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Toward the rational development of peptidomimetic analogs of the C-terminal endothelin hexapeptide: development of a theoretical model

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Abstract

In an early report on the structure–activity relationship of endothelin (ET) peptides, it was reported that the C-terminal hexapeptide ET(16–21), His-Leu-Asp-Ile-Ile-Trp, is the minimum ET fragment which maintains biological activity in some, but not all the tissues responding to ETs. Subsequently, other authors described a series of analogs of this peptide, in which the His16 residue was replaced by non-natural amino acids, characterized by bulky aromatic side chains. Among them, two well-characterized non-selective ETA/ETB antagonists were PD 142893 and PD 145065; interest in these potent ET antagonists was, however, reduced by their peptidic structure which was likely to lead to undesirable properties such as poor bioavailability and short duration of action. On the basis of these premises, our previous studies led to the development of a peptidomimetic ligand of ET receptors (compound **3**), based on the replacement of the His16 residue of ET(16–21) with an (*E*)-*N*-(benzyloxy)iminoacyl moiety; compound **3** proved to possess a certain affinity for ET receptors, albeit lower than that shown by PD 142893 and PD 145065. We report here on ETA/ETB binding affinity of compounds **4–12**, designed as a new series of ET(16–21) analogs. Compounds **4** and **5** were practically devoid of any affinity; derivatives **6–12** exhibited appreciable affinity indices for ETB receptors higher than that shown by **3**, even if still lower than that obtained for PD 145065. This paper also describes the development of a pharmacophoric model able to explain the ET receptor binding properties of our hexapeptide analogs compared with those of PD 142893 and PD 145065 and IRL2500, recently reported as a potent ETB selective endothelin antagonist.

Keywords: Endothelin; ET(16–21); ETA receptor; ETB receptor; Theoretical model

1. Introduction

Endothelins (ETs) are a family of 21-residue peptides recently discovered in the mammalian genome. Three isopeptides have been described so far, called ET-1, -2 and -3. They are characterized by two conserved disulphide bridges forming an N-terminal 15-residue bicyclic moiety, linked to a conserved, very hydrophobic C-terminal hexapeptidic tail [1]. ETs are endowed with a variety of biological actions [2], most notably at the cardio-vascular level: ET-1 is the most potent vasoconstrictor known [3]. These biological effects are exerted through interaction with specific membrane-bound receptors, belonging to the G-protein coupled, rhodopsin-like superfamily. At least two receptor subtypes have been cloned, sequenced and characterized in several species, including humans. They have been termed ETA, selective for ET-1, and ETB, unselective, and are widely distributed in several tissues and organs. The function and distribution of this receptor family have been found to vary within different species [1,4,5].

In previous studies on the structure–activity relationship (SAR) of ET peptides, we described the C-terminal hexapeptide ET(16–21), His-Leu-Asp-Ile-Ile-Trp, as the minimum ET fragment which maintains biological activity in some, but not all the tissues responding to ETs [6,7]. These results were subsequently developed by Cody and co-workers at Parke-Davis through an SAR study of this hexapeptide; in particular, it was reported that an analog in which the His16 residue of ET(16–21) is replaced by Ac-*D*-His (**1**) exhibited a marked increase in binding affinity

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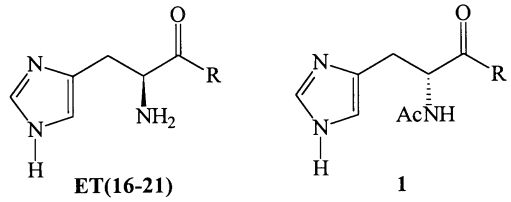
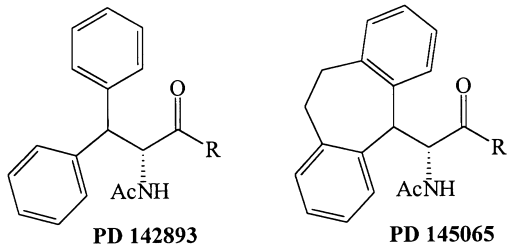
[8]; moreover the substitution of the Ac-*D*-His residue in the above compound **1** with bulky aromatic non-natural *D*-amino acids, led to compounds possessing interesting antagonistic properties towards ET receptors. Among them, two well-characterized non-selective ETA/ETB antagonists were PD 142893 and PD 145065, in which the His16 residue was replaced by an Ac-*D*-Dip (Dip = 3,3-diphenylalanine) and an Ac-*D*-Bhg (Bhg = 10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cycloheptenglycine) residue, respectively [8,9].

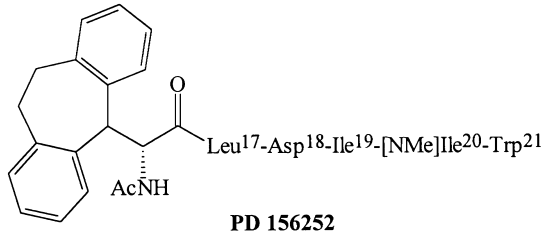
Interest in these potent ET antagonists was, however, reduced by their peptidic structure which was likely to lead to undesirable properties such as poor bioavailability and short duration of action [10]. On the basis of these premises, we thought it conceivable to further develop these studies through the design of peptidomimetic ligands of ET receptors derived from the ET(16–21) hexapeptide structure.

Apparently, the same strategy was followed also at Parke-Davis, as these authors very recently reported on a pseudopeptide analog of PD 145065, i.e. PD 156252, which is greatly stabilized with respect to proteolytic degradation by the introduction of an *N*-methyl amino acid in position 20 [11].





R = Leu[17]-Asp[18]-Ile[19]-Ile[20]-Trp[21]



Our first approach to these studies was the investigation of new analogs of ET(16–21) based on the replacement of the His16 residue with a non-amino acid moiety: thus, an analogue (**2**) characterized by the C-terminal pentapeptide ET(17–21), with the N-terminal amino function acylated by the bulky, aromatic 9-fluorenyl-methoxy-carbonyl (Fmoc) group, was found to antagonize ET-3 but not ET-1 in two different biological models [12,13]. Subsequently, we studied compound **3** in which the His16 residue of ET(16–21) was replaced with an (*E*)-*N*-(benzyloxy)imino-acyl moiety. This compound was designed on the basis of considerations about the high potency of PD 142893 and PD 145065: previous alanine scan studies of ET-1 indicated that the aromatic residues Tyr13 and Phe14 are essential for the interaction of ET-1 with its receptors [14,15]; these premises prompted us to hypothesize that the interesting activity shown by PD 142893 and PD 145065 might be related to the capacity of their N-terminal bulky aromatic side chains to occupy the receptor sites which normally interact with the aromatic moieties of the Tyr13 and/or Phe14 residues of ET-1. In order to test whether this hypothesis could be reasonable, simple molecular modeling techniques were employed: the backbone of the N-terminal bulky aromatic residues of **1** and PD 145065 were superimposed on the backbone of the His16 residue of the three-dimensional structure of ET-1 [16,17] and the distances of these aromatic portions from the aromatic rings of the Tyr13 and/or Phe14 residues were evaluated. The result of this superimposition (see Fig. 1) confirmed our hypothesis, as the antagonistic properties of **1** and PD 145065 were in fact related to the ability of their aromatic side chains to interact

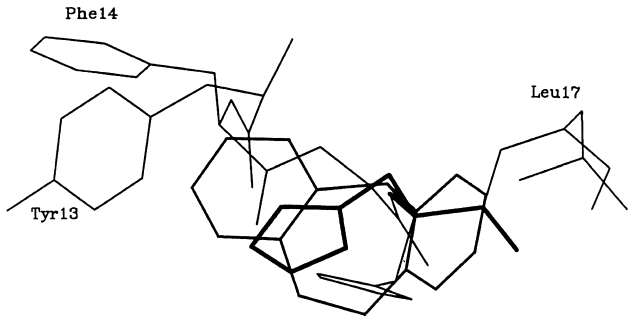


Fig. 1. Superimposition of residues 16–17 of **1** (the thickest line) and of PD 145065 (the medium line) on the ET(13-17) peptidic portion (the thinnest line).

