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RES-701-1, SYNTHESIS AND A REEVALUATION OF ITS EFFECTS ON THE ENDOTHELIN RECEPTORS

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Abstract: RES-701-1 is a cyclic peptide isolated from *Streptomyces* sp. RES-701 that has been reported to selectively inhibit the ET_B receptor subtype with an IC_{50} of 10 nM. Independently, we have prepared RES-701-1 and found that it only possesses micromolar affinity for either the human ET_A or ET_B receptors. The solid/solution phase synthesis is amenable to the multigram scale.

Introduction:

The endothelins (ETs, Figure 1a) are potent constrictors of vascular smooth muscle.[1,2] All ETs possess 21 amino acids, two disulfide bridges between positions 1-15 and 3-11 and a hydrophobic C-terminal hexapeptide. The cloning and expression of three endothelin receptor subtypes (ET_A, ET_B and ET_C) has been reported.[3-5] The ET_A receptor mediates vasoconstriction and is widely distributed.[5,6] The ET_B or non-selective receptor, possesses equal affinity for all the ET isopeptides and functions differently dependent upon species and tissue.[7,8] Recent studies have suggested the existence of additional ET_B receptor subtypes.[8,9] The ET_C receptor which is selective for ET-3 has been cloned and characterized from *Xenopus* melanophores and heart,[5] although no mammalian homolog has been cloned.

Many peptidic and nonpeptidic ET antagonists have been described (for recent reviews, see references 10,11). In general, these compounds are potent ET_A receptor selective antagonists or combined ET_A/ET_B receptor antagonists. Conversely, the availability of ET_B receptor selective antagonists has been quite limited, until recently. IRL 1038 was reported to exhibit low micromolar affinity for the ET_A receptor in a variety of species (e.g. rat aorta, guinea pig heart, pig aorta, and human umbilical vein) and low nanomolar affinity for ET_B receptor (e.g. rat cerebellum, guinea pig cerebellum, pig lung, human placenta).[12] However, it was recently reported that IRL-1038 possesses only low micromolar affinity for both the ET_A and ET_B receptor subtypes.[13,14] Also, BQ-788 is reported to be a highly selective ET_B receptor antagonist with an IC_{50} of 1.3 μM for ET_A (human neuroblastoma cell line) and 1.2 nM for ET_B (human girardi heart cells).[15]

RES-701-1 (cyclic (Gly^1-Asp^9)(Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp), Figure 1b), a peptide isolated from *Streptomyces* sp. RES-701 (compound 1), has been reported to bind selectively

to the ET_B receptor with 10 nM affinity, while it possesses greater than 5 μM affinity for the ET_A receptor.[16-18] RES-701-1 has been reported to inhibit ET-1 induced increase in cytosolic Ca^{2+} levels in transfected COS cells expressing the human ET_B receptor.[16] In order to further study the pharmacological role of the ET_B receptor, we have synthesized RES-701-1 (compound **2**) by a combined solution/solid phase synthetic strategy (Scheme **1**). Based upon our experience in the development of endothelin antagonists from its C-terminus, we also prepared several analogues of RES-701-1.[19,20]

Experimental:

The synthetic strategy for the preparation of RES-701-1 is illustrated in Scheme **1**. The C-terminal heptapeptide of RES-701-1 (compounds **4,5**) and the linear fragments (compounds **3,6-10**) were prepared using a standard Boc solid phase synthetic strategy[21] on an N^{α}-Boc-Trp-PAM[22] resin utilizing an Applied Biosystems Inc. (ABI) 430A instrument. The amino acid side chains were protected as follows:[22] BrZ(Tyr), Bzl(Asp, Thr), Bom(His). Individual amino acids were coupled as their 1-hydroxybenzotriazole (HOBt) activated esters. The peptide resin was Boc deprotected with trifluoroacetic acid (TFA) and indole (0.1 mg/mL) in dichloromethane (DCM, (1:1)) and subsequently, neutralized with 10% diisopropylethylamine (DIEA) in DCM. The linear N-terminal fragment was prepared using standard Fmoc solid phase synthetic protocols[23] on a Sasrin[24,25] resin. The amino acid side chains were protected as follows:[22] Trt(Asn), tBu(Thr, Asp), Bzl(His). N^{α}-Fmoc-Asp(tBu) was coupled to the resin with 1,3-diisopropylcarbodiimide (DIC, 3 equiv), HOBt (3 equiv) and 4-dimethylaminopyridine (DMAP, 0.3 equiv) in DMF. The remaining amino acids were coupled on an ABI 430A peptide synthesizer as their symmetrical anhydrides or HOBt activated esters. The peptide resin was Fmoc protecting group deprotected with piperidine (20% in *N,N*-dimethylformamide (DMF)). The protected peptide was cleaved from the resin with 1% TFA in DCM (3 x 15 min) and the solution was concentrated under reduced pressure. The linear protected peptide was dissolved in DMF (2 mL) and added dropwise to a solution of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 3 equiv); DIEA (6 equiv) in DMF (10 mL) at room temperature over 30 min.[26] The cyclization was complete after 30 min as determined by HPLC. The solution was concentrated under reduced pressure, dissolved in H_2O (50 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic solution was concentrated under reduced pressure. The crude cyclic peptide fragment (1.5 equiv) was dissolved in DMF (10 mL) and added in a portion to a solution of the protected heptapeptide resin (1 equiv); HBTU (1.5 equiv) and DIEA (3 equiv) in DMF (10 mL) on a manual shaker. The coupling was complete after 3 hours as verified by Kaiser test.[27] All final peptides (compounds **2-10**) were fully deprotected and cleaved from the resin with liquid HF/anisole (9:1), extracted into aqueous solution and lyophilized. All peptides were purified to homogeneity (>96.5% by analytical HPLC) by preparative reversed-phase HPLC on a Vydac C18 column (218TP1022, 2.2 X 25.0 cm, 15 mL/min) with a mobile phase of 0.1% TFA in H_2O and increasing concentrations of 0.1% TFA in acetonitrile. The structures of all peptides were determined

