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Endothelin Receptor Ligands. Replacement Net Approach to SAR Determination of Potent Hexapeptides

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Abstract: We determined the SAR of the potent hexapeptide endothelin ligand Ac-Dphe-Orn-Asp-Ile-Ile-Trp-OH[1] (**1**) through the systematic replacement of each residue with 50 amino acid substitutes. Multipin peptide synthesis methods allowed us to rapidly synthesize and then screen all 300 analogues.

Endothelin-1 (ET-1), the most potent vasoconstrictor yet discovered,[2] is an attractive target for antihypertensive drug discovery.[2,3] In addition, ET-1 antagonists may be potentially useful in the treatment of stroke,[4] atherosclerosis/restenosis,[5] ulcerative colitis,[6] and several other disorders.[7] Although the search for ET antagonists is understandably intense, few ET receptor antagonists have been reported.[1,8-14] A series of hexapeptide analogues of the C-terminal fragment of ET-1 (His-Leu-Asp-Ile-Ile-Trp) is among the most interesting leads reported to date.[1,12]

Since the discovery of ET-1, two closely related peptides (ET-2 and ET-3)[15] and two ET-specific receptor subtypes (ETR_A and ETR_B) have been identified.[16] The ETR_A receptor is highly specific for ET-1 and ET-2, relative to ET-3, and is involved in vasoconstriction.[17] The ETR_B receptor binds ET-1, ET-2, and ET-3 with equal affinity.[16] The ETR_B receptor is implicated in both vasoconstriction and vasodilation.[18] The only receptor antagonists thus far reported are ETR_A-selective .[1,8-14]

Analogues of the C-terminal hexapeptide common to ET-1, ET-2, and ET-3 bind with submicromolar affinities to the ETR_A receptor subtype.[1] We chose one analogue, Ac-Dphe-Orn-Asp-Ile-Ile-Trp-OH[1] (**1**, IC_{50} < 800 nM), as the basis for our SAR study. Herein we report some preliminary results of this study.

In a systematic approach designed to identify critical residues and to optimize the binding affinities at the ETR_A receptor, analogues of the lead peptide were synthesized in which each residue in turn was substituted with an amino acid from the set which comprised the twenty standard L-amino acids, the twenty isomeric D-amino acids, four N-substituted glycines (NSGs),[19] and six other amino acids: α-aminobutyric acid (Aaba), γ-aminobutyric acid (Gaba), ß-alanine (ß-ala), L-homophenylalanine (Hphe), L-norleucine (Norleu), L-norvaline (Norval), and L-ornithine (Orn). Also included were the five pentapeptides corresponding to the systematic deletion of each residue in the lead peptide.

Materials and Methods

We synthesized 300 analogues of compound **1** using the multipin approach described previously by Geysen, *et al.*[20] Basic cleavage of the peptides from the pins as described by Valerio, *et al.*[21] provided the free C-terminal acid required for activity.[1b] Compounds with an NSG at position 6 were prepared as the C-terminal amides to avoid diketopiperazine formation during synthesis.[19] Approximately 1 μmol of each peptide was synthesized. Compound **1** was included as an internal control (n=10), and was shown to be greater than 79% purity by amino acid analysis (n=3). All compounds were assayed without further purification at approximately 1 μM concentration as solutions in 0.25% Me_2SO/PBS/0.1% BSA buffer. No effort was made to identify the oxidation states of the Cys or Dcys derivatives. Selected compounds were purified by HPLC for IC_{50} determination. Finally, Ac-Dphe-Orn-Asp-Ile-Ile-Trp-OH (**1**), Ac-Dphe-Phe-Asp-Ile-Ile-Trp-OH (**12**), and Ac-Dphe-Nphe-Asp-Ile-Ile-Trp-OH (**4**) were re-synthesized using standard solid phase peptide chemistry for further *in vitro* testing.[22]

Receptor binding assays were performed using murine 3T3 cells which exhibit primarily the ETR_A receptor subtype.[23] All ligand binding competition studies were performed by incubating 3T3 cells grown on 96-well microtitre plates, 50 pM ^{125}I-ET-1, unlabeled test compound, and buffer at a volume of 200 μl for 2 hr at room temperature. Figure 1 shows data on the ability of compounds to displace binding of ^{125}I-ET-1 expressed as percent inhibition of control. All data shown in Figure 1 are based on single-point determinations of ligand binding. Table 1 shows the binding affinities for a subset of compounds of interest. In addition, compound **4** was resynthesized, purified, assayed on the ETR_A assay, and confirmed the results obtained in the initial screen.