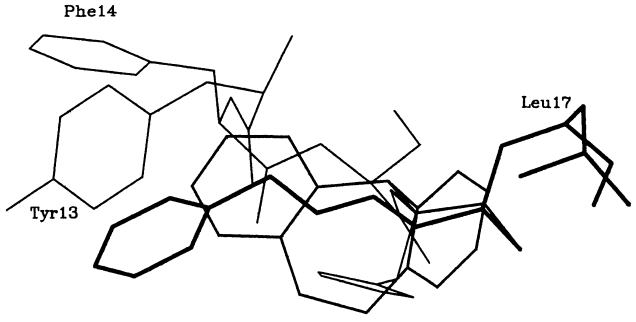
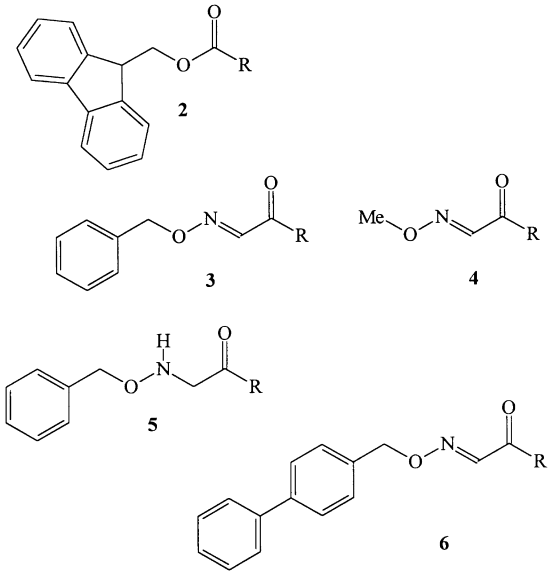


Fig. 2. Superimposition of residues 16-17 of 3 (the thickest line) and of PD 145065 (the medium line) on the ET(13-17) peptidic portion (the thinnest line).

with the spatial region occupied by the Tyr13 and Phe14 residues of ET-1.

On this basis, we thought that the replacement of the His16 residue of ET(16–21) with an (*E*)-*N*-(benzyloxy)iminoacyl moiety as in compounds **3** might lead to a further increase in affinity for ET receptors, as, according to our molecular model, its aromatic moiety proved to be capable of getting closer to the regions occupied by the aromatic ring of Tyr13 or Phe14 (see Fig. 2).



R = Leu^{17}-Asp^{18}-Ile^{19}-Ile^{20}-Trp^{21}

Binding affinity studies on ET receptors indicated that compound **3** possessed a certain affinity for ET receptors (see Table 2), albeit lower than that shown by PD 145065. Therefore, these results did not support the working hypothesis regarding the role played by the aromatic bulky groups of the N-terminal amino acid residue: in fact, even if in the proposed molecular model the aromatic moiety of **3** seemed to be able to interact with the spatial region occupied by the aromatic ring of Tyr13 or Phe14 better than the N-terminal bulky aromatic residue of PD 145065, the affinity of **3** for ET receptors resulted to be lower than that shown by PD 145065.

However, the fact that compound **3** still possessed a certain affinity for ET receptors might indicate that its lipophilic N-terminal non-amino acid (*E*)-*N*-(benzyloxy)iminoacyl moiety could play a certain role in the interaction with ET receptors. In order to verify this hypothesis, we synthesized compounds **4** and **5** in which the phenyl ring and the oxime group of the (*E*)-*N*-(benzyloxy)iminoacyl moiety of **3** were replaced respectively by a methyl group (**4**) and a saturated oxyamine moiety (**5**). Compounds **4** and **5** proved to be practically devoid of any affinity for ET receptors, thus confirming the hypothesis that the (*E*)-*N*-(benzyloxy)iminoacyl moiety of **3** could tentatively be considered as a pharmacophoric portion for the interaction with ET receptors. At this point of our research, we thought it reasonable to try to develop new ET inhibitors possessing this kind of moiety in their structure. We therefore proceeded to the elucidation of the role of each amino acid residue of the ET(17–21) peptidic portion of **3** 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 [18]. The results of these studies indicated that in compound **3**, only the C-terminal dipeptide Ile20-Trp21 including the free carboxylic function was important for the affinity and could be considered as a second pharmacophoric portion of **3**, while the central portion of the molecule seemed to have the role of maintaining the relative topography of the two termini. These results were in agreement with previous studies on ET in which the importance of the C-terminal dipeptide Ile20-Trp21 for interaction with ETs receptors had already been demonstrated [14,15].

As a continuation of these studies, we now report on the synthesis and ETA/ETB binding affinity of compounds **6–12** (see Table 1) designed as a new series of ET(16–21) analogs; compound **6** presents a phenyl ring in the *para* position of the N-terminal (*E*)-*N*-(benzyloxy)iminoacyl moiety of **3** and therefore might have made it possible to verify whether an increase in the lipophilicity characteristic of this hypothetical pharmacophore, as well as leaving the C-terminal dipeptide Ile20-Trp21 (i.e. the second pharmacophore) unchanged, could lead to an improvement in the affinity for ET receptors. In compounds **7–10**, the Leu17 or Asp18 residues of **3** and **6** were systematically replaced by a Phe residue, while in compound **11**, two Phe residues replace the Leu17 and Asp18 residues of **6**. These latter modifications were suggested by the fact that previous studies from Doherty and co-workers indicated that the ET receptor affinity in analogs of ET(16–21), in which the His16 residue is replaced by a *D*-Phe residue, increases when Leu17 or Asp18 residues are replaced with a Phe one [9]. Lastly, compound **12** differs from derivative **9** in the replacement of the Asp18-Ile19 dipeptidic portion with an aliphatic spacer (Ava) possessing the same length but without the substituents present on the side chain of the dipeptide. This type of modification diminishes the peptidic nature of these types of compounds and, at the same time, places greater emphasis on the two moieties which might be thought to act as the pharmacophoric portions of these types of structures, i.e. the lipophilic N-terminal portion and the Ile20-Trp21 dipeptidic C-terminal one.

