analogues, and to correlate their spatial geometry with the ET_A or ET_B receptor requirements. Thus, structural analyses using circular dichroism or nuclear magnetic resonance (NMR) spectroscopies, as well as X-ray crystallography, have described, for instance, an α-helical arrangement located between residues 9 and 15 (or 16) (Endo *et al.*, 1989; Saudek *et al.*, 1989; Perkins *et al.*, 1990; Aumelas *et al.*, 1991; Andersen *et al.*, 1992; Wallace & Janes, 1995; Katahira *et al.*, 1998; Van Der Walle & Barlow, 1998;

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Boulanger *et al*., 1999; Hewage *et al*., 1999; Orry & Wallace, 2000; Hewage *et al*., 2002). Furthermore, some of these studies have reported that the helical backbone would be stabilized by hydrogen bonds involving more particularly the amino-acid pairs Lys^9-Tyr^{13} and $Glu^{10}-Phe^{14}$. The conformational investigations also indicated that the N-terminal segment prevails in an extended geometry followed by type I β-turn between residues 5 and 8, while the C-terminal tail would be rather elongated and mobile. Nevertheless, no unambiguous conclusions are available about the C-terminal stretch, as Janes *et al*. (1994), (by means of X-ray diffraction) depicted for ET-1 an irregular α-helix extended up to the C-terminal residue, whereas other authors (using NMR spectroscopy) rather observed a random coil arrangement able to bend back and lie in close proximity with the core residues (Saudek *et al*., 1989; 1991; Andersen *et al*., 1992). Worthy of note is the revealing of defined secondary structures at the C-terminus of ET analogues, such as a γ-turn in IRL-1620 (Katahira *et al*., 1998) and a β-bend in biological active truncated ET derivatives (Boulanger *et al*., 1999).

Since the discovery of ET, many peptidic and nonpeptidic analogues were designed to determine the binding requirements of the receptor subtypes and to elucidate the pharmacophores underlying the receptor specificities (Kimura *et al*., 1988; Nakajima *et al*., 1989; Watanabe *et al*., 1991; Tam *et al*., 1994; Galantino *et al*., 1995; Rovero *et al*., 1998; Boss *et al*., 2002; Dasgupta *et al*., 2002). In particular, the role of the intramolecular S–S bonds was explored and it appeared that their structural modifications, as shown with the linear analogue $[Ala^{1,3,11,15}]$ET-1, promoted the production of ET_B-selective derivatives (Nakajima *et al*., 1989; Jones *et al*., 1991; Pelton & Miller, 1991; Saeki *et al*., 1992).

A similar preferential behavior towards the ET_B receptor subtype was described with many truncated ET-1 analogues, such as ET-1(16–21) (Rovero *et al*., 1990), N-Ac $[Ala^{11,15}]$ET-1(10–21) (Saeki *et al*., 1992), Suc-$[Glu^9, Ala^{11,15}]$ ET-1(8–21) known as IRL-1620 and $[Cys(Acm)^{3,11}, Trp(For)^{21}]$(3–11)-Ahx-(17–21)ET-1 (Forget *et al*., 1996). As a matter of fact, during the last decade, the development of agonists and competitive antagonists of ET_B receptors, as well as the design of specific ET_A antagonists were successful (Dasgupta *et al*., 2002). Nevertheless, no ET_A-selective agonists were described. Such compounds would be advantageous pharmacological tools in order to pursue the deeper characterization of the ET_A receptor pharmacophoric and structural requirements. In the present paper, we describe the design and the biological effects of substituted ET fragments exhibiting significant selectivity and activity towards the ET_A receptor subtype. Moreover, the data suggest that a single helical turn between residues Val^{12} and Cys^{15} favors the adoption of the right molecular arrangement of the Tyr^{13} phenolic side-chain, a major ET_A-related pharmacophore.