Results and Discussion

Ac-Xaa-Orn-Asp-Ile-Ile-Trp-OH analogues — Figure 1A

Among the five compounds that showed inhibition of greater than 50% in our initial screens were four analogues with aromatic D-amino acids (Dphe, Dhis, Dtyr, and Dtrp). The Dtyr (**4**) and Dtrp (**5**) analogues of compound **1** show the highest binding affinities (IC_{50}s are 30 nM and 15 nM, respectively) of position 1 analogues. Our results are in qualitative agreement with those of Cody, *et al.*,[12] who recently reported binding affinities for Ac-Dtyr-Leu-Asp-Ile-Ile-Trp-OH (IC_{50} = 400 nM) and Ac-Dtrp-Leu-Asp-Ile-Ile-Trp-OH (IC_{50} = 130 nM), assayed in rabbit renal artery tissue.

The fifth strongest binder in the initial screen (Gln, ≈ 60% inhibition) was inactive at 10 μM concentration in subsequent assays. It is important to note that all other compounds re-assayed showed good correlation between the IC_{50} and the results from the single point determinations.

The NSGs and the aromatic L-amino acids showed less than 30% inhibition in our single-point determinations, as did the deletion peptide Ac-Orn-Asp-Ile-Ile-Trp-OH.

Ac-Dphe-Xaa-Asp-Ile-Ile-Trp-OH analogues — Figure 1B

Only eight of the 50 Orn replacement analogues show *less than* 50% inhibition. Gly is well tolerated (75% inhibition), yet spacers like ß-ala (18%) and Gaba (32%) are not. Several D-amino acid analogues show no activity, including Dile (28%), Dglu (31%), Dlys (25%), while their L-counterparts all show greater than 70% inhibition. Dpro (9%) is the only N-substituted amino acid that is *inactive* at this position.

The L-Phe (**12**, purified material), D-Phe (**11**), and the Hphe (**7**) derivatives have IC_{50}s of 270 nM, 90 nM, and 70 nM, respectively. These all have affinities similar to the Ala derivative (**6**, IC_{50} = 60 nM), but significantly lower than the Nphe analogue (**4**, IC_{50} = 12 nM on purified material). Thus, this NSG apparently places a desirable side chain in a position that traditional amino acid monomers cannot probe.

Ac-Dphe-Orn-Xaa-Ile-Ile-Trp-OH analogues — Figure 1C

Only the Asp, Glu, Cys, and His analogues show greater than 50% inhibition, though the Trp (45%) and Tyr (47%) analogues show some activity. None of the NSGs shows activity, including Nasp and Nglu, both of which might be considered reasonable substitutions for Asp. Also, neither Pro nor Dpro are active, suggesting that an amide proton is important in this position for good binding affinity.

Ac-Dphe-Orn-Asp-Xaa-Ile-Trp-OH analogues — Figure 1D

Position 4 is tolerant to substitution by aliphatic or aromatic L-amino acids. The Ile can be replaced by Val (72%), Hphe (73%, **13**, IC_{50} = 114 ± 22 nM), and Trp (49%). The binding affinity decreases as the alkyl sidechain length increases in the series: Ala (66%), Aaba (67%), Norval (48%), Norleu (33%), and Met (18%). Like Norleu and Met, Leu (33%) does not serve as an Ile replacement. These data suggest the presence of a limited hydrophobic binding pocket, in which short linear and ß-branched amino acids are acceptable, but longer or γ-branched are not. The results also show the NSGs and all D-amino acids are inactive, as is the deletion peptide (Ac-Dphe-Orn-Asp-Ile-Trp-OH).

