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Synthesis and structure–activity relationship studies of new endothelin pseudopeptide analogues containing alkyl spacers

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Abstract

We replaced the Asp[18]–Ile[19] dipeptide of the C-terminal ET analogue Ph–Ph–CH_2–O–N=CH–CO–Phe–Asp–Ile–Ile–Trp–OH by alkyl spacers of various lengths to investigate the role of the aminoacidic central portion of the molecule and to define the N-terminal and C-terminal pharmacophoric regions of this analogue. The side-chains of the central dipeptide have been shown to be irrelevant for the binding of the molecule to the receptor, but the distance between the two postulated sites of interaction of the ligand with the ET_B receptor appears to be fundamental.

Keywords: Endothelin; ET(16–21); ET_B receptor; ω-amino acids

1. Introduction

Endothelins (ETs) are three isopeptides of 21 residues named ET-1, ET-2, and ET-3, structurally characterized by an N-terminal bicyclic moiety linked by two disulfide bridges among Cys^1 Cys^{15} and Cys^3 Cys^{11}, and by a conserved hexa-aminoacidic C-terminal tail [1]. ETs are potent vasoconstrictors involved in several physiological and pathological functions, especially at the cardiovascular level [2,3]. Their biological activities are exerted through the interaction with two cell membrane receptor subtypes, named ET_A and ET_B, belonging to the family of the G-protein coupled receptors characterized by seven transmembrane hydrophobic domains [4].

In early reports [5,6], we showed the importance of the C-terminal ET hexapeptide, ET(16–21) [H–His–Leu–Asp–Ile–Ile–Trp–OH]. Other authors reported that the substitution of the His^{16} residue of ET(16–21) with bulky, aromatic, unnatural amino acids led to compounds endowed with antagonist activity at ETs receptors [7]. We further expanded these observations, with the aim of the rational development of peptidomimetic analogues of this peptide antagonist.

Our first approach was the investigation of new analogues based on the replacement of the His^{16} residue with non-aminoacidic substituents [8–10]. Among them we found that compound **1** (Fig. 1), in which the His^{16}

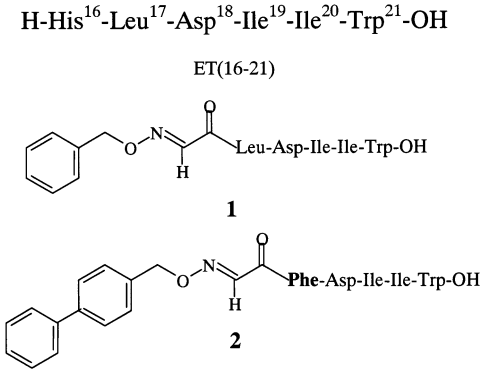

Fig. 1.

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residue was replaced by the (*E*)-*N*-(benzyloxy)-iminoacyl moiety, proved to possess a certain affinity for ET_A/ET_B receptors, while compounds in which the phenyl ring is lacking or the iminic double bond is saturated, were practically devoid of any affinity [10].

These results indicated that the contemporary presence of an oximethyl-ethereal group and an aromatic ring (as in the lipophilic N-terminal non-aminoacidic moiety of **1**) could play a certain role in the interaction with ET_A/ET_B receptors; therefore the (*E*)-*N*-(benzyloxy)iminoacetyl moiety of **1** could be tentatively considered as a pharmacophoric portion. Furthermore, in order to investigate the structure–activity relationship of this peptide, we proceeded to the elucidation of the role of each amino acid residue of the ET(17–21) peptidic portion of **1** in the ligand–receptor interaction, through an alanine scan of this pentapeptidic portion together with modification of the C-terminal Trp residue, i.e. amidation or deletion [9]. The results of these studies indicated that in compound **1**, only the C-terminal dipeptide Ile^{20}–Trp^{21}, including the free carboxylic function, was important for the affinity and could be considered as a second pharmacophoric portion of **1**, while the central portion of the molecule seemed to have the role of maintaining the relative topography of the two termini.

In a follow-up of this study [10], we found that compounds which differ from **1** in the insertion of phenyl moieties on the N-terminal residue and/or on the side-chain of amino acid residues 17 and 18, possessed an increased affinity, prevalently toward the ET_B receptor. In particular, the presence of a phenyl ring in the *para* position of the (*E*)-*N*-(benzyloxy)iminoacyl moiety of **1** and the replacement of Leu^{17} with a Phe residue as in **2**, seemed to be important for ET_B receptor interaction, as demonstrated by the higher affinity of **2** for such type of receptor with respect to **1**.

