BIOLOGICAL FUNCTION AND CHEMISTRY OF ENDOTHELIN. A REVIEW

Jan HLAVÁČEK[1,*] and Renáta MARCOVÁ

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Flemingovo nám. 2, Czech Republic; e-mail: [1] honzah@uochb.cas.cz

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The first part of this review deals with the biosynthesis and a biological function of strongly vasoactive peptides named endothelins (ETs) including vasoactive intestinal contractor. Where it was useful, snake venoms sarafotoxins which are structural endothelin derivatives, were also mentioned. In the second part, an attention is paid to structural basis of the ETs biological activity, with respect to alterations of amino acid residues in the parent peptides modifying the conformation and consequently the physico-chemical and biological properties in corresponding ETs analogs. Special attention is focussed on the area of ETs receptors and their interaction with peptide and non peptide agonists and antagonists, important in designing selective inhibitors of ETs receptors potentially applicable as drugs in a medicine. A review with 182 references.

Key words: Peptides; Endothelin; Sarafotoxin; Peptide synthesis; Vasoconstriction; Vasodilatation; Peptide antagonists; Non peptide antagonists; Conformation analysis.

1. INTRODUCTION

Vascular endothelium lines the inner walls of blood vessels, acts as modulator of vascular functions, and plays a role in the control of coagulation, lipid transfer, immunologic activity, and vascular tonus. Endothelial cells have been shown during the past few years to produce a number of vasodilating and vasoconstricting substances[1]. In 1980, Furchgott and Zawadski[2] were able to prove the presence of a locally acting vasodilating factor lacking prostaglandin character. It was named "endothelium-derived relaxing factor" (EDRF; ref.[3]) and identified as nitric oxide (NO). The isolation of EDRF enhanced the research of a balancing vasoconstricting factor. Pulmonary conversion of angiotensin I into angiotensin II was known by then[4] and the activity of acetylcholine esterase already detected in many blood vessels.

It has been demonstrated in 1985 that the vascular endothelium is the site of origin of additional vasoconstricting products one of them causing vasoconstriction lasting more than 60 min in contrary to the short effect of other vascular mediators[5,6]. This long-acting substance was isolated and sequenced by Yanagisawa *et al.*[7] in 1988 from a culture of endothelial cells and given the name of endothelin (ET). This peptide of unusual structure, composed of 21 amino acid residues, is the most potent vasoconstrictor so far identified. ET has been demonstrated a year later to be one of the three hydrophobic peptides – endothelins (ETs) found in the mammalian genome[8].

2. BIOSYNTHESIS OF ENDOTHELIN

ETs are synthesized in various tissues, only ET-1 was detected in vascular endothelium. ET-2 and ET-3 are produced in other tissues, such as brain, kidney, suprarenal glands, and intestines. ET-3 is relatively abundant in nervous tissues and it seems to be the nerve form of ET. ET-1 is also produced by nonvascular tissues, such as brain, kidney, lungs, and others. Vascular smooth muscles also produce ET-1 *in vitro* yet in amounts 100 times lower than endothelial cells[9].

Similarly to other peptide hormones and neurotransmitters the ETs arise from a specific prohormone by a proteolytic process (Fig. 1). The precursor of the ETs is preproendothelin, a polypeptide containing over 200 amino acid residues. Human preproendothelin consists of 212 amino acid residues, contains characteristic N-terminal hydrophobic secretion sequences and this seems to indicate that the prepro form is transported through the cell membrane for further processing. This form undergoes proteolytic

cleavage yielding a 38 residue peptide, the so called big endothelin[8]. A neutral endopeptidase, the ET-converting enzyme (ECE), subsequently splits the Trp[21]–Val[22] bond in big ET thus releasing the final peptide. This process is necessary for its full biological activity[10,11] since the activity of big ET-1 is 100 times lower *in vitro* than the activity of ET-1. Big ET-1 is specifically converted by ECE into ET-1 in human brain and this has led to the conclusion that the complete synthesis of ET-1 takes place in this organ[11–13].