Methods

Peptide synthesis and purification

Three sets of truncated analogues of endothelin-1 were derived from its 8–21, 9–21 and 10–21 fragments, and were particularly designed in order to evaluate the effects of a restricted mobility of the core residues, through a $Cys^{11}-Cys^{15}$ disulfide bridge. Thus, all peptides of these series, including endothelin-1 and sarafotoxin 6c (STX_{6c}), were synthesized using a semiautomatic homemade solid-phase synthesizer, according to a protocol using Boc chemistry and a procedure previously described (Forest *et al*., 1990). Briefly, a Boc-Trp(For)-aminoacyl-resin was used as the solid support and subsequent couplings of Boc-amino acids were performed in dimethylformamide (DMF) in the presence of BOP reagent and diisopropylethylamine. N-acetylation of analogues was achieved through a 20-min reaction with 50 equivalents (eq) of acetic anhydride in DMF. Peptide resins were cleaved from their solid support with hydrofluoric acid (HF – 10 ml g^{-1}) using dimethylsulfide and *m*-cresol as scavengers. The reaction was carried out for 1 h at 0°C. HF was rapidly evaporated and the resin was washed with diethylether. The crude synthetic peptides were extracted with TFA.

Crude peptides were purified by means of preparative reverse-phase HPLC using a Waters PrepLC500A system equipped with a model 441 absorbance detector and a Phenomenex Jupiter C_{18} (300 Å, 15 μm, 250 × 21.2 mm) column. Peptide were eluted with a 2 h linear gradient from A (0.05% aqueous NH_4OH) to B (40% CH_3CN in solvent A). Flow rate was maintained at 20 ml min^{-1} and detection was at 229 nm. Collected fractions were analyzed using analytical reverse-phase HPLC. Fractions corresponding to the desired product were pooled and lyophilized. Cyclization was achieved by iodine oxidation (50 eq) in 80% aqueous acetic acid at a peptide concentration of 0.5 mg ml^{-1}. The reaction was monitored by analytical HPLC and mass spectrometry, and was usually completed within 2 h. Cyclization was stopped by adding ascorbic acid until discoloration. This solution was diluted with water (1 : 10) and injected onto a preparative HPLC column and purified as described above.

Deformylation of the indole moiety of Trp^{21} was obtained by shaking the peptide (0.3 mg ml^{-1}) in 0.1 N piperidine at 0°C for 15 min. Then, the solution was diluted with water (1 : 10) and purified by preparative reverse-phase HPLC. Peptide purity was assessed with analytical reverse-phase HPLC and molecular weight was confirmed with MALDI-TOF mass spectrometry.

Biological activity studies

The contraction induced by ET-1 and its truncated analogues was measured in two different preparations: rat thoracic aorta rings (Nguyen *et al*., 1993) and guinea-pig lung parenchyma strips (Wong *et al*., 1992). Male Sprague–Dawley rats (250–300 g) were anaesthetized and the thoracic aorta was removed, cleaned of connective tissues and its endothelium was detached by gentle rubbing. Rings (4 mm wide) were cut and mounted under an initial tension of 1 g, in water-jacketed organ baths containing oxygenated Krebs buffer maintained at 37°C. Each preparation was allowed to equilibrate for 1 h and contractions were recorded using a Grass 7E Polygraph equipped with force–displacement transducers. Contractile responses were measured for concentrations of ET-1 or analogues ranging from 10^{-10} M to 10^{-5} M. The ET_A nature of the contraction was demonstrated by the inhibition of the biological response when the tissues were in the presence of the ET_A-selective antagonist BQ-123 (10^{-6} M). The biological activity was expressed as a percentage of the effect produced after the addition of KCl (80 mM).

Male Hartley guinea-pigs (300 – 350 g) were anaesthetized and the lungs were removed. The parenchyma was dissected in strips that were mounted in the system described above. Contractile reponses were measured for concentrations of ET-1 analogues ranging from 10^{-10} M to 10^{-5} M, in the presence of BQ-610 (10^{-7} M), an ET_A-selective antagonist. ET_B activity of tissues was also assessed using sarafotoxin 6c, a selective ET_B agonist. The biological activity was expressed as a percentage of the effect produced after the addition of histamine (10^{-6} M).

Concentration – response curves were analyzed using a nonlinear least-squares regression obtained with the Prism 3.0 software. The results are expressed as mean ± s.e.m. and *n* varied from four to 12 animals.