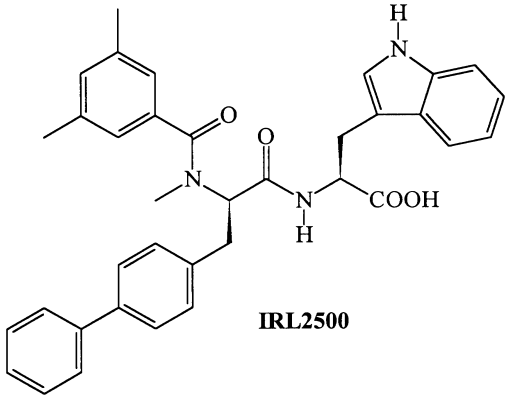
This paper also reports on the development of a pharmacophoric model able to explain the ET receptor binding properties of our hexapeptide analogs compared with those of PD 142893 and PD 145065. For this purpose, the new compound IRL2500, reported to be a potent ETB-selective endothelin antagonist [19], proved to be particularly useful: its structure, simpler and less flexible than those of the ET(16–21) analogs, allowed us to simplify the definition of the pharmacophoric model. For this

Table 1
Sequences and analytical characterization of compounds **3–12**

No.	Compound[a]	Purity (%)[b]	R_t[c]	M[d]	ES/MS $[M + H]^+$
3	R-Leu-Asp-Ile-Ile-Trp	99	19.9[e]	820.1	821
4	R′-Leu-Asp-Ile-Ile-Trp	98	17.0[e]	744.3	745
5	R″-Leu-Asp-Ile-Ile-Trp	98	18.2[e]	821.8	823
6	R‴-Leu-Asp-Ile-Ile-Trp	99	22.4[e]	895.7	897
7	R-Phe-Asp-Ile-Ile-Trp	97	20.0[e]	854.3	855
8	R-Leu-Phe-Ile-Ile-Trp	98	22.4[e]	851.6	853
9	R‴-Phe-Asp-Ile-Ile-Trp	98	17.2[f]	930.3	931
10	R‴-Leu-Phe-Ile-Ile-Trp	98	19.5[f]	928.0	929
11	R‴-Phe-Phe-Ile-Ile-Trp	96	19.3[f]	961.9	963
12	R‴-Phe-Ava-Ile-Trp	99	16.8[f]	801.3	802

[a] R = Ph—CH_2—O—N=CH—CO. R′ = CH_3—O—N=CH—CO. R″ = Ph—CH_2—O—NH—CH_2—CO. R‴ = Ph—Ph—CH_2—O—N=CH—CO. Ava = NH—CH_2—CH_2—CH_2—CH_2—CO.
[b] Chromatographic purity, calculated from peak areas in analytical HPLC.
[c] Retention time in analytical HPLC.
[d] Calculated molecular weight.
[e] Chromatographic conditions: method A (see Section 6).
[f] Chromatographic conditions: method B (see Section 6).

reason the conformation of IRL2500 was studied by means of theoretical calculations and compared with those of PD 142893, PD 145065 and the most significant of our new compounds.



IRL2500

2. Chemistry

Compounds **3**, **5** were synthesized as previously reported [20]. Compounds **4**, **6–12** were synthesized by the continuous-flow solid phase method, using standard Fmoc chemistry [21] on a Novasyn PA500 resin. Boc and t-Bu protecting groups were used for the side chains of Trp and Asp, respectively. Couplings were performed using HBTU/HOBt/NMM activation and a five-fold excess of amino acids. The N-terminal amino group was acylated with the following acids: (*E*)-*N*-(Benzyloxy)iminoacetic acid [22] (compounds **7**, **8**), (*E*)-*N*-(methoxy)iminoacetic acid [23] (compound **4**), and (*E*)-*N*-(*p*-phenyl-benzyloxy)iminoacetic acid (compounds **6**, **9–12**), prepared by treatment of *O*-[(4-phenylphenyl)methyl]hydroxylamine hydrochloride with glyoxylic acid. Each acid was coupled to the N-terminal residue of the relevant peptide moiety, still on the resin, using five-fold excess and HBTU/HOBt/NMM activation. Peptides were cleaved from the resin and deprotected from the side-chain protecting groups with TFA/phenol/anisole (92:4:4, v/v/v; 1 h at room temperature) [20]. Crude peptides were purified to homogeneity by preparative reverse-phase HPLC. The final products were characterized by analytical HPLC and ES-MS (see Table 1) [24].

3. Biological results

The affinity of derivatives **6–12** towards ETA/ETB receptors was checked by binding tests on rat uterus membranes for ETA receptors and on rat cerebellar membranes for ETB receptors using [^{125}I]ET-1 as the specific ligand for ETA/ETB receptors. The results of these tests are reported in Table 2, together with those obtained for compound **3** and PD 145065, taken as reference compounds. The binding affinity for ETA/ETB receptors was expressed as percentage inhibition at a concentration of 1 μM, and, for compounds possessing an inhibition percentage $> 50\%$ at this concentration, as K_i.

All the newly synthesized compounds exhibited appreciable affinity indices for ETB receptors. Compound **6**, which differs from **3** in the presence of a phenyl ring in the *para* position of the N-terminal (*E*)-*N*-(benzyloxy)iminoacyl moiety, proved to possess an affinity for ETB receptors higher than that shown by **3**, with a K_i value of 0.96 μM. A slight increase in the affinity was obtained for compounds **7–10**, in which the Leu17 or Asp18 residues of **3** and **6** were systematically replaced by a Phe residue; their K_i values for ETB receptors ranged from 0.35 to 0.60 μM. Compound **11**, in which both the Leu17 and Asp18 residues of **6** were replaced by two Phe residues proved to be the most active

Table 2
Displacement of [^{125}I]ET-1 bound to ET-A and ET-B receptors by compounds **3**, **6–12**

No.	Compound[a]	ET-A[b]	ET-B[d]	
		% Inhibition (1 μM)[c]	% Inhibition (1 μM)[c]	K_i (μM)[e]
3	R-Leu-Asp-Ile-Ile-Trp	25.2 ± 1.8	21.8 ± 3.3	–
6	R‴-Leu-Asp-Ile-Ile-Trp	20.0 ± 2.1	57.7 ± 0.4	0.96 ± 0.30
7	R-Phe-Asp-Ile-Ile-Trp	8.8 ± 0.3	60.7 ± 0.6	0.35 ± 0.07
8	R-Leu-Phe-Ile-Ile-Trp	14.9 ± 2.6	62.0 ± 2.1	0.58 ± 0.10
9	R‴-Phe-Asp-Ile-Ile-Trp	18.6 ± 4.3	66.3 ± 2.5	0.60 ± 0.08
10	R‴-Leu-Phe-Ile-Ile-Trp	7.6 ± 2.2	53.9 ± 1.3	0.46 ± 0.09
11	R‴-Phe-Phe-Ile-Ile-Trp	17.2 ± 1.5	69.0 ± 2.5	0.27 ± 0.04
12	R‴-Phe-Ava-Ile-Trp	18.1 ± 4.9	59.7 ± 5.2	0.48 ± 0.03
	PD145065	NT[f]		0.011[g]

[a] R = Ph—CH_2—O—N=CH—CO. R‴ = Ph—Ph—CH_2—O—N=CH—CO. Ava = NH—CH_2—CH_2—CH_2—CH_2—CO.
[b] Displacement of specific [^{125}I]ET-1 binding to rat uterus membranes.
[d] Displacement of specific [^{125}I]ET-1 binding to rat cerebellar membranes.
[c] Percentage of inhibition at the concentration of 1 μM. Values represent the means ± SE of three experiments.
[e] Binding data were computer-analyzed by non-linear least squares analysis (GraphPad Prism Softwares, San Diego, CA) and IC_{50} determined. The IC_{50} values were converted to K_i (inhibition constant) values by the Cheng and Prusoff equation [30]. Values represent the means ± SE of three experiments.
[f] Not tested.
[g] This K_i value represents the average of two experiments.

for ETB receptors with a K_i value of 0.27 μM. Lastly compound **12**, which differs from derivative **9** in the replacement of the Asp18-Ile19 dipeptidic portion with an aliphatic spacer (Ava), proved to possess an affinity for ETB receptors similar to that of derivatives **6–11**.