Some authors believe that they will be able to control the level of ET in the body[14]. One of the logical lines of approach toward this goal is the manipulation with receptors, *e.g.* the use of a specific antagonist. Since this is a

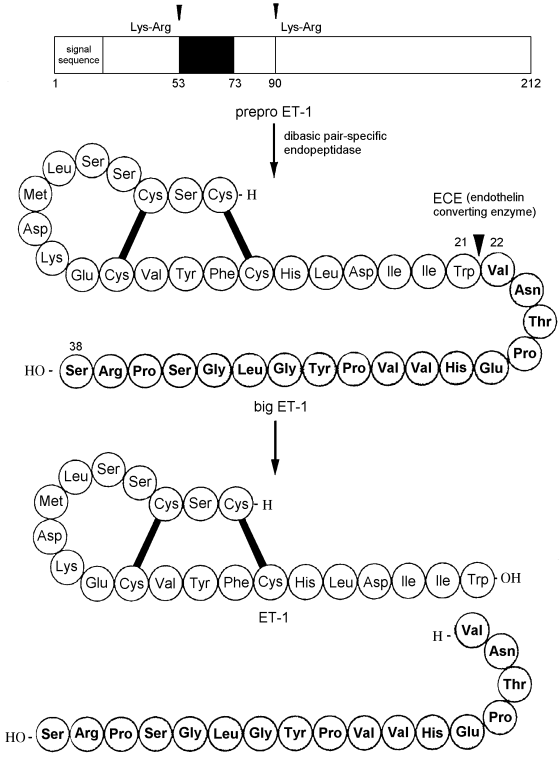


FIG. 1
Release of endothelin from its polypeptide precursor. Copyright 1996 Jpn. J. Pharmacol.

rather difficult task the inhibition of the precursor-cleaving enzyme could be an alternative and successful approach.

The production of ET, *i.e.* the synthesis of ET mRNA can be induced in endothelial cells by adrenaline, thrombin, and a Ca^{2+} ionophore, also by vasoactive hormones (*e.g.* by angiotensin II, arginine-vasopressin), cytokinins and growth factors, by physical stimulation such as hypoxemia or increased tension in the blood vessels, action of free radicals and endotoxin. Glucocorticoids increase ET-1 production in a cell culture of smooth muscles of blood vessels by 500% yet being without any effect on ET-1 production in endothelial cells. The production of ET is inhibited by NO and nitro vasodilators supplying NO, and also by atrial natriuretic factor (ANF) decreasing the concentration of intracellular cyclic GMP (ref.[9]).

3. BIOLOGICAL FUNCTION OF ENDOTHELIN

ETs constrict all types of smooth muscles by activation of specific receptors, however, they can also relax the muscles, in some cases either by direct receptor activation or indirectly by releasing a specific compounds from the endothelium and epithelium such as are NO and eicosanoids. Even though the ET concentration in plasma is low, ET behaves as a circulating hormone and can locally influence cells which are close to ET-producing tissues. ETs modulate both chronotropy and inotropy, bronchoconstriction, neurotransmission and act as regulators of other hormones and neurotransmitters. When applied intravenously, regulation of blood pressure (both pressor and depressor effect), of local blood flow and of kidney function are observed; renin, vasopressin and ANF levels can also be increased. ETs also show a strong hyperplastic and hypertrophic effects which become effective during the normal growth and development of the organism yet which can also bring about neoplastic growth after its injury[15].

These observations initiated interest in the research on ET action under physiological and pathophysiological conditions (Table I). Numerous studies have focused on the finding of stimuli responsible for gene expression and/or production of the peptide. An increased level of free ET has been detected *in vivo* under various pathological conditions of the organism, such as in particular essential hypertension[7,16], pulmonary hypertension[17], ischemic heart diseases[18], brain hemorrhage[19], acute and chronic kidney failures[20,21] and glaucoma[22,23]. Elevated ET levels have been detected in human patients with acute severe asthma[24–26]. The ET level in blood of healthy individuals and patients suffering from various diseases[27–36] has been examined in experiments aimed at the determination of changes in