by electron-spray mass spectrometry (ES-MS) and amino acid analyses (AAA). The endothelin receptor binding (ET_A and ET_B) and functional assays (AAR_A and AAR_B) were performed as previously described.[11]

Figure 1a:

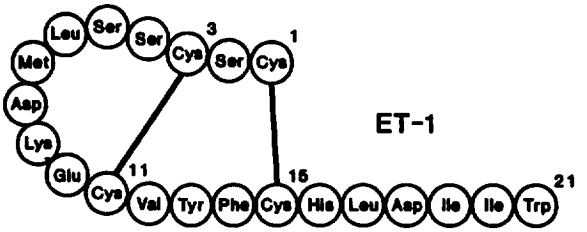
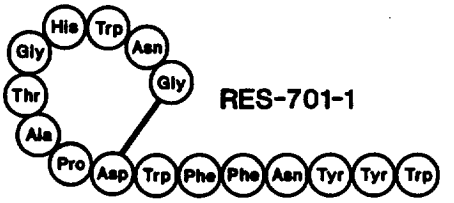


Figure 1b:



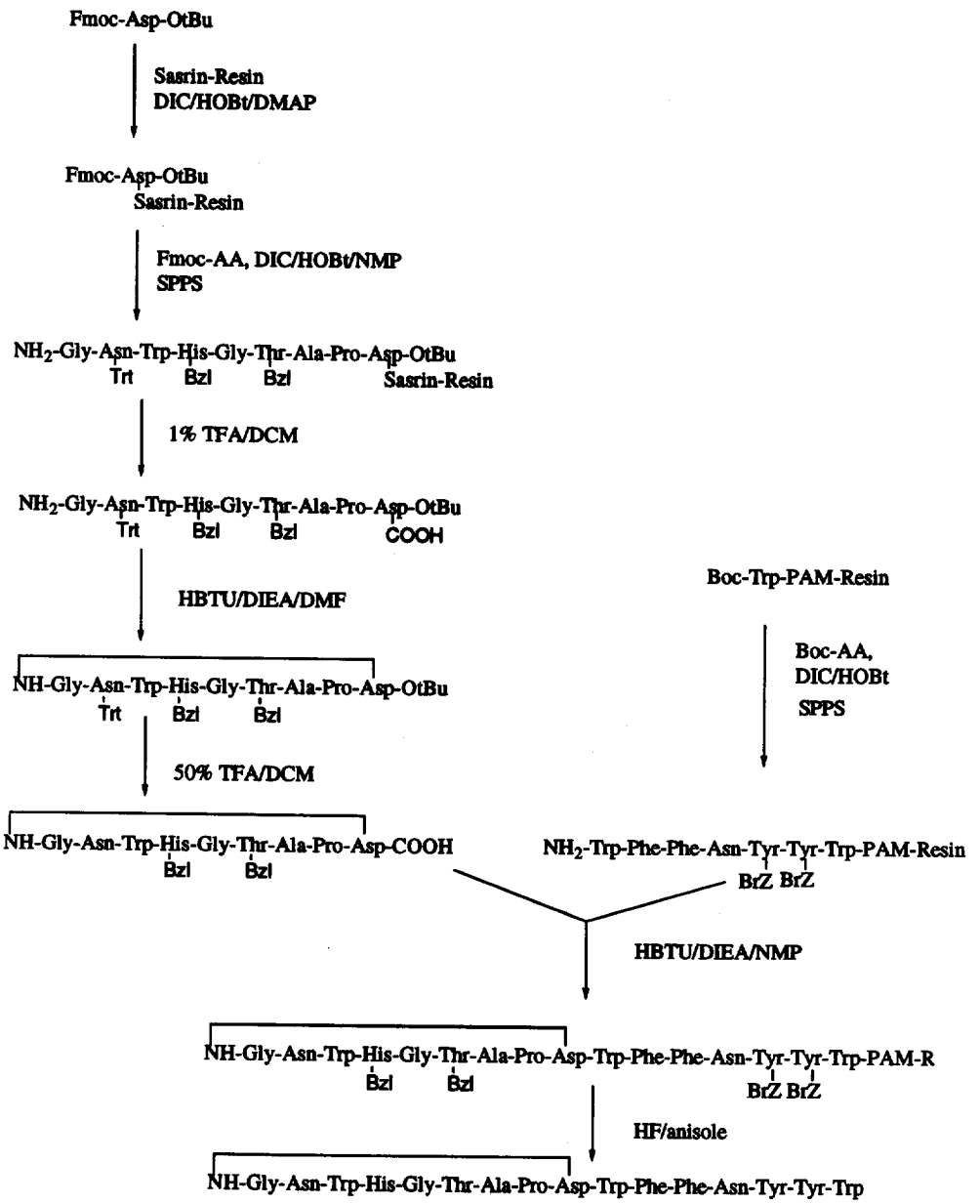
Results and Discussion:

We have developed an efficient solid/solution phase synthetic strategy for the preparation of RES-701-1 (Scheme 1). Several attempts at the preparation of RES-701-1 by standard solid phase approaches with formation of the lactam bridge between the α-amine of Gly and the β-carboxylate of Asp on the solid support or in solution, were unsuccessful. The synthetic strategy outlined in Scheme 1 provides RES-701-1 in a highly pure form (>99%) and is amenable to the multiple gram scale.

Upon examining the pharmacological properties of synthetic RES-701-1 (compound 1) we found that it was not selective for the human ET_B receptor over that of the ET_A receptor. In fact, this peptide only possesses low micromolar affinity for both receptor subtypes with IC_{50}'s of 7.8 and 6.5 μM for ET_A and ET_B, respectively.[28] A careful examination of the experimental protocols utilized for the ET_B receptor binding assay reveals that our assay is similar to the literature report[16-18] and we are both using the human cloned ET_B receptor stably expressed in Chinese hamster ovary (CHO) cells. Therefore, it is not readily apparent why this discrepancy in binding affinities at the ET_B receptor subtype exists. In addition, we have examined the functional activity of this preparation of RES-701-1 by measuring its ability to block ET-1 and sarafotoxin-6c (SRTX-6c) stimulated arachidonic acid release in rabbit renal vascular smooth muscle cells (AAR_A) and in CHO cells expressing the recombinant rat ET_B receptor (AAR_B).[11] In both, both cases only micromolar activity was observed (IC_{50} = 7.6 (AAR_A) and 16.0 μM (AAR_B)) which correlates well with the binding results. We must assume that our synthetic approach yielded the compound described as RES-701-1, since all of the synthetic intermediates and the final product has been fully characterized for homogeneity (HPLC) and structural integrity (ES-MS and ^{1}H-NMR (where appropriate)). Since the previous preparation was not synthetic, but an isolate from the culture broth of *Streptomyces* sp. two possibilities exist; 1) the preparation may be contaminated with a small amounts (<1%) of a highly potent compound, or 2) that the structure as reported for RES-701-1 is incorrect.[28]

We have previously reported on the preparation of several potent antagonists of ET-mediated vasoconstriction from the C-terminal hexapeptide of ET-1 by the incorporation of D-amino acids and N-terminal

Scheme 1. Synthesis of RES-701-1:



capping groups.[19,20] We attempted to apply this methodology to the C-terminus of RES-701-1 (compounds **3-8**). In all cases, compounds with weak to minimal binding affinities without receptor selectivity resulted. Also it has been reported that potent and selective agonists for the ET_B receptor can be developed from the 8-21 sequence of ET-1 (IRL 1620 and related analogues).[29] With this in mind, we prepared the corresponding linear analogue of RES-701-1 (compound **9**). This compound had only >2.5 μM affinity for either of the ET receptors. This additional data on linear C-terminal fragments of RES-701-1 further supports our pharmacological results with this synthetic preparation of RES-701-1.

Table 1: Analytical Data and Receptor Binding Affinities of RES-701-1 and Related Analogues.[30]

	Compound	HPLC (purity (%))	ES-MS $(m/z)^+$	Binding (IC_{50} (μM)) ET_A[a]	ET_B[b]
1	RES-701-1 (isolated)[c]			>5	0.010
2	RES-701-1 (synthetic)	>99	2045.1 (M+H)	7.8	6.5
3	FFNYYW	98.2	939.5 (M+H)	>25	>25
4	WFFNYYW	>99	1125.5 (M)	2.4	15
5	Ac-WFFNYYW	96.5	1168.7 (M+H)	0.25	7.6
6	Ac-wFFNYYW	>99	1168.5 (M+H)	8.1	14
7	bhg-FNYYW	>99	1041.5 (M+H)	9.5	13
8	Ac-bhg-FNYYW	>99	1082.5 (M)	1.9	4.3
9	WHGTAPDWFFNYYW	98.2	1900.7 (M+H)	>2.5	>2.5
10	GNWHGTAPDWFFNYYW	97.6	2082.9 (M+Na)	>2.5	>2.5

[a]Binding data in Ltk cells stably transfected with the human ET_A receptor. [b]Binding data in CHO cells stably transfected with the human ET_B receptor. IC_{50} values were derived from single competition experiments in which data points were measured in triplicate. Binding data was computer-analyzed by nonlinear least squares analysis giving the best fit for a one site model. [c]See references 16, 17 and 18.

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