As regards the affinity for ETA receptors, compounds **6–12** displayed inhibition percentages lower than those on ETB receptors and very similar to that obtained for **3**.

Furthermore, compounds **6–12** exhibited an affinity for ETA/ETB receptors lower than that obtained for PD 145065, even if the selectivity for ETB receptors was found to be slightly more marked.

4. Theoretical calculations

With the aim of rationalising the results obtained for compounds **6–12** and of understanding the difference of affinity between them and the more potent PD compounds, a comparative conformational analysis was carried out by means of molecular mechanics and dynamics on all these compounds [25]. The ET(16–21) hexapeptide portion of endothelin was previously found to possess quite a high conformational freedom [26]. NMR spectroscopy and X-ray diffraction on endothelin did not reveal any evident structuring of this hexapeptide portion [26].

As this conformational freedom should undoubtedly be present also in the ET(16–21) analogs and such compounds display a considerable molecular complexity, it was not possible to perform an exhaustive conformational analysis on them. We therefore carried out a high-temperature molecular dynamics simulation for each molecule considered; some conformations were then regularly sampled and minimized by means of molecular mechanics calculations.

Fig. 3 shows the results of conformational analysis for compounds PD 142893 and PD 145065, **6** and **11** (the results regarding compounds **3**, **7–10** and **12** are completely analogous). For each compound, the figure shows the minimal energy conformation and the superimposition of the five lowest energy conformations, together with the graphs plotting the distance between the alpha carbon of the C-terminal Trp21 residue and the center(s) of the phenyl ring(s) of the non-natural residue 16, against the conformational energy. The results of conformational analysis indicated that all compounds considered had a really considerable conformational freedom. However, it was also found that the backbone of all the molecules (but especially of PD 142893 and PD 145065) has the tendency to assume a common folded conformation, which makes the aromatic systems of the two terminal residues quite close. In any case these aromatic rings of the terminal residues can move freely. This observed conformational trend seems to be consistent with a very recent report of a ^{1}H NMR and molecular dynamics simulation study on PD 145065 [11].

A comparison of the conformational data for compounds PD 142893 and PD 145065 with those of **3** and **6–12** suggests that the latter possess a greater conformational freedom, as demonstrated by the fact that in **3** and **6–12** there is a larger number of conformations with similar energies than in PD 142893 and PD 145065 (see Fig. 3).

However, this difference does not seem to be sufficiently significant to rationalize the great difference in potency between PD 142893 and PD 145065 with respect to **3** and **6–12**.

Moreover the conformational freedom described above, together with the fact that PD 142893 and PD 145065 in their preferred conformations are not superimposable at all,

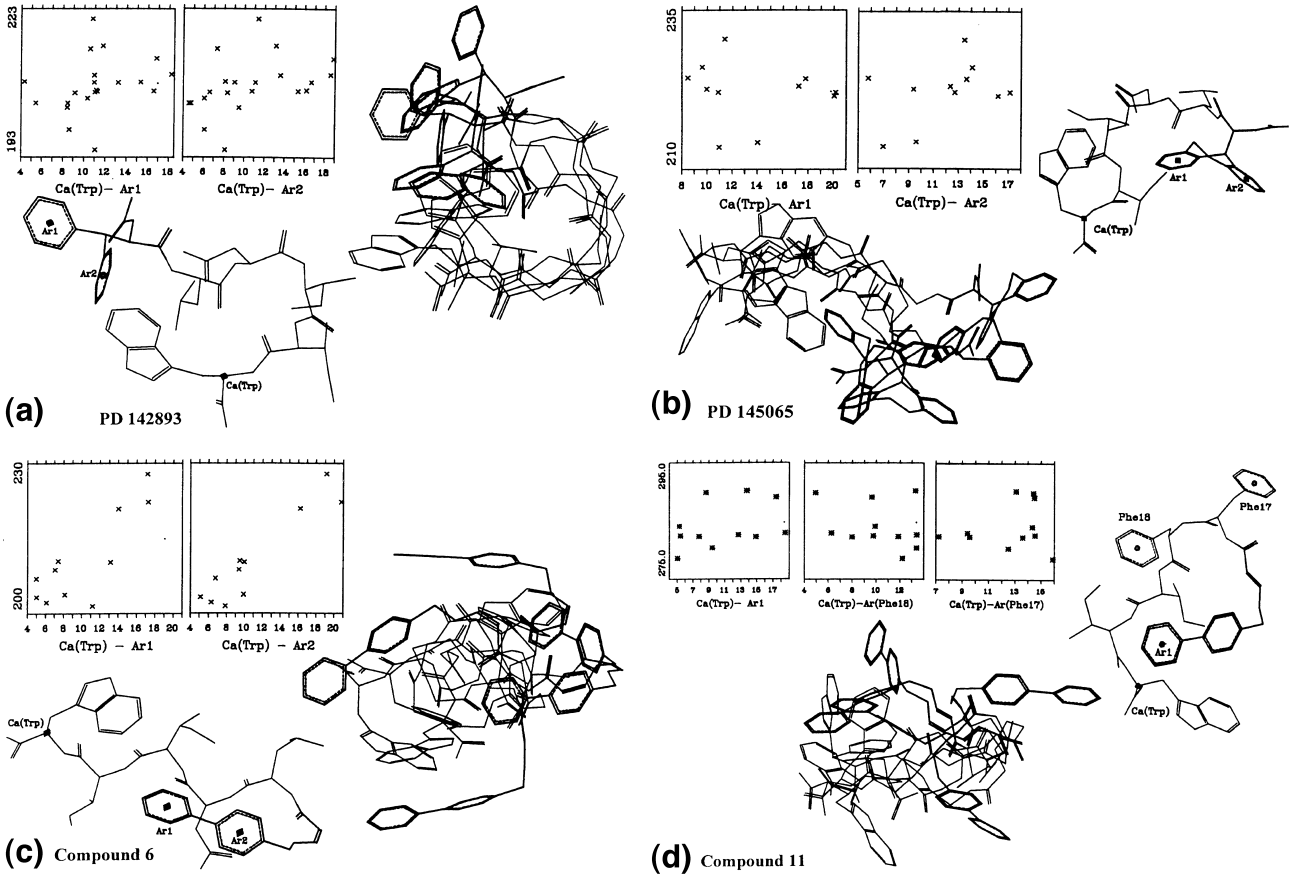


Fig. 3. Conformational analysis of (a) PD142893, (b) PD 145065, (c) compound **6** and (d) compound **11**. The conformational energy (kcal/mol) is plotted against the distance between the alpha carbon of the Trp21 [Ca(Trp)] and the centers of the phenyl rings of the non-natural residue 16 (Ar1 and Ar2) and, in the case of compound **11**, also the centers of the phenyl ring of residues 17 and 18. The lowest energy conformation is shown together with the superimposition of the five lowest energy conformations; in this case, for residues 17–20, only the backbone is shown.

does not allow us to use these potent compounds as starting points for the building of a reliable pharmacophore model for interaction with ETA/ETB receptors.

