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IRL 2500: A POTENT ET_B SELECTIVE ENDOTHELIN ANTAGONIST

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Abstract: Combination of a glycine substitution scan on the C-terminal dodecapeptide analog of ET-1 and a substance P antagonist screen on the basis of a homology study of the rhodopsin superfamily of seven-transmembrane receptors yielded in the development of IRL 2500, a potent ET_B selective endothelin antagonist.

Endothelins (ETs) are a family of potent vasoactive peptides originally isolated from the conditioned medium of cultured endothelial cells.[1] These peptides (ET-1, ET-2 and ET-3) have been suggested to play a role in the pathophysiology of a large number of diseases such as renal failure, vasospasm, hypertension and asthma.[2] The pharmacological actions of the ETs are mediated by two distinct subtypes of ET receptors.[3] The ET_A receptor is characterized by distinct selectivity for ET-1 and ET-2 over the isopeptide ET-3, and two important biological actions mediated by the ET_A receptor are vasoconstriction and vascular smooth muscle cell proliferation.[4] In contrast the ET_B receptor shows equivalent affinity towards ET-1, ET-2 and ET-3, and mediates vasodilatation as well as vasoconstriction and bronchoconstriction.[4] The development of potent endothelin antagonists, selective as well as non-selective, is necessary to determine the pathophysiological role of the ETs and their receptor subtypes. Various peptidic compounds have been reported[5] as ET-receptor antagonists which are selective for ET_A (BQ-123 and FR139317), ET_B (BQ-788 and RES-701-1) or exhibit dual ET_A / ET_B antagonism (PD 142893, PD 145065 and TAK-044). Non peptidic ET antagonists have also been reported.[5,6] These include the ET_A selective antagonists (A-127722, BMS 182874 and PD 156707) and the dual ET_A / ET_B antagonists (A-158112, L-754142, PD 160874, Ro 47 0203 (bosentan), and SB 209670).

In this report we describe the development of the new low molecular weight ET_B selective ET antagonist IRL 2500. In our approach we first tried to identify amino acid sequences of ET responsible for the strong receptor binding affinity. As the C-terminal tryptophan has been already identified as an important residue for binding,[7] we concentrated our efforts on the hydrophobic C-terminus of ET. The dodecapeptide IRL 1543,[8] the shortest C-terminal ET fragment with very strong binding potency (Ki (ET_B) = 0.077 nM) was submitted to a glycine substitution scan. After one amino acid was replaced by glycine at a time, each inhibition of [^{125}I]ET-3 binding to the ET_B receptor as well as [^{125}I]ET-1 binding to the ET_A receptor was determined.[9] The results are summarized in Table 1.

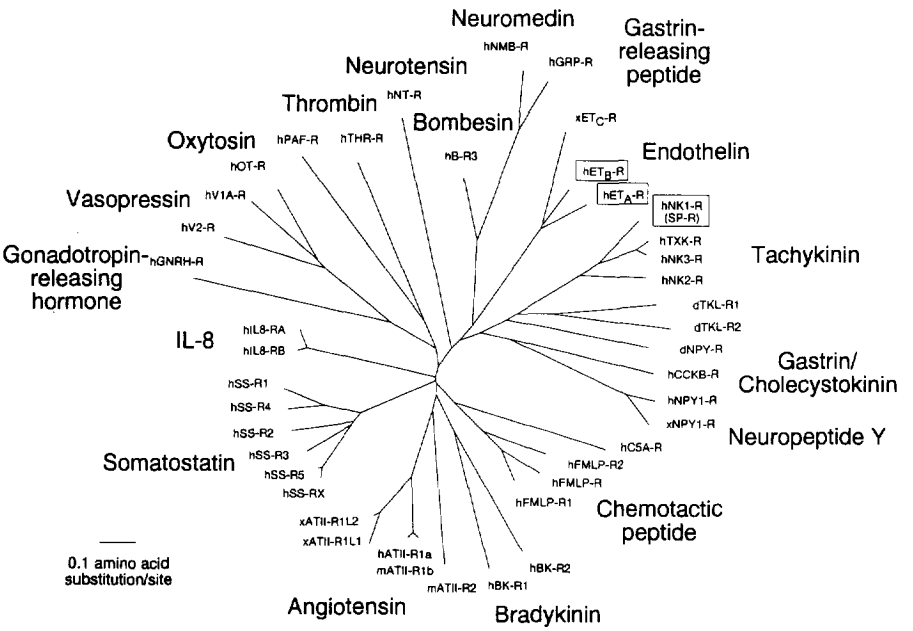
Table 1: Glycine substitution scan of the C-terminal dodecapeptide fragment of ET-1.