TABLE I
Pharmacological responses induced by ET-1 and ET-3 in various tissues

Responses	Tissue and organ	Effect, assay
ET-1 > ET-3 responses	Vascular smooth muscle	Pressor effect *in vivo*, contraction, prostanoids release, mitosis of endothelial, fibriblast and smooth muscle cells, release of tissue type plasminogen activator and von Willebrand factor, binding assay
	Bronchial smooth muscle (human)	Contraction
	Smooth muscle of gall bladder (human)	Contraction
	Lymphatic vessel (human)	Constriction
	Uterine smooth muscle (rat)	Contraction, binding assay
	Heart	Positive inotropic and chronotropic effect, ANP release, binding assay
	Adrenal gland (bovine)	Release of aldosterone, cathecholamine release increase, binding assay
	Fibroblast cells (human, mouse)	Ca^{2+} transport, binding assay
	Osteoblast cells (rat)	DNA synthesis, binding assay
ET-1 = ET-3 responses	Kidney (human, rat)	Vasoconstriction of renal artery, inhibition of renin release, Na^{+} absorption decrease, mitosis and contraction of mesangial cell, diuresis and natriuresis
	Mesenetric artery (rat)	Transient relaxation, EDRF release
	Stomach and illeum	Constriction, ulcerogenicity
	Whole animal (rat)	Initial depressor response *in vivo*
	Whole animal (rabbit)	Inhibition of *in vitro* platelet aggregation
	Liver	Glycogenolysis
	Central nervous system (rat)	Increase in substance P, LH, FSH release
	Astrocytes (rat)	Ca^{2+} transport
	Eye	Rise in intraocular pressure

ET levels under pathophysiological conditions. ET was proposed to have an important role in endometrial repair and regeneration following menstruation. It may act to constrict basal arterioles following menstruation to halt menstruation bleeding[37]. Recent studies have shown an involvement of the ET system in cirrhosis with marked relationships to liver dysfunction and splanchnic and systemic haemodynamics[38]. A function of ETs as autocrine regulators of tumor cell growth was described using ovarian carcinoma cells[39]. The RIA measurement was carried out with ET-1, ET-3, and big ET (refs[40,41]). The results of the tests have shown that the basic ET level of human plasma is 1.5–2 pg/ml increasing several times under pathophysiological conditions. The results, however, can be influenced by several factors:

1) ET is strongly bound to its receptors and the dissociation of the resulting complexes is relatively slow; hence, the amount of free ET need not necessarily represent the correct response to the biological activity of the system.

2) The increase in local ET concentration in target organs can be higher than in plasma.

3) Big ET-1 is functionally less active than ET-1 yet it is metabolically more stable and can thus be a possible source of additional ET-1 release.

4) Local ET degradation can occur. The functional importance of increased ET concentration in plasma is thus subject to speculations[14].

Sarafotoxins (SRTXs), strongly toxic snake venoms, are clear ET analogs from the chemical viewpoint and can thus bring about similar effects. They cause strong vasoconstriction of coronary arteries and death after intoxication with these venoms being the result of heart ischemia or infarction[42].