Recently, a new compound, IRL2500, was found to be active as a potent selective ETB antagonist [19]. IRL2500 has a tripeptide-like structure, which therefore should have a better defined conformational trend than hexapeptide analogs like PD's. Moreover the SAR of IRL2500 can also be well-defined: four molecular portions can be identified as fundamental for the eliciting of the high potency: the indole system, the carboxylic function of the Trp residue, the xylene ring and the diphenyl moiety.

Fig. 4 reports the results of the conformational analysis performed on this compound; it confirms that IRL2500 has quite a well-defined conformation: substantially there are two preferred conformations with a similar energy, which differ only in the position of the indole ring, which is close to the xylene ring or the diphenyl system, respectively.

In order to find a common pharmacophoric model able to explain the high potency of PD 142893, PD 145065 and IRL2500 we tried superimposing the pharmacophoric groups of IRL 2500 on the corresponding groups of the PDs; the chemical characteristics of their groups suggested superimposing (a) the terminal Trp residues of all compounds together and (b) the diphenyl moiety of IRL2500 on the bulky aromatic groups of PD 142893 and

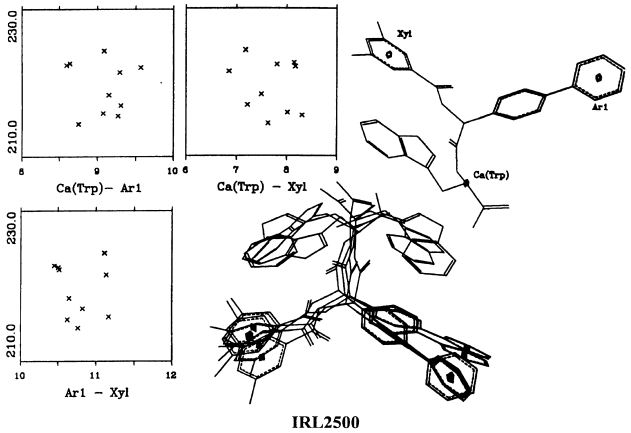


Fig. 4. Conformational analysis of IRL2500. The conformational energy (kcal/mol) is plotted against the distance between the alpha carbon of the Trp21 [Ca(Trp)] and the centers of the aryl rings Ar1 and Xyl.

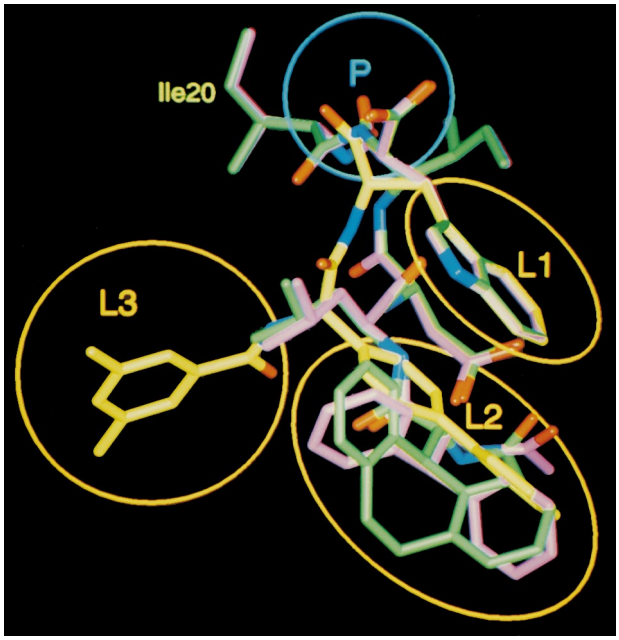


Fig. 5. Superimposition of IRL2500, PD 142893 and PD 145065. The carbon atoms of IRL2500, PD 142893 and PD 145065 are colored yellow, magenta and green, respectively; the oxygen atoms are colored red and the nitrogen ones blue; the hydrogen atoms are hidden. The molecular regions circled are those which should interact with: (P) the positive-charged site for interaction with the C-terminal carboxylate moiety; (L1) the lipophilic pocket able to host the indole side chain of the C-terminal Trp; (L2) the lipophilic pocket able to host the bulky aromatic moiety; (L3) the lipophilic pocket able to host the xylene moiety of IRL2500.

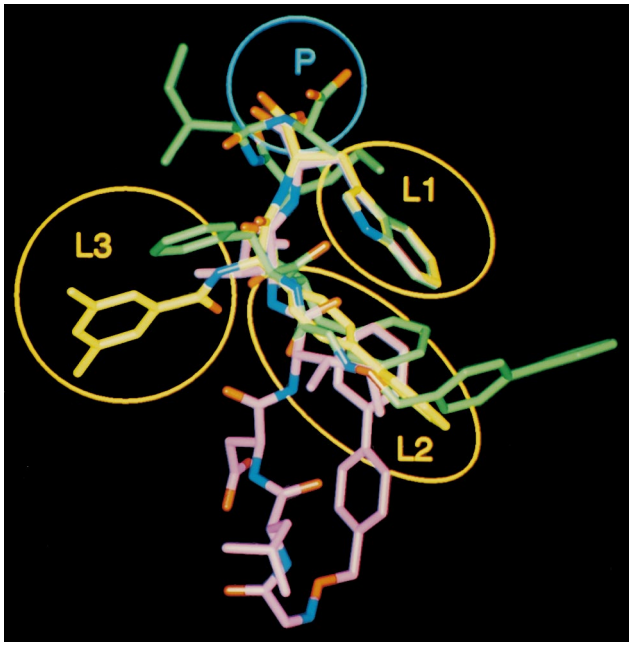


Fig. 6. Superimposition of IRL2500, compounds **6** and **11**. The carbon atoms of IRL2500, **6** and **11** are colored yellow, magenta and green, respectively; the oxygen atoms are colored red and the nitrogen ones blue; the hydrogen atoms are hidden. As in Fig. 5.

PD 145065. The xylene ring of IRL2500 was therefore considered to be a supplementary pharmacophore peculiar to this drug.