	$K_i(ET_B)$	Ki(ETA)	
Met(Asp)-Leu - Ser - Ser - Cys - Ser - Cys -NH_2 (S–S) Lys - Glu - Cys - Val - Tyr - Phe - Cys - His - Leu - Asp - Ile - Ile - Trp -COOH	0.008 nM	0.04 nM	**ET-1**
Suc - Glu - Ala - Val - Tyr - Phe - Ala - His - Leu - Asp - Ile - Ile - Trp -COOH	0.077 nM	620 nM	**IRL 1543**
Suc- - Gly -COOH	**>10000 nM**	>10000 nM	**1**
Suc- - Gly - -COOH	**5200 nM**	>10000 nM	**2**
Suc- - Gly - -COOH	180 nM	>10000 nM	**3**
Suc- - Gly - -COOH	0.25 nM	96 nM	**4**
Suc- - Gly - -COOH	5.9 nM	>10000 nM	**5**
Suc- - Gly - -COOH	1.0 nM	250 nM	**6**
Suc- - Gly - -COOH	5.3 nM	2000 nM	**7**
Suc- - Gly - -COOH	**260 nM**	>10000 nM	**8**
Suc- - Gly - -COOH	**11 nM**	5000 nM	**9**
Suc- - Gly - -COOH	0.91 nM	1300 nM	**10**

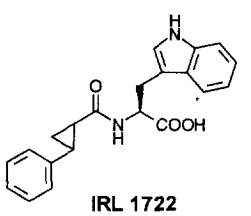
While in all of the above modifications of IRL 1543 the binding potency decreases, there are two areas which are more sensitive towards an amino acid exchange than others. The Ki (ET_B) values of compounds **1**, **2**, **3** and **8**, **9** suggest that the C-terminal -Ile-Ile-Trp- and the aromatic -Tyr-Phe- are the most important areas of ET-1 for receptor binding. Based on this evidence a small series of simple analogs having an aromatic moiety attached through a spacer to the amino group of tryptophan was synthesized. Out of this series of weak inhibitors N-*trans*-2-phenylcyclopropanoyl-tryptophan IRL 1722[10] was identified as the first lead structure with weak inhibitory activity (Ki (ET_B) = 16 μM).

At this point an extremely helpful input came from a very different direction. An extensive homology study[11] of the rhodopsin superfamily of seven-transmembrane receptors showed greatest homology of the ET-receptors with bombesin receptors and tachykinin receptors, suggesting a close evolutionary relationship between these receptors. Both subtypes of the ET receptors, together with two subtypes of bombesin receptors (BB_1 and BB_2 receptors) and three subtypes of tachykinin receptors (NK_1 (substance P), NK_2 and NK_3 receptors), are part of a cluster distinct from other clusters in a phylogenetic tree of the superfamily (Fig. 1). In general, the tertiary structure of a protein is more conserved than the primary structure during evolution. It is also thought that this general rule may be applicable to the structure of the ligand-binding domain of the receptor. Thus, recognition of the close evolutionary relationship between ET receptors and tachykinin receptors prompted us to a limited random screening of Ciba compounds with substance P antagonistic activity.

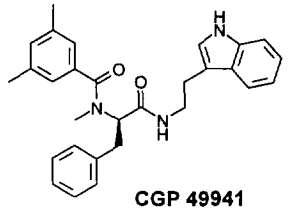
Fig. 1. Unrooted phylogenetic tree of peptide receptors of the rhodopsin superfamily.[11]



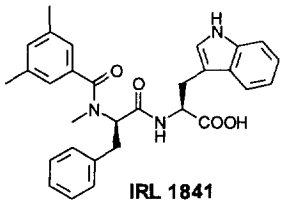
Out of randomly selected 140 compounds with substance P antagonistic activity three compounds showed weak ET_A or ET_B receptor binding activity (Ki = 5 ~ 20 μM). Among them, CGP 49941[12] was found to show the highest affinity for the ET_B receptor (Ki (ET_B) = 5 μM). The combination of the structural elements of CGP 49941 and IRL 1722, the above mentioned weak lead structure, resulted in IRL 1841[13] with a notable