We also reported that compound **4**, which differs from **2** for the replacement of the Asp^{18}–Ile^{19} dipeptidic portion with an aliphatic spacer (Ava) possessing the same length but without the substituents present on the side-chain of the dipeptide, proved to maintain the ET_A/ET_B affinity profile found for compound **2**. This result underlined the two moieties which might be thought to act as the pharmacophoric portions of these structures, i.e. the lipophilic N-terminal portion and the C-terminal Ile^{20}–Trp^{21}. Furthermore, this type of modification diminishes the peptidic nature of the lead compound and could be regarded as the first effective step toward the rational design of a true peptidomimetic analogue of ET(16–21).

In this paper, we want to point out the importance of these pharmacophoric regions, by replacement of the less significant residues, Asp^{18} and Ile^{19}, by alkyl spacers of various lengths. The use of ω-amino acids as building blocks replacing two or more residues in bioactive or model peptides has been described in the literature [11]. We have previously successfully used the same approach for the design of modified analogues of the two kinins, bradykinin and kallidine, and their antagonists [12].

The modification of natural peptides by introduction of alkyl spacers and iminoacyl moieties is directed toward the discovery of peptidomimetic molecules, with long-lasting biological effects and simplified chemical structure, that are of great pharmaceutical interest (Table 1).

2. Results and discussion

Analogues **1** and **2** are the reference compounds and have been previously described [9]. In the analogues **3**–**7** the dipeptide Asp^{18}–Ile^{19} of compound **2** was replaced by the following alkyl spacers: 4-aminobutyric acid (Abu) in analogue **3**, 5-aminovalerianic acid (Ava) in analogue **4** [9], 6-aminohexanoic acid (Ahx) in analogue **5**, 8-aminooctanoic acid (Aoc) in analogue **6** and 12-aminododecanoic acid (Ado) in analogue **7**. In ana-

Table 1
Sequence and analytical characterization of the peptides [a]

Number	Sequence	Yield (%) [b]	Purity (%) [c]	M [d]	$ES/MS_{[M+H]}$
2	R–Phe–Asp–Ile–Ile–Trp–OH	47	98	930.0	931
3	R–Phe–Abu–Ile–Trp–OH	49	99	787.6	788
4	R–Phe–Ava–Ile–Trp–OH	33	99	801.3	802
5	R–Phe–Ahx–Ile–Trp–OH	30	94	814.6	815
6	R–Phe–Aoc–Ile–Trp–OH	30	99	842.6	844
7	R–Phe–Ado–Ile–Trp–OH	41	95	898.6	900
8	R–Phe–Phe–Ava–Trp–OH	50	98	835.0	836

[a] R = Ph–Ph–CH_2–O–N = CH–CO.
[b] Yield of the crude mixture, referred to the substitution level of the resin.
[c] Chromatography purity, calculated from the peak areas in analytical HPLC of the purified products.
[d] Calculated molecular weight.

Table 2
Effects of peptides on [^{125}I]ET-1 binding to ET_B receptor

Number	Sequence	% Inhib. [a] ET_B
2	R–Phe–Asp–Ile–Ile–Trp–OH	66.3 ± 2.5
3	R–Phe–Abu–Ile–Trp–OH	29.1 ± 2.1
4	R–Phe–Ava–Ile–Trp–OH	59.7 ± 5.2
5	R–Phe–Ahx–Ile–Trp–OH	33.2 ± 1.3
6	R–Phe–Aoc–Ile–Trp–OH	36.0 ± 3.3
7	R–Phe–Ado–Ile–Trp–OH	16.9 ± 0.6
8	R–Phe–Phe–Ava–Trp–OH	18.1 ± 0.4

[a] Peptides were tested at a concentration of 1 μM. Results are presented as mean ± S.E. of three independent experiments.

logue **8** we replaced the dipeptide Ile^{19}–Ile^{20} of compound **2** with the spacer Ava in order to further investigate the role of Ile^{20} residues in a peptidomimetic structure, and to possibly extend the replaced sequence from two to three residues. Concomitantly, Asp^{18} was replaced by Phe, to increase the overall hydrophobicity of the N-terminal portion, in line with our previous observations [10]. Thus, the structure of compound **8** is quite far from the primitive sequence of ET(16–21), the only conserved residue being the C-terminal Trp^{21}.

The biological activity of these analogues is reported in Table 2. The effect on [^{125}I]ET-1 specific binding to ET_B receptor of the reference compound and its modified analogues **3**–**8** is shown. The same peptides were modest inhibitors of [^{125}I]ET-1 binding to ET_A receptor (data not shown).