Unfortunately this kind of superimposition was not possible for any of the three compounds in their minimal energy conformations; therefore we decided to take into account also other low energy conformers. We found a good fit between PD 142893 in its minimal energy conformation and IRL2500 in a conformation which differed from the minimal energy one in the position of the indole ring and was unfavored only by about 0.8 kcal/mol. In the case of PD 145065 we found a conformer which fitted quite well with the considered conformers of PD 142893 and IRL2500 and was unflavored by about 8 kcal/mol with respect to its minimal energy conformation. The superimposition of the three compounds in these conformations (see Fig. 5) was used as the basis for the proposal of a pharmacophoric model for the interaction of these compounds with the ETA/ETB receptors. In this model, the negative carboxylate group of the terminal Trp residue (charged at physiological pH), the indole ring and the bulky lipophilic aromatic moiety (the diphenyl system of IRL2500, the Ac-*D*-Dip moiety of PD 142893 and the Ac-*D*-Bhg one of PD 145065) represent three common pharmacophoric features. The lipophilic xylene ring of IRL2500 is not superimposable on a corresponding group of PD 142893 and PD 145065 (in this spatial region these drugs have the side chain of a Leu residue, lipophilic but much less bulky) and therefore it could be considered as responsible for the ETB selectivity of IRL2500. Another reason for the ETB selectivity of IRL2500 could be assigned to the lack of a lipophilic group placed in the region occupied by the Ile20 side chain of PD 142893 and PD 145065. This Ile20 residue is generally considered to be important for the activity of endothelin and of the ET(16–21) analogs [14,15].

According to the characteristics of this model, it should be hypothesized that both the ETA and ETB receptors possess a positive-charged site (P) for interaction with the C-terminal carboxylate moiety and two lipophilic pockets able to host the indole side chain of tryptophan (L1) and the bulky aromatic moiety (L2), respectively; moreover the ETB receptor might also possess an additional lipophilic pocket (L3) able to host the xylene moiety of IRL2500 (see Fig. 5).

Finally, this proposed model was used in order to try to explain the lower activity of the series of compounds here synthesized with respect to IRL2500, PD 142893 and PD 145065 and the differences of activity within the series itself.

For this purpose, we inserted compounds **3** and **6–12** into the model. Fig. 6 illustrates how it is possible to superimpose compounds **6** and **11** on IRL 2500: the diphenyl moiety of **6** and **11** does not seem to be able to occupy effectively the lipophilic pocket L2, occupied by the same moiety of IRL2500 and by the bulky aromatic portions of PD 142893 and PD 145065; this observation might justify the lower

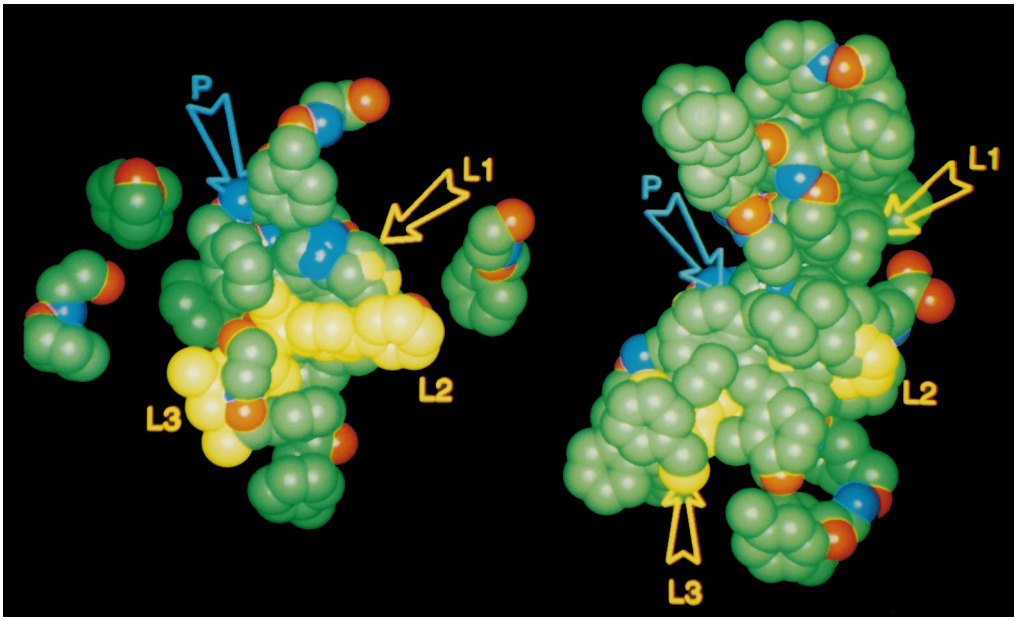


Fig. 7. Superimposition of IRL2500 on the ten lowest energy conformations of compound 3 (on the left) and those of compound **11** (on the right); all the hydrogen atoms are hidden. IRL2500 is completely colored yellow. As regards compounds **3** and **11**, only the C—O—N=C—Ph portion of residue 16 and the whole of residue Trp21 are shown; in the case of **11**, also the phenyl rings present on residues 17 and 18 are shown. Carbon, oxygen and nitrogen atoms of both **3** and **11** are colored green, red and blue, respectively.

ETA/ETB receptor affinities found for derivatives **6** and **11** with respect to PD 142893 and PD 145065. However, it should be pointed out that, as a consequence of their high conformational freedom (see Fig. 3), the possibility cannot be excluded of a certain favorable interaction between the diphenyl portion and the lipophilic pockets L2 (in agreement with the appreciable, albeit low, receptor affinity of these compounds) and L3 (in agreement with their ETB selectivity). The presence in **11** of Phe residues in position 17 and 18, is not able to change this situation significantly; however, it may be observed that these lipophilic residues might improve the interaction with both the lipophilic pockets L2 and L3 (in agreement with the slightly higher affinity of **11** for ETB receptors with respect to **6**) (see Fig. 6).

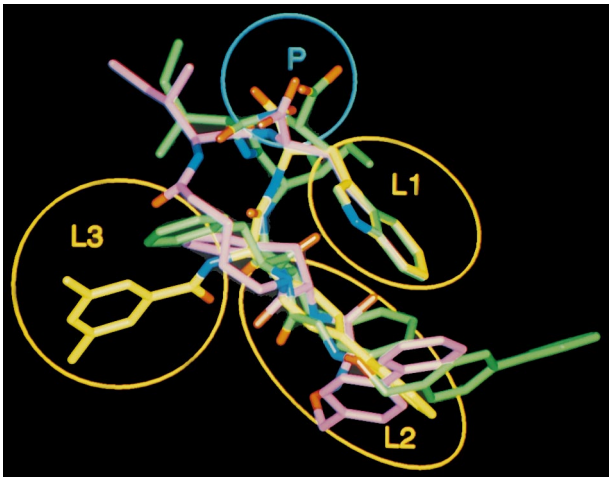


Fig. 8. Superimposition of IRL2500, compounds **11** and **12**. The carbon atoms of IRL2500, **11** and **12** are colored yellow, green and magenta, respectively; the oxygen atoms are colored red and the nitrogen ones blue; the hydrogen atoms are hidden. As in Fig. 5.

This fact might also explain why compound **3** possesses the lowest ETB binding affinity and the lowest ETB selectivity. In Fig. 7, the superimposition of ten conformers of **3** with IRL2500 together with the superimposition of ten conformers of **11** again with IRL2500 is shown: in the case of compound **3**, there are only conformers for which the phenyl ring of residue 16 can only partially occupy the pocket L2, while, in the case of compound **11,** there is the possibility that the pocket L2 or L3 can be occupied by the phenyl rings of its residues, even if no conformation of **11** exists that is able to simultaneously occupy both the pockets, in agreement with its lower binding affinity with respect to IRL2500.