Results

The biological activity of these peptides was measured in the rat thoracic aorta and the guinea-pig lung parenchyma bioassays, two pharmacological preparations showing predominant populations of ET_A and ET_B receptors, respectively. Figures 1 and 2 show the concentration – response curves of the active compounds and Table 1 summarizes the results. ET-1 was active on both tissues while STX_{6c}, as expected, induced a contraction only in the guinea-pig lung parenchyma. The first group of derivatives (analogues derived from segments 8 to 21 of ET-1), containing a putative central helical turn stabilized with a Cys^{11} – Cys^{15} disulfide bridge, focused on Asp^8, with or without a free N-terminus (compounds 3 – 6). None of the peptides produced a potent biological response in the aorta or the lung parenchyma. Similarly, using ET-1 fragments, a small set of analogues (derived from segment 10 – 21 of ET-1) explored the usefulness of the Lys^9 and Glu^{10} residues while keeping intact the 11 – 15 bond in the molecule (compounds 27 and 28). Again, no activity was recorded in the ET_A preparation. However, cyclo$^{11-15}$ ET-1(10 – 21) provoked a contraction of the guinea-pig lung parenchyma with a threshold concentration at about 10^{-7} M and an EC_{50} value estimated at slightly less than 10^{-5} M.

The third series describes fragments 9 – 21 of endothelin-1 with various substitutions or chemical modifications within their parent molecules (compounds 7 – 26). Among those analogues, the cyclic fragment 9 – 21 was inactive in both preparations (compound 7). Nonetheless, the N-terminal capping by acetylation of this peptide led to a weak ET_A agonist (compound 8; EC_{50}: 1.4×10^{-6} M), whereas the inversion of the chirality of residue 9 ([D-Lys^9]cyclo$^{11-15}$ ET-1(9 – 21); compound 9) produced an analogue with a significant potency in the ET_A paradigm (EC_{50}: 2.3×10^{-7} M) but with a very weak activity in the guinea-pig lung parenchyma tissue (about 1000-fold less potent). N-acetylation of compound 9 (compound 10) did not improve the biological activity related to the ET_A receptor subtype.

The function of the cyclic structure was investigated by capping the Cys^{11} and Cys^{15} side chains of the analogues with acetamidomethyl groups ($CH_3CONHCH_2$ – Acm), thus lead-

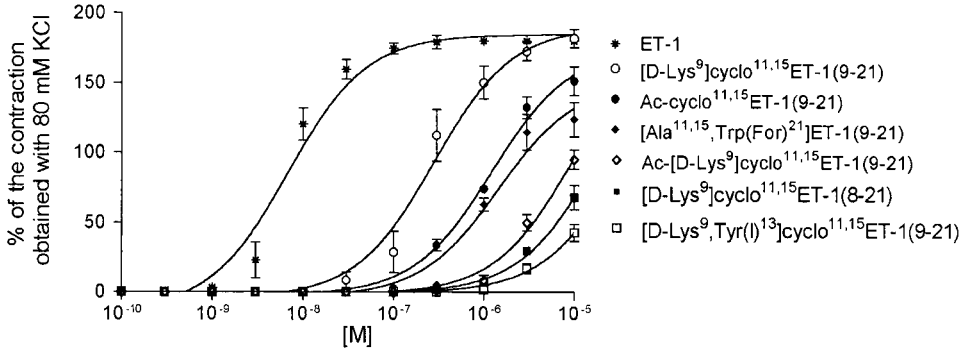

Figure 1 Concentration – response curves of ET-1 and analogues (from 10^{-10} to 10^{-5} M) obtained in the rat thoracic endothelium-denuded aorta ring bioassay. Results are expressed as percentage of the contractile response of 80 mM KCl.