Ac-Dphe-Orn-Asp-Ile-Xaa-Trp-OH analogues — Figure 1E

Position 5 is more tolerant of large hydrophobic monomers than is position 4. Here, Ile (69%), Leu (58%), Val (61%), Met (62%), Norleu (62%), Norval (62%), and Aaba (45%) all bind with reasonable affinity. In addition, lipophilic D-amino acid replacements, such as Dile (66%) and Dleu (52%) show activity.

Ac-Dphe-Orn-Asp-Ile-Ile-Xaa-OH analogues — Figure 1F

Our results at the C-terminus confirm what is known from the literature.[1] Only L-Trp derivatives are active at position 6. No other derivative at this position showed significant binding. The NSG analogues have C-terminal amides, suggesting that a free C-terminal acid might also be required for activity when this new class of monomers is used at the C-terminus.

Conclusion

In the past, systematic peptide SARs were hindered by the cost of the required peptides and the length of time required for their synthesis. As a result of multipin peptide synthesis methods, we were able to rapidly synthesize and assay 300 analogues of a lead compound in less than 4 months. This information will be useful in the design and optimization of endothelin receptor ligands. A full account of this research, as well as the design, synthesis, and testing of other potent endothelin ligands, will be published in due course.

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Table 1: Binding affinities of selected hexapeptide analogues.

Compound	Sequence								ETR_A IC_{50} (nM)[a]
1	Ac	Dphe	Orn	Asp	Ile	Ile	Trp	OH	230. ± 40.[b,c]
2	Ac	***Dtrp***	Orn	Asp	Ile	Ile	Trp	OH	15. ± 5.
3	Ac	***Dtyr***	Orn	Asp	Ile	Ile	Trp	OH	26. ± 4.
4	Ac	Dphe	***Nphe***	Asp	Ile	Ile	Trp	OH	12. ± 3.[b]
5	Ac	Dphe	***Pro***	Asp	Ile	Ile	Trp	OH	45. ± 11.
6	Ac	Dphe	***Ala***	Asp	Ile	Ile	Trp	OH	57. ± 14.
7	Ac	Dphe	***Hphe***	Asp	Ile	Ile	Trp	OH	70. ± 16.
8	Ac	Dphe	***Asn***	Asp	Ile	Ile	Trp	OH	75. ± 18.
9	Ac	Dphe	***Tyr***	Asp	Ile	Ile	Trp	OH	77. ± 15.
10	Ac	Dphe	***Trp***	Asp	Ile	Ile	Trp	OH	79. ± 22.
11	Ac	Dphe	***Dphe***	Asp	Ile	Ile	Trp	OH	98. ± 26.[c]
12	Ac	Dphe	***Phe***	Asp	Ile	Ile	Trp	OH	270. ± 40.[b,c]
13	Ac	Dphe	Orn	Asp	***Hphe***	Ile	Trp	OH	114. ± 22.

a) Measured using murine 3T3 cells. b) Assayed using purified compound.
c) compound previously reported. See Reference 1.

References and Notes

Abbreviations used in this manuscript: ETR_A = Endothelin receptor subtype A, ETR_B = endothelin receptor subtype B. PBS = Phosphate Buffered Saline, BSA = Bovine Serum Albumin, Ac = acetyl, Gaba = γ-aminobutyric acid, Aaba = α-aminobutyric acid, Hphe = L-homophenylalanine, Norval = L-norvaline, Norleu = L-norleucine, NSG = N-substituted glycine, Nphe = N-benzylglycine, Nleu=N-isobutyl glycine, Nasp=N-carboxymethyl glycine, Nglu=N-carboxyethyl glycine. D-amino acids are refered to as four-letter codes, e.g. Dphe = D-Phe.