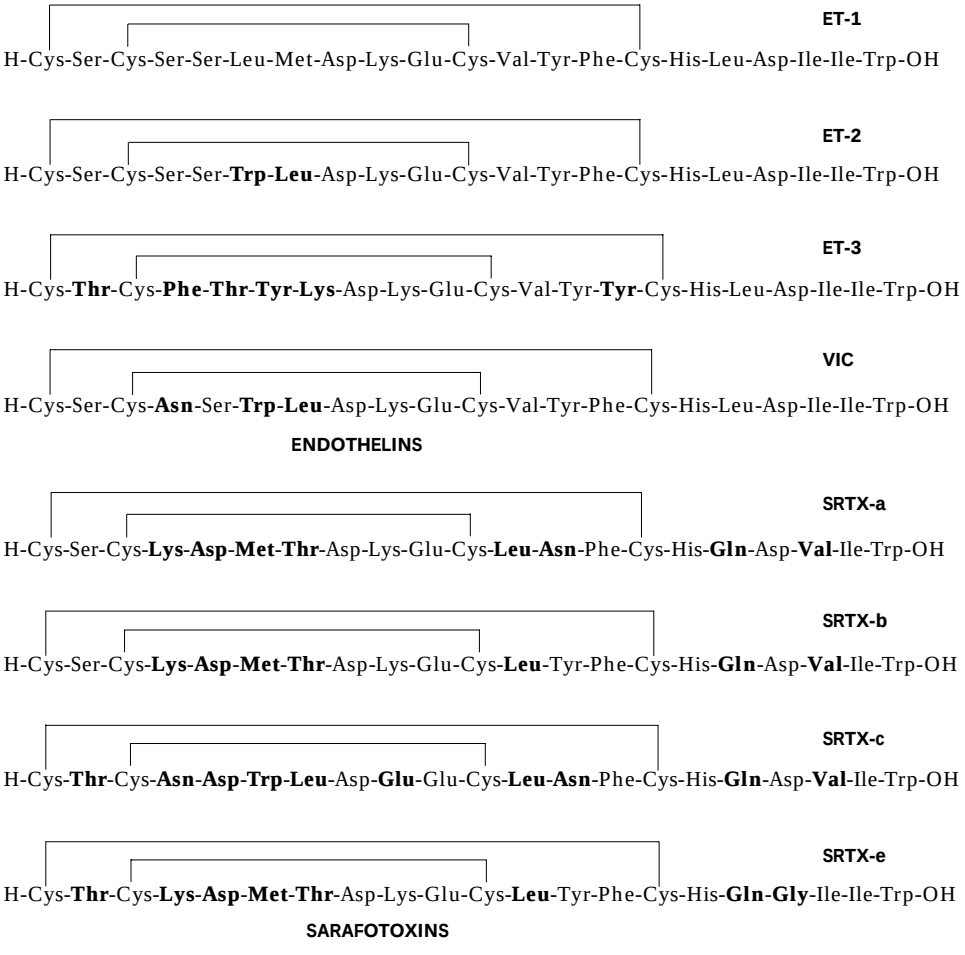
4. ENDOTHELIN STRUCTURE

ET is a peptide composed of 21 amino acids. Two disulfide bonds provide its structure with two rings, the more flexible one being located closer to the C-terminus of the peptide. The original peptide detected in the culture of endothelial cells, now known as one of the three endothelins, was named ET-1. The existence of ET-2 and ET-3 was predicted after subsequent isolation of the ET-1 encoding genes. The amino acid sequence of ET-1, originally isolated from porcine aorta (pET-1), is identical with human ET-1 (hET-1) and dog, rat, and mouse ET-1. Human ET-2 is identical with dog ET-2 and human ET-3 with the rat peptide[43].

Similarities in structure existing between ET and several peptide neurotoxins, such as the bee venom apamin or α-scorpion toxin, have been already noted in the original study by Yanagisawa *et al.*[7]. Both contain

several disulfide bonds. It has been demonstrated that SRTX (refs[44,45]) a venom of the Israeli snake *Atracta engadensis*, is structurally very similar to ET. ETs and also SRTXs (SRTX-6a, -b, -c, -e) are two structurally related groups of efficient vasoactive peptides. Another member falling into the endothelin group is the "vasoactive intestinal contractor" (VIC, ref.[46]), known as endothelin-β. ETs, SRTXs, and VIC display similar pharmacological activities.

At present, eight naturally occurring peptides of the ET-SRTX group are known. Although they are derived from different sources, they show a high degree of sequential homology. Each of the peptides contains four cysteine



residues and about 60–70% of their 21 amino acid residues are identical. They contain normally two disulfide bonds, a hydrophobic C-terminus (residues 16-21) and three polar side chains (residues 8-10).

5. RECEPTORS FOR ENDOTHELIN

Receptors are usually classified with respect to their affinity to antagonists. ET receptors are probably the first group of G-protein pair receptors whose classes have been defined by molecular biology methods prior to the discovery of their specific agonists or antagonists[47]. No types of selective antagonist groups were originally defined for the ET receptor system and the first two specific representatives of antagonists became known at the time the classification was in progress.

The receptors from bovine lungs were cloned and synthesized[48] in COS cells in 1990. These receptors were selective for both ET-1 and ET-2 compared to ET-3 (K_{is} ET-3/ET-1 = 980) whereas the receptors cloned from rat lungs and also synthesized in COS cells were unable to distinguish between these peptides[49]. The receptors selective for ET-1 were designated ET_A whereas the nonselective ones were marked ET_B (Table II). The ET_A receptors reside generally on smooth muscle cells and mediate vasoconstrictor responses, whereas the endothelial cells express the ET_B receptors (Fig. 2) which mediate vasodilator effects *via* the ET-induced release of endothelium-derived relaxing factors[50]. However, ET_B receptors have also been

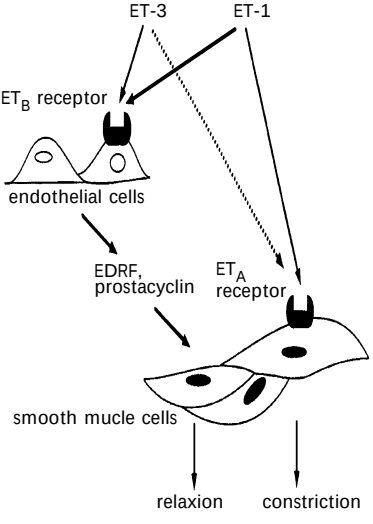


FIG. 2
Schematic presentation of vasodilatation and vasoconstriction induced by endothelin-1 (ET-1) or endothelin-3 (ET-3). Copyright 1996 Jpn. J. Pharmacol.

shown to reside on vascular smooth muscle cells and mediate vasoconstriction[51]. Although only one gen was cloned for ET_B receptors[49], the presence of ET_{B1} and ET_{B2} receptors was described using anatomical, functional and pharmacological profiles[52–57].