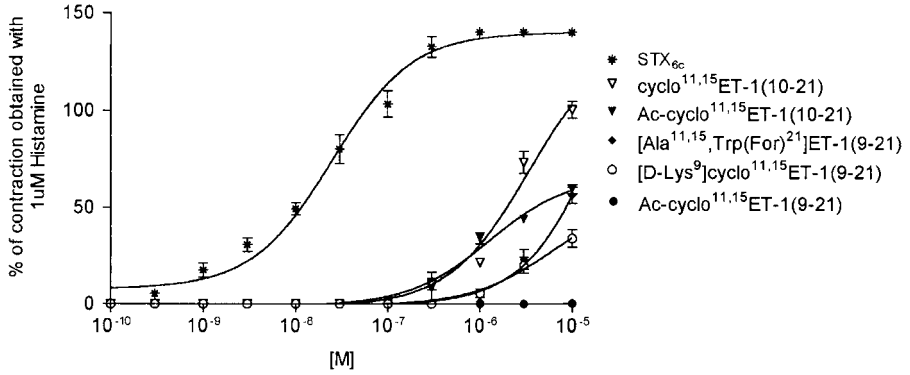

Figure 2 Concentration – response curves of ET-1 and analogues (from 10^{-10} to 10^{-5} M) obtained in the guinea-pig lung parenchyma strip bioassay. Results are expressed as percentage of the contractile response induced with 10^{-6} M histamine.

Table 1 Biological activities of human endothelin-1, sarafotoxin 6c and ET-1 analogues in the rat thoracic aorta (ET_A) and guinea-pig lung parenchyma (ET_B) bioassays

	Peptide	EC_{50}–ET_A[a] (M)	EC_{50}–ET_B[a,b] (M)
1	ET-1	6.8×10^{-9}	9.0×10^{-9}
2	STX_{6c}	NA	2.6×10^{-8}
3	Cyclo$^{11-15}$ET-1(8–21)	NA	NA
4	Ac-cyclo$^{11-15}$ET-1(8–21)	NA	NA
5	[D-Lys^{9}]cyclo$^{11-15}$ET-1(8–21)	$> 10^{-5}$	NA
6	Ac-[D-Lys^{9}]cyclo$^{11-15}$ET-1(8–21)	NA	NA
7	Cyclo$^{11-15}$ET-1(9–21)	NA	NA
8	Ac-cyclo$^{11-15}$ET-1(9–21)	1.4×10^{-6}	NA
9	[D-Lys^{9}]cyclo$^{11-15}$ET-1(9–21)	2.3×10^{-7}	$\gg 10^{-5}$
10	Ac-[D-Lys^{9}]cyclo$^{11-15}$ET-1(9–21)	$\approx 10^{-5}$	NA
11	[Cys(Acm)^{11,15}]ET-1(9–21)	NA	NA
12	Ac-[Cys(Acm)^{11,15}]ET-1(9–21)	NA	NA
13	[Ala^{11,15}]ET-1(9–21)	NA	NA
14	Ac-[Ala^{11,15}]ET-1(9–21)	NA	NA
15	[Cys(Acm)^{11,15}, Trp(For)^{21}]ET-1(9–21)	NA	NA
16	Ac-[Cys(Acm)^{11,15}, Trp(For)^{21}]ET-1(9–21)	NA	NA
17	[Ala^{11,15}, Trp(For)^{21}]ET-1(9–21)	3.0×10^{-6}	$> 10^{-5}$
18	Ac-[Ala^{11,15}, Trp(For)^{21}]ET-1(9–21)	NA	NA
19	Ac-[Gln^{10}]cyclo$^{11-15}$ET-1(9–21)	NA	NA
20	Ac-[Tyr(I)^{13}]cyclo$^{11-15}$ET-1(9–21)	NA	NA
21	[D-Lys^{9},Tyr(I)^{13}]cyclo$^{11-5}$ET-1(9–21)	NA	NA
22	Ac-[Ala^{16}]cyclo$^{11-5}$ET-1(9–21)	NA	NA
23	[D-Lys^{9}, Ala^{16}]cyclo$^{11-15}$ET-1(9–21)	NA	NA
24	Ac-[Asn^{18}]cyclo$^{11-15}$ET-1(9–21)	NA	NA
25	Ac-[Gly^{18}]cyclo$^{11-15}$ET-1(9–21)	NA	NA
26	[D-Lys^{9},Gly^{18}]cyclo$^{11-15}$ET-1(9–21)	NA	NA
27	Cyclo$^{11-15}$ET-1(10–21)	NA	$\approx 10^{-5}$
28	Ac-cyclo$^{11-15}$ET-1(10–21)	NA	$> 10^{-5}$