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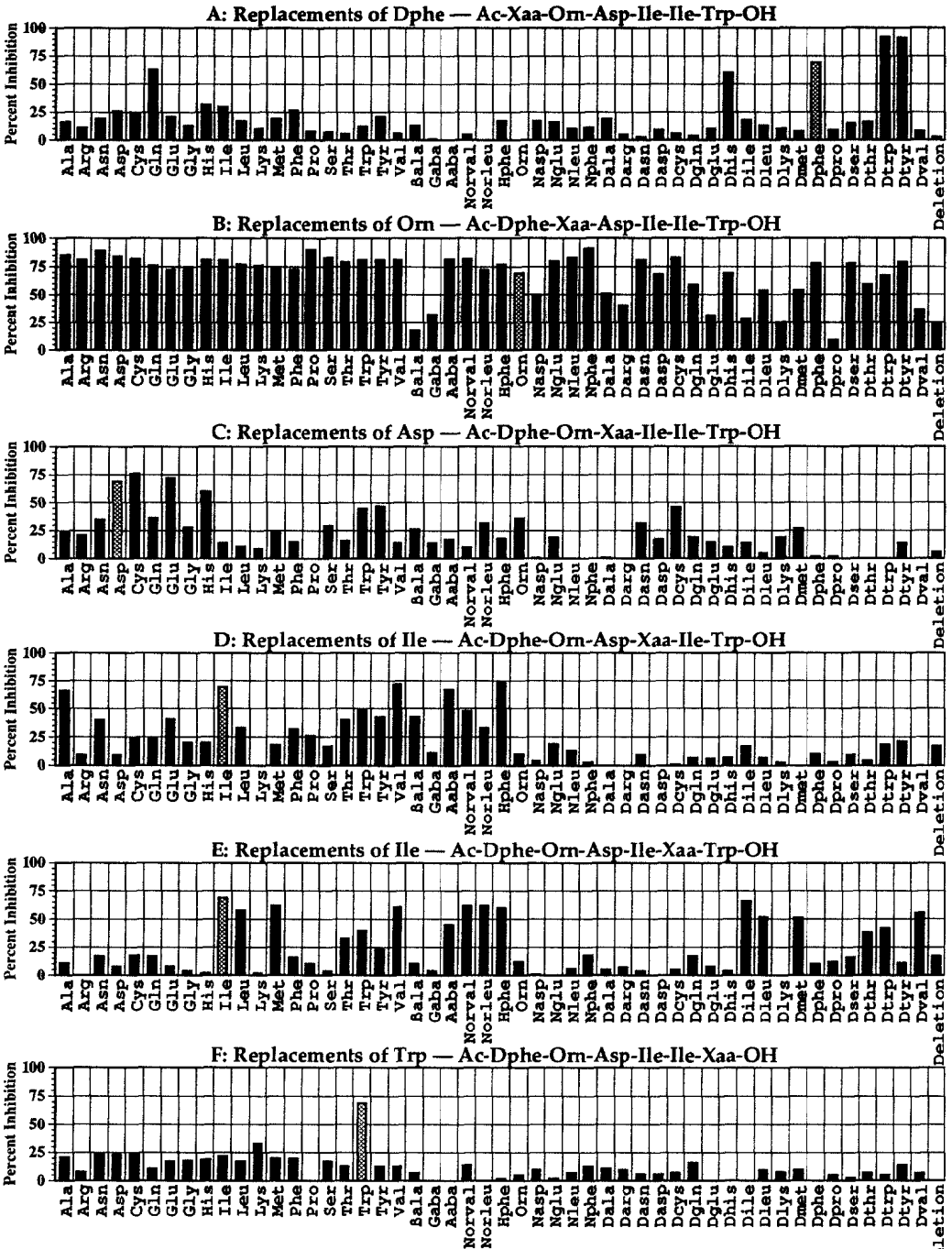

Figure 1: Plots showing percent inhibition of control for analogues of Ac-Dphe-Orn-Asp-Ile-Ile-Trp-OH. Single point assays were performed on unpurified material at an estimated concentration of 1 μM. The parent is shown in hashed bars in each of the plots.