Reynolds *et al.* suggested the possibility of subclassification based on the species difference[58]. On the other hand, Hara *et al.*[59], when examining the affinities of several ligands to ET_B receptors in living cells and their membrane preparations, have found no difference between rat and human ET_B receptors, probably due to destruction of the intact cell membrane structure possibly influencing the conformation of receptors in cell membrane, which is very flexible[60]. The agonist selectivity can be defined in terms of binding affinity only and not on the basis of a reaction in cell or tissue which is dependent on receptor population. The agonist efficiency can depend on the receptor factor and it is therefore possible that ET-1 has a higher efficiency than ET-3 in tissues containing all receptors of the ET_B type. Tissues and cells containing a mixture of ET_A and ET_B receptors can still show selectivity for ET-1 in terms of efficiency. The need for the use of affinity values for a defined receptor type and directly obtained from binding data is generally overlooked and data recorded in literature thus become largely biased. On the other hand, the advantage of this designation is the possibility of defining ET_A receptor subgroups since the degree of selectivity or ET-1 has not been defined[61].

TABLE II
Endothelin receptor characterization

Endothelin	Receptors	
	ET_A	ET_B
Agonist potency	ET-1 > ET-2 >> ET3	ET-1 = ET-2 = ET-3
Tissue	Aorta	Coronary artery
B		Endothelium
Biological activity	Constriction	Constriction Dilatation
Selective agonists	None	ET-3 (500-fold specific
		Sarafotoxin S6c (3.104-fold specific)
Antagonists	BQ-123	BQ-788

The distribution of different receptor types has been studied with the aid of cloned receptors. Even though data derived from various sources somewhat differ, the main model remains essentially the same. Brain contains almost exclusively ET_B receptors whereas some tissues with a broad structure of blood vessels, such as placenta or lungs, contain a large amount of receptors of both types. Heart is probably the only tissue showing a high content (both relative and absolute) of ET_A receptors[62]. Even though the brain is well supplied with blood, its capillaries mostly lack smooth muscles and this may account for the absence of ET_A receptors. Kidneys also contain mostly ET_B receptors. A small yet significant amount of receptors of both types occurs in liver where ET-3 is responsible for the induction of glycogenolysis and also for the constriction of the hepatic vein (portal vein). The isolation of a receptor specifically binding ET-3 has been also reported. This receptor was cloned from the skin of the tropical frog *Xenopus* and designated ET_C (refs[14,61]). Detection of ET receptors in vascular smooth muscle cells of a human coronary artery was described recently[63].

The amino acid sequence of ET_A and ET_B receptors has been elucidated by sequencing of cloned cDNA. It is a single chain structure containing seven hydrophobic domains penetrating through the membrane, with α-helical structure and a relatively long extracellular N-terminus. Approximately, 25% of the sequence is identical with sequences of other peptide receptors[9]. The amino acid sequence homology of receptors ET_A and ET_B is 60%, the sequences of the transmembrane regions are very similar yet there is no homology in the N-terminal extracellular segment.

One of the characteristic features of binding of ETs to various tissues is the extremely slow dissociation of the receptor–ligand complex. Thus, *e.g.* this binding is almost irreversible in cells of rat vascular smooth muscles[64]. The half-life of free ET in the rat kidney is about two minutes and thus in essential contrast to the extremely long-lasting functional effect. This obvious discrepancy can be partly due to a very slow progress of dissociation of the receptor–ligand complex[14].

The slow association of ET to receptors and its dissociation from ET–receptor complexes is not basically surprising since this ligand is relatively long, containing a flexible C-terminus, essential for the binding to the receptor, and the principles of binding of small neurotransmitters to G-protein receptors pair apply here. It has been proposed that ET receptors interact *via* G-protein linkages with membrane ion channels and membrane phospholipases[65–67]. Galron *et al.*[68,69] was able to find differences between the mode of signal transfer by ETs and SRTXs even though these peptides interact with the same receptor. This may indicate that these pep-

tides possess a certain core domain of their structure through which they interact with the same receptor, and with another portion of sequence through which various receptor conformations are induced[61].