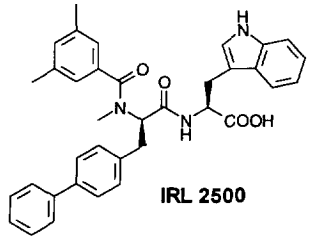
IRL 1722



CGP 49941



IRL 1841



IRL 2500

ET_B-selective binding affinity (Ki (ET_B) = 36 nM; Ki (ET_A) = 11000 nM). A further chemical optimization provided the *p*-phenyl-phenylalanine analog IRL 2500[14] with very high ET_B-affinity (Ki (ET_B) = 1 nM; Ki (ET_A) = 440 nM). These are selective ET-antagonists and showed only weak affinity to substance P receptors. While CGP 49941 inhibited the ^{3}H-substance P binding to bovine retina[15] with an IC_{50} = 800 nM, IRL 1841 showed a weaker binding (IC_{50} = 2 μM) and IRL 2500 was even weaker (IC_{50} > 10 μM).

Functional characteristics of IRL 2500 were investigated using isolated guinea pig trachea and rat thoracic aorta denuded of epithelium and endothelium, respectively.[16] The guinea pig tracheal smooth muscle expresses both ET_A and ET_B receptors[17] and rat aortic smooth muscle possesses predominantly the ET_A receptor.[18] IRL 2500 showed no agonistic activity in both tissues at least up to 30 μM. In the guinea pig tracheal tissue, IRL 2500 concentration-dependently antagonized the ET_B-mediated contraction induced by ET-3 which was hardly affected by an ET_A specific antagonist, BQ-123, of 10 μM (Fig. 2). On the other hand, IRL 2500 had no effect on the ET-1-induced contraction of the rat aorta up to 30 μM (data not shown). These results indicate that IRL 2500 behaves as an antagonist selective to the ET_B receptor.

In conclusion, a small molecular weight compound, IRL 2500, was rationally developed as a potent and highly selective antagonist for the ET_B receptor. The rational approach, based on the structure-activity correlation of ET-1 fragments and structural homology of the rhodopsin superfamily of receptors, successfully aided the quick discovery of early lead compounds. With an increase in evidence for the involvement of the

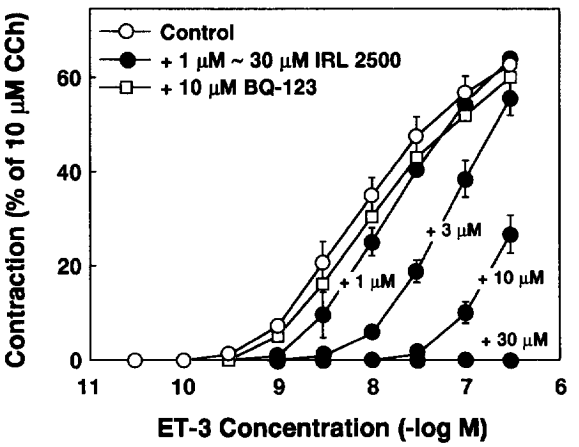


Fig. 2. Effect of IRL 2500 on ET-3-induced contraction in epithelium-denuded guinea pig trachea. ET-3 was added cumulatively in the absence or presence of IRL 2500 of 1 μM to 30 μM or of BQ-123 of 10 μM. IRL 2500 or BQ-123 was applied 30 min or 20 min before addition of ET-3, respectively. The contractile response is represented as a percentage of the contraction induced by 10 μM carbachol (CCh). Each point represents the mean ± S.E.M. of 3 ~ 5 experiments.

ET_B receptor in a multitude of ET actions, IRL 2500 will be a useful tool to further clarify the role of ET_B-mediated responses in physiological and pathological processes.

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9. These binding affinities for the two subtypes of ET receptor, ET_A and ET_B, were examined in porcine lung membranes. For a detailed description of the binding assay see ref. 8.
10. Synthesis of IRL 1722: (L)-tryptophan methyl ester was coupled with *trans*-2-phenyl-1-cyclopropane carboxylic acid in DMF in the presence of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide and hydroxybenztriazole. The methylester was hydrolized with lithium hydroxide in THF/water (1:1) at 0°C. - mp: 124-126°; $[\alpha]_D$ = -5° (c = 1.15, ethanol); HPLC (Chiralcel OD, hexane/isopropanol/TFA 900:100:3) ee > 95%.
11. In collaboration with the *Biomolecular Engineering Research Institute, Osaka, Japan* a multiple alignment of the deduced amino acid sequences of about 100 members of the superfamily was constructed on a FACOM-supercomputer. For further methods see Toh, H.; Ichikawa, A.; Narumiya, S. *FEBS Lett.*, **1995**, *361*, 17.