[a]EC_{50}: concentration of peptide giving 50% of the maximal effect. s.e.m. on the data ± 0.5.
[b]Measured in the presence of 10^{-7} M BQ-610, a specific ET_A antagonist.
ET-1: human endothelin-1; STX_{6c}: sarafotoxin 6c, a specific ET_B agonist; n.a.: not active at 10^{-5} M.

ing to linear and stable peptides (compounds 11 and 12). Substitutions of residues 11 and 15 with L-alanine were also carried out (compounds 13 and 14). Analogues practically devoid of ET_A or ET_B activity were obtained following these modifications. Formylation of the linear analogues was applied (compounds 15 – 18) and, interestingly, the incorporation in [Ala^{11,15}]ET-1(9 – 21) of a formyl (For) group (CHO) on the indole moiety of Trp^{21} generated a weak agonist in the rat endothelium-denuded aorta (compound 17). The other three substances (compounds 15, 16 and 18) were inactive.

Finally, using the active peptide fragments as templates, we carefully examined the effects of alterations targeting residues believed to play a key role for activity and/or affinity at the receptor level (compounds 19 – 26). We noticed that amidation of Glu^{10} (compound 19) completely abolished the activity of the 9 – 21 fragment, as well as the iodination of the Tyr^{13} side chain (compounds 20 and 21). The loss of contractile effects was also observed after amidation of Asp^{18} (compound 24) or substitutions of His^{16} with L-alanine (compounds 22 and 23), or Asp^{18} with glycine (compounds 25 and 26).

Discussion

Structure – activity studies described various linear and/or truncated ET-1 analogues as potent agonists in ET_B pharmacological preparations (Doherty & Patt, 1997; Pelton, 1997; Dasgupta *et al*., 2002). In parallel, the data collected in ET_A paradigms suggested that the entire structure was likely required to induce a potent response via the ET_A receptor subtype. In fact, some fragments were shown to bind weakly to tissues containing ET_A receptors, but no activity nor antagonistic effects were measured in pharmacological preparations, thus leading to the speculation that these peptides may not be acting at ET receptors (Doherty *et al*., 1991; Pelton, 1997). In addition, the disulfide links, and more particularly the outer loop formed by the Cys^{1} – Cys^{15} linkage, appeared as important features for ET_A action. A direct function at the receptor level, involving a disulfide exchange mechanism, has even been proposed for the exterior bridge (Spinella *et al*., 1991). Conformational studies agree that the central core of ET and possibly the segment 9 – 21 adopts a helical organization (Endo *et al*., 1989; Saudek *et al*., 1989; Perkins *et al*., 1990; Aumelas *et al*., 1991; Andersen *et al*., 1992; Wallace & Janes, 1995; Orry & Wallace, 2000). This secondary structure would be stabilized by the cystine residues (Heitz *et al*., 1999) and would be, for instance, characterized by the alignment of the Tyr^{13} and His^{16} side chains (Orry & Wallace, 2000). We hypothesized that these residues, in this precise geometry, are key elements for the ET_A activity and that the whole molecule is therefore not essential. Thus, we developed human ET-1 analogues containing only one disulfide bridge, assembled in the non-natural 11 – 15 configuration. Such a disulfide arrangement restricts efficiently the mobility of the 11 – 15 segment and stabilizes the putative helical turn found within the limits of the loop, in a similar mode to intramolecular amide bridges linking (i, $i+4$)-spaced residue pairs (Taylor, 2002).