Analogue **4**, containing the Ava spacer, whose length corresponds to that of the dipeptide backbone, maintains quite the same affinity as compound **2**, confirming that the side-chains of the two residues are not important for the binding interactions of the reference compound, as previously reported [10]. Compounds **3** and **5**, containing spacers characterized by one methylene unit less (Abu) and one methylene unit more (Ahx), respectively, than the original backbone, had a decrease in binding affinity of about 50%. Analogue **6**, with the longer Aoc spacer, showed a binding affinity to the ET_B receptor comparable to that of the previous analogues **3** and **5**. Analogue **7**, in which was introduced the longer, more hydrophobic and flexible, Ado spacer, gave a drop of affinity at the ET_B receptor. We hypothesize that a high mobility of the two pharmacophoric regions penalizes the binding interaction of the antagonist with the ET_B receptor. All together, these data indicate that the side-chains of the dipeptide Asp^{18}–Ile^{19} are not involved in the interaction with the ET_B receptor and that they can be replaced by an alkyl spacer. In particular, they show that the distance maintained by the dipeptide backbone among the two pharmacophoric portions is fundamental for the correct interaction of the ligand with the ET_B receptor. Compound **8**, where the Ile^{19}–Ile^{20} dipeptide was replaced by the Ava spacer, was also found to be inactive, thus confirming the importance of the Ile^{20} side-chain in the C-terminal dipeptide.

In conclusion, we have confirmed that the Asp^{18}–Ile^{19} residues are not fundamental for the binding interaction of the lead compound with the ET_B receptor and that they can be replaced by simplified structures, such as alkyl spacers.

More importantly, we have shown the necessity of the six atom backbone of the natural dipeptide for a good and effectual orientation of the two pharmacophoric portions in the ligand–receptor interaction. Finally, we have obtained a simplified compound **4**, which could be a useful tool for further investigation, and an important step toward a rational design of pseudopeptide ligands for ET_B receptor.

3. Experimental

3.1. Chemistry

Protected standard amino acids, Fmoc–Abu–OH and Fmoc–Ahx–OH, were purchased from Novabiochem; Fmoc–Ado–OH was from Chemtech. Fmoc–Trp(Boc)–Novasyn PA500 resin, HOBt and HBTU were from Novabiochem. Analytical grade CH_3CN, DMF and MeOH were obtained from Lab-Scan and all other reagents were from Sigma, Aldrich, and Acros.

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra were obtained with a Bruker AC-200 instrument in ca. 2% solution of $CDCl_3$ using Me_4Si as the internal standard. Analytical TLCs were carried out on 0.25 mm layer silica gel plates containing a fluorescent indicator (Macherey-Nagel Alugram® SilG/UV254 Art. Nr. 81813) using the following eluants: 1 = $CHCl_3$/EtOH 9:1; 2 = 1-BuOH/CH_3COOH/H_2O 3:1:1; 3 = toluene/EtOH 7:1; 4 = AcOEt/petroleum ether 1:3. Spots were detected under UV light (254 nm). Evaporations were made in vacuo (rotating evaporator); $MgSO_4$ was always used as the drying agent. Elemental analyses were performed in our analytical laboratory and agreed with the theoretical values to within ± 0.4%.

3.1.1. General procedure for solid phase peptide synthesis with Fmoc strategy, automated synthesis

Peptides were synthesized following the classical continuous-flow solid phase method on a Milligen 9050 Automatic Synthesizer using standard Fmoc/^{t}Bu chemistry [13]. All the syntheses were performed on the Fmoc–Trp(Boc)–Novasyn PA500 resin using dimethylformamide (DMF) as the solvent. The activation reagents 2-(1H-benzotriazolyl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*-hydroxybenzo-

triazole (HOBt)/*N*-methylmorpholine (NMM) and protected aminoacids or iminoacyl group [10] in four-fold excess were recycled in the coupling step over a time of 50 min at a flow rate of 4 ml/min. Fmoc-deprotection was performed with 20% (v/v) piperidine in DMF, 9 min at 4 ml/min.