12. Synthesis of CGP 49941: N-Methyl-(D)-phenylalanine was acylated with 3,5-dimethylbenzoyl chloride in dioxane/THF/aqueous 1N sodium hydroxide at 0°C and coupled with tryptamine in dichloromethane in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, 1-hydroxybenzotriazole and N-methylmorpholine. - mp: 160-161°C; NMR ($CDCl_3$, 400 MHz) δ [ppm] 8.29 (s), 8.11 (s), 7.59 (d, J = 7.5 Hz), 7.33-6.90 (m), 6.81 (br), 6.69 (br), 6.52 (s), 5.95 (br), 5.86 (s), 5.35 (dxd, J = 7, 9.5 Hz), 4.2 (br), 3.76 (m), 3.62 (dxd, J = 7, 13 Hz), 3.52 (m), 3.35 (dxd, J = 7, 15 Hz), 3.15 (dxd, J = 10, 15 Hz), 3.05-2.8 (m), 2.72 (s), 2.22 (s), 1.97 (s).
13. Synthesis of IRL 1841: N-BOC-N-methyl-(D)-phenylalanine was coupled with (L)-tryptophan methyl ester in DMF in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 1-hydroxybenzotriazole. After TFA mediated cleavage of the BOC group the dipeptide was acylated with 3,5-dimethylbenzoyl chloride and the methylester hydrolized with lithium hydroxide in MeOH/water (2:1) at 0°C. - mp: 91-94°C; FAB-MS m/e 498 $(M+H)^+$; $[\alpha]_D$ = -46° (c = 1.1, ethanol); HPLC (Chiralcel OD, hexane/isopropanol/TFA 900:100:3) ee > 95%; NMR ($CDCl_3$, 400 MHz) δ [ppm] 8.29 (s), 8.15 (s), 7.56 (d, J = 7.8 Hz), 7.47 (d, J = 7.8 Hz), 7.3-6.7 (m), 6.48 (s), 5.92 (s), 5.41 (dxd, J = 6.8, 9.7 Hz), 4.84 (dxd, J = 5.8, 13.2 Hz), 4.33 (dxd, J = 2, 7.2 Hz), 3.4-2.75 (m), 2.70 (s), 2.18 (s), 1.91 (s).
14. Synthesis of IRL 2500: (D)-(4-phenylphenyl)alanine methyl ester hydrochloride was reacted with freshly distilled cyclopentadiene in THF in the presence of formalin. The bicyclic intermediate[19] was treated with TFA and triethylsilane in chloroform to give N-methyl-(D)-(4-phenylphenyl)alanine methyl ester. The amino group was acylated with 3,5-dimethylbenzoyl chloride and the ester hydrolized with lithium hydroxide in MeOH/water. Coupling with (L)-tryptophan methyl ester hydrochloride and ester hydrolysis gave IRL 2500: FAB-MS m/e 574 $(M+H)^+$; $[\alpha]_D$ = +2.5° (c = 1.0, ethanol); HPLC (Chiralcel OD) ee > 95%; NMR ($CDCl_3$, 400 MHz) δ [ppm] 8.32 (s), 8.22 (s), 7.6-6.8 (m), 6.93 (s), 6.8 (m), 6.51 (s), 5.97 (s), 5.46 (t, J = 8 Hz), 4.85 (q, J = 6 Hz), 4.36 (m), 3.4-2.8 (m), 2.73 (s), 2.15 (s), 1.85 (s).
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16. Functional Assays: Epithelium- and endothelium-denuded ring preparations (~2 mm width) from guinea pig trachea and rat thoracic aorta, respectively, were placed into a 4.0-ml organ bath containing the oxygenated (95% O_2 - 5% CO_2) Krebs-Henseleit solution (composition, mM: 113.0 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 5.5 glucose and 0.01 EDTA; pH 7.4; 37°C) for isometric tension measurement at a resting tension of 1 g. Each preparation was first stimulated with 10 μM carbachol for the trachea or with 100 nM norepinephrine for the aorta to use as a reference standard for the responses to ET-3 for the trachea or ET-1 for the aorta. IRL 2500 was dissolved in dimethyl sulfoxide, the solution was diluted to 1/1000 with the Krebs-Henseleit solution, and then the whole volume of the Krebs-Henseleit solution in the organ bath was replaced with the solution containing IRL 2500.
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