The study looked at three series of cyclic and linear compounds corresponding to 8 – 21, 9 – 21 and 10 – 21 fragments of ET-1. This choice was made in order to evaluate the effects of a restricted mobility of the core residues, and to explore the consequence of a variation in the total net charge at the N-terminus, following the successive deletions of Asp^8 and Lys^9. The ionic character of the N-terminus was also investigated with the removal of the N-terminal positive charge by capping the amine function with an acetyl group. Among the 8 – 21 and 10 – 21 sets (compounds 3 – 6, as well as 26 and 27), only cyclo$^{11-15}$ET-1(10 – 21) (compound 27) exhibited a very weak response in the guinea-pig lung parenchyma. This was somewhat surprising since the ET_B receptor is very tolerant towards structural modifications of its ligands. Moreover, it has been previously described that Ac-[$Ala^{11,15}$]ET-1(10 – 21) retained a good affinity (12 nM) in an ET_B binding preparation (Saeki *et al*., 1992; Pelton, 1997), and that the replacement of the middle segment of the ET molecule, Val^{12}-Tyr^{13}-Phe^{14}, with an aliphatic spacer (amino-hexanoic acid: Ahx) led to selective ET_B agonists (Forget *et al*., 1996). Therefore, these results might be indicative of a structural ET_B requirement favoring a loose and stretched arrangement of the central core of its ligands.

The second series of this study gave rise to a more exhaustive biological analysis since active and selective fragments were obtained following structural alterations. First, Ac-cyclo$^{11-15}$ET-1(9 – 21) (compound 8) showed biological activity in the rat endothelium-denuded thoracic aorta. Steric effects generated by the incorporation of the N-terminal acetyl group, more than the exclusion of the N-terminal positive charge, are believed to be responsible for this result. As a matter of fact, acetylation of the active analogue [D-Lys^9]cyclo$^{11-15}$ET-1(9 – 21) decreased substantially the ET_A activity (compound 10). If charge neutralization had been at the origin of the activity of compound 8, a major improvement of the EC_{50} of [D-Lys^9]cyclo$^{11-15}$ ET-1(9 – 21) (compound 9) would have been expected after its capping. Taking into account that molecular dynamics simulations (Hempel *et al*., 1994) proposed, as a preferred ET conformer, a helical stretch with the anionic amino acids Glu^{10} and Asp^{18} adjacent in space, and considering that NMR suggested a folding back of the C-terminal portion of ET-1 towards the central core of the molecule, it is believed that the inclusion of an N-terminal acetyl moiety in cyclo$^{11-15}$ET-1(9 – 21) favors the proper orientation of the flanking Lys^9 side chain towards the negatively charged Asp^{18}. This interaction would give a compact molecular arrangement of the analogue and would jut out the Glu^{10} carboxylic function that appears to be essential for ET_A, as suggested by the complete loss of activity following its amidation (compound 19). Also, accordingly, previous studies reported that specific ET_A receptor antagonists require, at the receptor level, an interaction with a putative cationic site surrounded by a hydrophobic environment (Astles *et al*., 1998a, b). Thus, the negative charge of Glu^{10} would probably interact directly with this putative cationic site found on ET_A receptors.

Another fact supporting the claim about a Lys^9 – Asp^{18} salt-bridge is that the inversion of chirality of Lys^9 (compound 9), which conferred a four-fold increase of ET biological activity (Galantino *et al*., 1995), produced the selective ET_A agonist [D-Lys^9] cyclo$^{11-15}$ET-1(9 – 21). The new orientation of the Lys^9 side chain appears to facilitate the intramolecular interaction of the respective anionic and cationic nuclei of Asp^{18} and D-Lys^9. Also, it was shown that the elimination of the negative charge of Asp^{18}, by amidation (compound 24), or its substitution with glycine (compound 25 and 26), provided peptide derivatives inactive in both ET_A and ET_B receptor bioassays. Glycine was used for the substitution because Katahira *et al*. (1998) reported that the introduction of a Gly moiety at position 18 of IRL-1620, a specific ET_B agonist, gave a new analogue showing biological properties in an ET_A paradigm.