As regards compounds **7–10,** the conformational trend is analogous to that of **6** and **11**, characterized by a conformation not easy to define (due to the rotational freedom) and by the ability of the Phe residues in position 17 or 18 to improve the interaction with L2 and L3 (in agreement with the ETA/ETB binding affinity profile of **7–10** similar to that found for **6** and **11**).

Finally, Fig. 8 shows the superimposition of a suitable low-energy conformer of **12** on **11** and IRL2500: it may be seen that the aliphatic spacer (Ava) of **12** may be able to replace the residues 18 and 19 without significantly modifying the relative spatial position of residues 16, 17, 20 and 21 with respect to **11**, and consequently to the other hexapeptide analogs **6–10**; this is in agreement with the similar ETA/ETB binding affinity profile of **12** with respect to **6–11**.

5. Conclusions

Compounds **6–11**, which differ from **3** in the insertion of phenyl moieties on the N-terminal non-amino acid residue and/or on the side chain of amino acid residues 17 and 18, proved to possess an increased affinity, prevalently toward the ETB receptor: these results indicate that an increase in the lipophilic characteristics of the proposed N-terminal pharmacophore might play a certain role in the interaction with ET receptors.

In particular, the presence of a phenyl ring in the para position of the (*E*)-*N*-(benzyloxy)iminoacyl moiety of **3** as in **6**, seems to be important for ETB receptor interaction, as demonstrated by the higher affinity of **6** for such type of receptor subtype with respect to **3**. Moreover, the compound which proved to possess the best affinity for ETB receptors was **11**, in which the highest number of aromatic residues are present in the N-terminal portion.

Derivative **12**, in which the Asp18-Ile19 dipeptidic portion is replaced by an aliphatic spacer (Ava), possessing the same length but without the substituents present on the side chain of the dipeptide, maintains the ETA/ETB affinity profile found for compounds **6–11**; this fact underlines the two moieties which might be thought to act as the pharmacophoric portions of these types of structures, i.e. the lipophilic N-terminal portion and the Ile20-Trp21 dipeptidic C-terminal one. Furthermore, compound **12** has a significantly simplified structure with a far less peptidic nature and could be regarded as the first step toward the rational design of a true peptidomimetic of ET(16–21).

The ET receptor binding properties of derivatives **6–12** were rationalized by means of a pharmacophoric model, constructed on the basis of a comparative conformational analysis of PD 142893, PD 145065 and IRL2500: according to the characteristics of this model, the ETA and ETB receptors seem to possess a positive-charged site (P) and two lipophilic pockets (L1, L2); the ETB receptor might also possess an additional lipophilic pocket (L3). The proposed model was able to explain the high potency of PD 142893, PD 145065 and the ETB selectivity of IRL2500 and at the same time the lower ETA/ETB affinity as well as the ETB selectivity of derivatives **6–12**.

Most importantly, the information obtained from these theoretical studies might lead to further development of ET(16–21) peptidomimetic analogs with a greater affinity and selectivity for ET receptors.

6. Experimental

6.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra for comparison of compounds were taken as paraffin oil mulls or as liquid films on a Mattson 1000 Series FTIR Spectrometer. ^{1}H NMR spectra of compounds 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); 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%.

6.1.1. Synthesis of O-[(4-phenylphenyl)methyl]-hydroxylamine hydrochloride [27]

A solution of *N*-hydroxyphthalimide (0.885 g, 5.42 mmol), triphenylphosphine (2.13 g, 8.12 mmol) and *N*,*N*-diethylazodicarboxylate (1.41 ml, 8.95 mmol) in anhydrous THF (27.0 ml) was treated with 4-biphenylmethanol (1.0 g, 5.43 mmol) and the resulting mixture was stirred for 36 h at room temperature. Evaporation of the organic solvent gave a crude residue, which, after crystallisation with $CHCl_3$/hexane, yielded pure *N*-(4-phenylbenzyloxy)phthalimide (1.6 g, 89%): m.p. 158–160°C; ^{1}H NMR δ 5.17 (s, 2H, CH_2O), 7.5 (m, 13H, 2xC_6H_4 + C_6H_5). *Anal.* for $C_{21}H_{15}NO_3$ (C, H, N).

N-(4-Phenylbenzyloxy)phthalimide (1.3 g, 3.94 mmol) was suspended in absolute EtOH (40 ml) and, after the addition of hydrazine monohydrate (0.276 ml, 5.7 mmol), was refluxed for 30 h. The solvent was evaporated and the residue was then dissolved in Et_2O (40 ml) and washed with 3% aqueous K_2CO_3 solution (3 × 30 ml). The organic phase was then dried ($MgSO_4$) and evaporated to give a crude oil. This was dissolved in Et_2O and acidified to pH 3 at 0°C by the addition of a saturated Et_2O/HCl solution to give *O*-[(4-phenylphenyl)methyl]hydroxylamine hydrochloride (0.596 g, 64%) as a white solid: m.p. 170–172°C; ^{1}H NMR δ 5.09 (s, 2H, CH_2O), 7.55 (m, 9H, C_6H_4 + C_6H_5). *Anal.* for $C_{13}H_{14}NOCl$ (C, H, N).

6.1.2. Synthesis of (E)-N-(4-phenylbenzyloxy)iminoacetic acid

A suspension of *O*-[(4-phenylphenyl)methyl]hydroxylamine hydrochloride (0.20 g, 0.85 mmol) in acetonitrile (20 ml), was treated with glyoxylic acid monohydrate (0.094 g, 1.02 mmol) and the resulting mixture was stirred for 24 h at 40°C. The solvent was evaporated and the crude residue was purified by crystallization with $CHCl_3$/hexane to yield pure (*E*)-*N*-(4-phenylbenzyloxy)iminoacetic acid (0.175 g, 81%) as a white solid: m.p. 156–158°C; ^{1}H NMR δ 5.30 (s, 2H, CH_2O), 7.45 (m, 9H, C_6H_4 + C_6H_5), 7.62 (s, 1H, CH=N). *Anal.* for $C_{15}H_{13}NO_3$ (C, H, N).

6.1.3. Peptide synthesis

All reagents and solvents for peptide synthesis were of reagent grade and used without any further purification. Peptides were synthesized by the continuous-flow solid phase method on a Milligen 9050 Automatic Synthesizer

using standard Fmoc chemistry. Fmoc-Trp(Boc)-Novasyn PA500 resin was used for all syntheses; couplings were performed using HBTU/HOBt/NMM activation and a five-fold excess of protected amino acid, recycling for 50 min in the column at a flow rate of 3 ml/min. The same protocol was used to incorporate 5-[*N*-(9-fluorenylmethyloxy)carbonyl]aminovaleric acid [28]. Fmoc deprotection was obtained with 20 % (v/v) piperidine in DMF, for 9 min at 3 ml/min. The N-terminal residue was acylated on the resin using a five-fold excess of the relevant acid and HBTU/HOBt/NMM activation. Peptides were cleaved from the resin and deprotected from the side-chain protecting groups with TFA/phenol/anisole (92:4:4, v/v/v; 1 h at room temperature), precipitated with cold ether, filtered, suspended in water and lyophilized. Peptides were subsequently analyzed by RP-HPLC (Beckman System Gold apparatus) under the following conditions: Vydac C_{18} column (0.46 × 15 cm); eluant A, water/0.1% TFA; eluant B, acetonitrile/0.1% TFA; gradient from 10% to 85% B over 25 min (method A) or from 20% to 95% B over 25 min (method B); flow 1 ml/min; UV detection, 210 nm. Crude peptides were purified to homogeneity by preparative HPLC on a Beckman System Gold apparatus; Vydac C_{18} column (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 R_t and chromatographic purities observed are reported in Table 1. All final products were examined by fast ion bombardment mass spectrometry; the measurements were performed on triple quadrupole instrument (VG Quattro, Fisons Instruments, Manchester, UK). The analysis conditions were 10 keV Cs^+ ion beam and 3-nitrobenzyl alcohol as the liquid matrix. The acquired mass spectra contained substantial singly and doubly protonated molecules, as well as a number of characteristic fragment ions. The measured mass to charge (*m/z*) ratios of singly charged ions are reported in Table 1.

