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Endothelin Receptor Ligands. Multiple D-Amino Acid Replacement Net Approach

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Abstract: All possible single and multiple L- to D- amino acid replacements of a potent hexapeptide endothelin receptor ligand were synthesized and tested. While most of these 64 analogues were inactive on the ETR_A receptor, three showed submicromolar activity. Interestingly, two of these contain 5 D-amino acids and may be stable to proteolysis.

The proteolytic stability of a peptide can be increased by substitution of L-amino acids with D-amino acids.[1-3] Typically, more than one D-amino acid is needed to confer sufficient stability to enhance bioavailability. Historically, the cost of synthesizing large numbers of peptides and the inability to predict which peptides will tolerate single or multiple D-amino acid replacements have limited the use of systematic multiple replacement studies of peptide ligands. Recent studies have shown that a parallel synthetic approach to multiple stereoisomeric replacements in a polypeptide chain can yield ligands with good affinity.[3]

Endothelin-1 (ET-1), the most potent vasoconstrictor yet discovered,[4] is an attractive target for antihypertensive drug discovery.[4,5] Since the discovery of ET-1, two closely related peptides (ET-2 and ET-3)[6] and two ET-specific receptor subtypes (ETR_A and ETR_B) have been identified.[7] The ETR_A receptor is highly specific for ET-1 and ET-2, relative to ET-3, and is involved in vasoconstriction.[8] The ETR_B receptor binds ET-1, ET-2, and ET-3 with equal affinity.[7] The ETR_B receptor is implicated in both vasoconstriction and vasodilation.[9]

Single D-amino acid substitutions of the C-terminal hexapeptide common to ET-1, ET-2, and ET-3 (His-Leu-Asp-Ile-Ile-Trp) have been shown to enhance binding to the ETR_A receptor subtype.[10-12] Several other analogues of the C-terminal hexapeptide have also been reported.[10-12,13] We report here the results of a systematic study of single and multiple D-amino acid replacements in a potent analogue of the C-terminal hexapeptide, Ac-Dphe-Orn-Asp-Ile-Ile-Trp-OH[10] (**1**, IC_{50} = 230 nM).

Materials and Methods

Sixty-four stereoisomers of compound **1** were synthesized using the multiple peptide pin approach described previously by Geysen, *et al.*[14] All peptides were cleaved with 0.1 M NaOH in 40% MeCN/H_2O for 1 hr with sonication to provide approximately 1 μmol of free peptide.[15] The basic solutions were neutralized with 0.166 M acetic acid in 1 M HCl to give an final pH of *ca.* 5. The peptides were freeze dried and then reconstituted in 0.25% Me_2SO/PBS/0.1% BSA buffer.

All peptides were assayed without further purification at approximately 1μM concentration. Five duplicate syntheses of compound **1** were included as an internal controls for the binding affinity experiments. Amino acid analysis and HPLC indicated that one copy of compound **1** was of *ca.* 85% purity. HPLC analysis also shows the purity of compounds **2** and **3** to be > 85%. IC_{50} values were determined for the three most potent compounds (Table I). Receptor binding assays were performed using murine 3T3 cells, which exhibit the ETR_A receptor subtype.[16] Ligand binding competition studies were performed by incubating 3T3 cells grown on 96-well microtiter 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 lists data on the ability of the peptides to displace binding of [^{125}]I-ET-1 expressed as percent inhibition of control. Data shown in Figure 1 are based on single-point determinations of ligand binding. Compound **1** was also synthesized using standard solid phase peptide chemistry methods[17] and purified by HPLC.

Results and Discussion

Only three of the 64 analogues synthesized show greater than 60% inhibition at 1 μM concentration (Figure 1). All five syntheses of compound **1** used as an internal control show 77% to 82% inhibition, providing an error estimate for the assay. The peptide Ac-Dphe-Dorn-Dasp-Dile-Ile-Dtrp-OH (**2**) binds to the ETR_A receptor with an IC_{50} of 250 nM (Table 1), equipotent with the parent peptide (**1**, IC_{50} = 230 nM). Ac-Dphe-Dorn-Asp-Dile-Dile-Dtrp-OH (**3**) binds with an IC_{50} of 900 nM. The peptides with six D-amino acids or six L-amino acids are inactive.

All of the peptides with appreciable activity have the D-configuration at the N-terminal amino acid. The chirality of no other residue can be easily classified as critical for binding. This is quite surprising and contrasts with similar work on substance P analogues in which residues near the C-terminus were found to be more sensitive to stereoisomeric substitution than those near the N-terminus.[3] In this study, we are unable identify any combination of two or more residues which have stereospecific requirements.