The contribution of the cyclic geometry of the analogues was also verified by capping the Cys^{11} and Cys^{15} side chains with Acm groups, and by replacing the cysteine residues with L-alanine (compounds 11 – 14). Following these conversions, the biological activity was totally abolished. Such results strongly suggested that the stability of the putative helical turn, involving residues 11 – 14 or 12 – 15, is a critical parameter for the activity towards the ET_A receptor subtype. However, our previous investigation with truncated linear analogues of ET-1 (Forget *et al*., 1996) showed that formylation of linear analogues was a chemical modification favorable for biological activity in the guinea-pig lung parenchyma preparation. This strategy was then applied to the linear peptides and gave compounds 15 – 18. Interestingly, the incorporation in [$Ala^{11,15}$]ET-1(9 – 21) of a formyl group (CHO) on the indole moiety of Trp^{21}, generated a weak agonist in the rat endothelium-denuded aorta (compound 17), thus suggesting that a ligand – receptor interaction implicating cysteine residues, as hypothesized before (Spinella *et al*., 1991), is unlikely. Conformational studies using NMR spectroscopy (Boulanger *et al*., 1999) showed that the truncated – formylated ET peptide derivatives giving rise to biological responses in the lung parenchyma, all contained a reverse turn involving the C-terminal residues. Likewise, Katahira *et al*. (1998) also reported a γ-bend at the C-terminus of IRL-1620. We do not know yet if a similar geometry is found in compound 17. Nevertheless, the incorporation, in the indole function of Trp^{21}, of a small chemical moiety such as a formyl group promoting intramolecular hydrogen bonding might be, as described before for ET_B-selective analogues (Forget *et al*., 1996; Boulanger *et al*., 1999), a suitable scheme for improving the potency of ET_A-selective agonists.

Finally, iodination of Tyr^{13} and substitutions of His^{16} also established that those residues play a prime function in the ET_A paradigm (compounds 20 – 23). Introduction of an iodine atom in the Tyr^{13} side chain of ET fragments was achieved in order to check the feasibility of producing new ET_A-selective radioligands. Unexpectedly, the strategy did not succeed in generating active ligands. Furthermore, the compounds did not show any apparent affinity for the ET_A or ET_B receptors since they did not display, at a concentration up to 10^{-6} M, any antagonistic effects on the ET-1 or STX_{6c} response, in the rat or guinea-pig tissues, respectively. Taking into consideration that [Tyr^{13}(^{125}I)]ET-1 is a nonselective radioprobe exhibiting an excellent affinity (Davenport *et al*., 1993), the results indicate that the large iodine atom disturbs, through steric hindrance, the central arrangement of the ET_A-selective agonists, thus demonstrating the key role of the Tyr^{13} side chain for the ET_A receptor activation and/or recognition phenomena. The histidine residue at position 16 was replaced with L-alanine in both active ET fragments and this exchange gave analogues devoid of any biological activity. These data

suggest that the His^{16} side chain participates actively in the biological and/or binding effects of the analogues. An identical substitution was carried out by Tam *et al*. (1994) in the whole ET molecule and this peptide appeared as a highly potent analogue in the rabbit vena cava bioassay, a pharmacological preparation containing a population of ET_A receptors. The switch from an imidazole side chain (His^{16}) to a small methyl moiety (Ala^{16}) decreases the steric hindrance and therefore should facilitate the adoption of the proper secondary structure. Thus, the histidine residue might be more biologically significant than what was concluded before with endothelin. These divergent results might reflect the variations in both paradigms, especially regarding the homogeneity of the receptor populations. In our ET_A assay, the biological activity of ET-1 and its active analogues was totally abolished in the presence of BQ-123, a specific ET_A antagonist. This demonstrated, as reported before in the literature, that the rat thoracic aorta contains a homogeneous population of ET_A receptors.

In conclusion, this structure – activity study using ET_A and ET_B receptor paradigms led to the development of [D-Lys^9]cyclo$^{11-15}$ ET-1(9 – 21), an ET_A agonist showing a significant potency. To the best of our knowledge, this compound is the first ET_A agonist exhibiting an unambiguous selectivity towards the ET_A receptor, with an ET_A/ET_B ratio of about 1000-fold. All inactive compounds were also checked for their potential antagonistic properties and none demonstrated any affinity for the ET_A or ET_B receptors. This result proves that the central core of the ET-fragment agonists is not tolerant to modifications causing probably a change in the molecular organization of the pharmacophores found within the 11 – 15 loop. Moreover, the data also suggest that the positive charge of the Lys^9 side chain would participate in an intramolecular salt-bridge with the carboxylate function of Asp^{18}. This conformation favors the clustering of the hydrophobic amino acids Tyr^{13}, Phe^{14}, Leu^{17} and Trp^{21}, as suggested by structural studies (Orry & Wallace, 2000).

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