Peptides were cleaved from the resin and deprotected from the side-chain protecting groups with a solution of trifluoroacetic acid (TFA)/phenol/anisole [14] (92/4/4 v/v) at room temperature for 1 h under magnetic stirring, precipitated with cold ether and filtered. The filtrate was dissolved in methanol and treated for 72 h at room temperature to convert the carbamate on the indole moiety of the tryptophan to the desired products; the total deprotection was observed by analytical high performance liquid chromatography (HPLC). The methanol was removed under vacuum and the residue was suspended in water and lyophilized. Crude peptides were analyzed by analytical RP-HPLC on a Beckman System Gold apparatus in the following condition: Vydac C_{18} column, 0.46×15 cm; eluant A: 0.1% TFA in H_2O, eluant B: 0.1% TFA in CH_3CN; gradient from 20 to 95% B over 25 min; flow 1 ml/min; UV detection at 210 nm.

All peptides were purified by preparative RP-HPLC on a Beckman System Gold apparatus; column Vydac C_{18} (2.2×25 cm); eluants as above; gradient from 20 to 40% B over 100 min, flow 8 ml/min; UV detection 210 nm.

The characterization of peptides was performed by electrospray ionization mass spectrometry (ES/MS) [15], (see Table 1).

3.2. Synthesis of Fmoc-8-aminooctanoic acid

The amino acid (1 equiv.) was suspended in water, dissolved under reflux and pH adjusted to 9 with 10% Na_2CO_3 solution. A solution of Fmoc–OSu (1 equiv.) in dioxane was then added dropwise over 20 min and the mixture was stirred under reflux for 24 h. The reaction mixture was then diluted with water, acidified to pH 3 with HCl and extracted twice with CH_2Cl_2. The organic layer was washed, dried over Na_2SO_4, and the solvent removed to dryness. Yield: 85%; m.p. 118–119°C; $Rf_1 = 0.51$, $Rf_2 = 0.92$, $Rf_3 = 0.29$, $Rf_4 = 0.05$; ^{1}H NMR (1×10^{-2} M $CDCl_3$) δ 7.80–7.26 (m, 8H, Fmoc aromatic CH), 4.79 (s, 1H, NH), 4.41 (d, 2H, Fmoc CH_2), 4.22 (t, 1H, Fmoc 9-CH), 3.16 (m, 2H, Aoc αCH_2), 2.35 (t, 2H, Aoc βCH_2), 1.68–1.24 (m, 10H, Aoc CH_2).

3.3. Pharmacology

Male Sprague-Dawley rats (160–250 g) were killed by decapitation. The cerebellum was removed and homogenized using a Polytron homogenizer in 10 volumes (w/v) of ice-cold buffer containing 0.32 M sucrose, 20 mM Hepes-Tris (pH 7.4), 5 mM EDTA and protease inhibitors (0.1 mM bacitracin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml leupeptin). The homogenated was centrifuged at $48\,000 \times g$ for 15 min at 4°C. The pellet was resuspended in 10 volumes of 20 mM Hepes-Tris (pH 7.4), 5 mM EDTA (buffer HT) containing protease inhibitors. The membrane homogenated was centrifuged at $48\,000 \times g$ for 15 min at 4°C. The tissue preparation was used either immediately or stored in aliquots at −80°C.

3.3.1. Radioligand binding assay

Binding assays were performed as described by Cody et al. [16], with some modifications. Briefly, cerebellar (5 μg proteins) membranes were incubated in 0.25 ml of T_1 buffer (20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 0.1 mM bacitracin, 0.1 mM PMSF, 1 μg/ml leupeptin, and 5 μg/ml aprotinin) with 15 pM [^{125}I]ET-1 (2000 Ci/mmol) for 2 h at 37°C. Reactions were terminated by addition of 3 ml of ice-cold T_2 buffer (50 mM Tris–HCl, pH 7.3, 0.1 mM bacitracin) and rapid filtration of samples through GF/C glass fiber filters which had been soaked in T_2 buffer containing 0.2% bovine serum albumin (BSA) for 24 h. The filters were then washed four times with 3 ml of ice-cold T_2 buffer. Assays were done in duplicate and non-specific binding was checked in the presence of 100 nM ET-1. [^{125}I]ET-1 was diluted in T_1 buffer plus 1 μg/ml BSA, while ET-1 and ET-3 were dissolved in the same buffer without BSA. Peptides were diluted in T_1 buffer plus 1 mg/ml BSA and 1% dimethyl sulfoxide (DMSO) to a concentration of 5 mM.

Saturation binding experiments (EDBA/LIGAND Software, Cambridge, UK, and GraphPad Prism Software, San Diego, CA) allowed the calculation of equilibrium dissociation constant (K_D) and binding capacity (B_{max}) values for the binding site in cerebellar membrane that were 54 pM and 2.23 pM/mg proteins, respectively.

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