6.2. Radioligand binding assay

[^{125}I]ET-1 (2000 Ci/mmol) was obtained from Amersham International plc (Little Chalfont, Buckinghamshire, UK). ET-1 and ET-2 were purchased from Alexis Corporation (Läufelfingen, Switzerland). Leupetin and aprotinin were obtained from Boehringer-Mannheim (Mannheim, Germany). Bacitracin, benzamidine and PMSF (phenylmethylsulfonyl fluoride) were products of Fluka Chemie AG (Buchs, Switzerland). Other agents and reagents were from standard commercial sources.

Male and female Sprague–Dawley rats (160–300 g) were killed by cervical dislocation. The cerebellum was homogenized using a Polytron homogenizer in 10 vols. (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 benzamidine, 0.1 mM bacitracin, 0.1 mM PMSF, and 1 μg/ml leupeptin). The homogenate was centrifuged at 1000 × *g* for 5 min at 4°C. The supernatant was centrifuged at 48 000 × *g* for 15 min at 4°C. The pellet was resuspended in 10 vols. of 20 mM Hepes-Tris (pH 7.4), and 5 mM EDTA (buffer HT) containing protease inhibitors. The membrane homogenate was centrifuged at 248 000 × *g* for 15 min at 4°C. The tissue preparation was either used immediately or stored in aliquots at –80°C. The uterus was homogenized using a Polytron homogenizer in 10 vols. (w/v) of ice-cold buffer HT containing protease inhibitors. The homogenate was centrifuged at 48 000 × *g* for 15 min at 4°C. The resulting pellet was resuspended in 20 vols. of buffer HT containing protease inhibitors. The membrane homogenate was filtered through 3 layers of cheesecloth and centrifuged at 48 000 × *g* for 15 min at 4°C. The tissue preparation was either used immediately or stored in aliquots at –80°C.

Binding assays were performed as described by Cody et al. [29], with some modifications. Briefly, either cerebellar (5 μg of proteins) or uterus (10–20 μg of proteins) membranes were incubated in 0.25 ml of buffer T_1 (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 the addition of 3 ml of ice-cold buffer T_2 (50 mM Tris–HCl, pH 7.3, 0.1 mM bacitracin) and rapid filtration of samples through GF/C glass fibre filters which had been soaked in buffer T_2 containing 0.2% BSA (bovine serum albumin) for 24 h. The filters were then washed four times with 3 ml of ice-cold buffer T_2. Assays were performed in duplicate. Non-specific binding was defined in the presence of 100 nM ET-1. [^{125}I]ET-1 was diluted in buffer T_1 containing 1 mg/ml BSA while ET-1 and ET-3 were dissolved in the same buffer without BSA. Test compounds were diluted in buffer T_1 containing 1 mg/ml BSA and 1% DMSO to a concentration of 5 mM. In order to investigate the saturability of specific binding, equilibrium binding studies were performed by diluting [^{125}I]ET-1 (∽ 15 pM) with increasing concentrations of ET-1 (12.5–500 pM). Analysis of binding data (EBDA/LIGAND, Elsevier-Biosoft, UK; GraphPad Prism Softwares, San Diego, CA) as saturation experiments made it possible to calculate the equilibrium dissociation constant (K_D) and binding capacity (B_{max}) values for binding sites in cerebellar (ET-B receptor) and uterus (ET-A receptor) membranes. The K_D and B_{max} for ET-B binding sites were 54 pM and 2.23 pmol/mg proteins, respectively, while ET-A binding sites exhibited a K_D of 110 pM and a B_{max} of 0.6 pmol/mg protein. Competition binding studies were performed by incubating either cerebellar or uterus membranes in buffer T_1 with [^{125}I]ET-1 (∽ 15 pM) and up to 9 concentrations of displacing drugs ranging from 0.01 to 100 nM. Data were analyzed as competition curves using the non-linear regression analysis of the GraphPad Prism program. The IC_{50} values were converted to K_i values by the Cheng and Prusoff equation [30]. The agonists, ET-1 and ET-3, displayed K_i values of approximately 169 and 119 pM, respectively, at ET-B binding sites. K_i values were 176 pM and 4.7 nM for ET-1 and ET-3, respectively, at the ET-A binding sites.

6.3. Computational details

Molecular mechanics (MM) and molecular dynamics (MD) calculations were performed by means of the program Discover [25] using the CVFF forcefield and a value for the dielectric constant equal to 4 (distance-dependent). In all molecules, the carboxylic groups were considered in their negative charged form. The program does not explicitly take into account the possibility of a hydrophobic folding which should have a certain importance in determining the conformational behaviour of rather flexible molecules endowed with hydrophobic tails as those here considered.

A cut-off threshold of 16 Å was used and no constraint was applied.

The MM minimization procedure used a convergence criterion of 0.0001 kcal/mol on the maximum derivative; the MD simulation used a 1 fs step.

Superimpositions and molecular graphics representations were performed using the program Insight II [25].

6.3.1. Superimposition of *1*, PD 145065 and *3* on endothelin

Residue 17 of **1**, PD 145065 and **3** was placed in the same conformation as the same residue of endothelin [16,17], superimposed on it and fixed; then the conformation of residue 16 of **1**, PD 145065 and **3** was fully optimized.

6.3.1.1. Conformational analysis of 3, 6-12, PD 142893, PD 145065 and IRL2500

A 100 ps MD simulation at 1500 K was performed after a 10 ps initialization step; 11 conform-ations (one every 10 ps) were sampled and then fully mini-mized. In the case of PD 142893 and PD 145065, additional MD simulations were performed in the same way in order to check whether the conformational search could be considered exhaustive. We verified that no significant information was obtained from the additional calculations about the con-formational trend of the molecules and, therefore, this could be considered a validation of the procedure.

6.3.2. Superimposition of IRL2500 on all other molecules

The side chains of the Trp21 residue of all the molecules considered were superimposed. In some cases it was necessary to adjust the conformation of these side chains and re-minimize the new conformation thus obtained in order to optimize the superimposition; however these arbitrary adjustments were justified by the rotational freedom of the side chain, with the result that the new rotamers obtained differed by less than 1 kcal/mol from the original ones.

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