The utility of this type of systematic replacement method is clearly demonstrated in this experiment. We could not have predicted the outcome of this experiment based on past literature work.[3,10-12,13] Furthermore, this type of study should aid in the identification of more proteolytically stable peptides and in the quest for new pharmaceutical agents.

Table 1: Binding Affinities of Selected Hexapeptide Stereoisomers.

Compound	Sequence	ETR_A IC_{50} (nM)[a,b]
1	Ac-Dphe-Orn-Asp-Ile-Ile-Trp-OH	230. ± 40.[c,d]
2	Ac-Dphe-Dorn-Dasp-Dile-Ile-Dtrp-OH	250. ± 55.
3	Ac-Dphe-Dorn-Asp-Dile-Dile-Dtrp-OH	900. ± 220.

a) Measured on murine 3T3 cells. b) HPLC analysis shows purity of compounds **1-3** to be > 85%. c) Assays performed with purified compound. d) Compound previously reported. See reference 10.

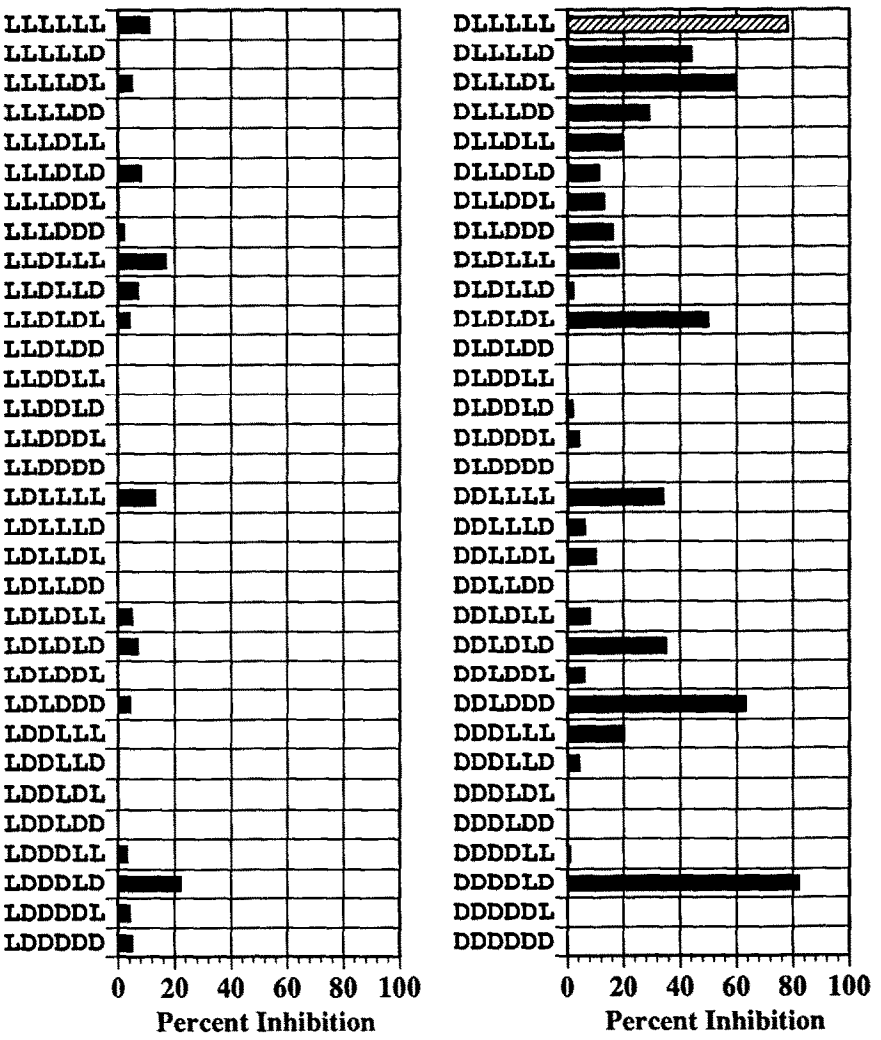

Figure 1: Plots showing percent inhibition of control for stereoisomeric analogues of Ac-Dphe-Orn-Asp-Ile-Ile-Trp-OH.[10] Entries are indexed with an "L" to indicate an L-amino acid at a specific position. Likewise, a "D" indicates that a D-amino acid is used. Single point assays were performed on unpurified material at an estimated concentration of 1 μM. The parent is shown in hashed bars at the upper right.

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References and Notes

Abbreviations used in this manuscript: Ac = acetyl, PBS = Phosphate Buffered Saline, BSA = Bovine Serum Albumin. ETR_A = Endothelin receptor subtype A, ETR_B = Endothelin receptor subtype B, Dphe = D-phenylalanine, Dorn = D-ornithine, Dasp = D-aspartic acid, Dile = D-isoleucine, Dtrp = D-tryptophan.

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