6. CHEMISTRY OF ENDOTHELIN

6.1. Space Structure and Position of Disulfide Bonds

6.1.1. Alanine and D-Amino Acid Scans

The experiments with Ala and D-amino acid ET-1 scans involved the synthesis of a large series of analogs in which all amino acid residues with the exception of Cys were stepwise replaced by Ala or a D-amino acid[70].

The contribution of the side chain can be effectively assessed only on the condition that the analog retains the conformation of the naturally occurring molecule. Ala is thus more suitable than Gly showing a tendency to loosen the secondary structure.

ETs refolding always leads to the 1 : 3 ratio of the 1,3 isomer and 1,4 isomer (Fig. 3). The analogs prepared were subjected to random oxidation and

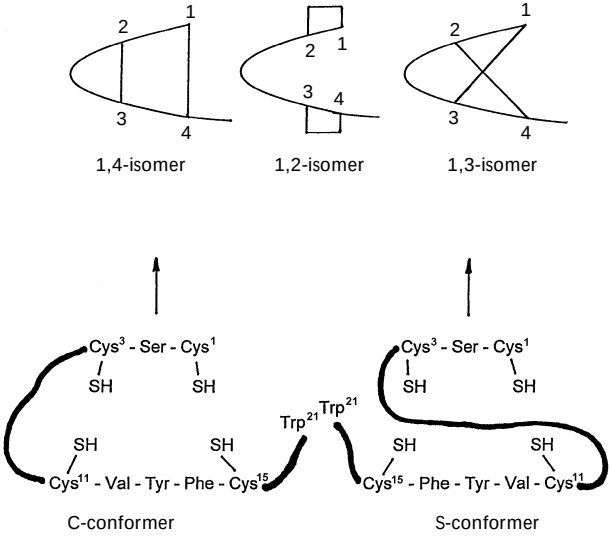


FIG. 3
Disulfide isomers of endothelin.

the ratios of the isomers formed were examined. The same ratio, *i.e.* with the dominant 1,4 isomer was obtained with all analogs of the Ala scan.

The results obtained with the D-amino acids were, however, different. Almost one half of the analogs showed special preference for each individual isomer. The analogs with the D-Ser^{2}, D-Lys^{9}, and D-Val^{12} yielded exclusively the 1,4 isomer whereas that with the D-Ser^{4} the 1,3 isomer only. The analogs containing D-Ser^{5} and D-Leu^{6} showed the isomer ratio of 1 : 1. The distribution of the individual isomers was examined by CD. From the results of these measurements, the D-amino acids can be classified with respect to their effect on isomer formation as follows:

a) D-Amino acids adjacent to Cys residues which show isomer selectivity.

b) D-Amino acids, such as D-Lys^{9}, which stabilize the terminus of the α-helical segment.

c) D-Amino acids destabilizing bends of the structure.

Ala scan: From conclusions of binding studies carried out *in vitro* with cells of vascular smooth muscles, the greatest biological importance has been ascribed to Asp^{8}, Tyr^{13}, Phe^{14}, Leu^{17}, and Trp^{21}. Four of these five positions are occupied by hydrophobic amino acids. The Ala analogs occupying positions of aromatic amino acid residues (13, 14, 21) decrease the binding affinity for the receptor below 2% of that of ET and this indicates that these positions are important for the contact with the receptor. Replacement of Asp^{8} and Leu^{17} yields analogs deprived of more than 90% of agonistic activity of ET yet retaining almost its original receptor affinity. These amino acids therefore are responsible for the signal transfer. The other three positions, Val^{12}, Asp^{18}, and Ile^{20}, adjacent to the biologically important amino acids (Tyr, Leu, Trp) partly tolerate the substitution with Ala retaining 14 to 50% of the potency of ET. The Ala analogs of the 1,3 isomers are always less active than the corresponding 1,4 isomer.

The opinion on the role of positions 8 and 14 is slightly different in the light of the conclusions of another study (Hunt *et al.*[71]). However, this can be accounted for by differences in the techniques of biological and binding testing and especially by the fact that the experiments were carried out solely with monocyclic analogs with the external ring closed, differing from bicyclic analogs in the three-dimensional structure.

D-*Amino acid scan:* Four of the seventeen analogs could be isolated in one isomeric form only[72]. This is most likely due to the thermodynamic disadvantage accompanying the process of refolding of one of the isomers. In three cases (Ser^{2}, Ser^{4}, and Val^{12}), the amino acids involved were those adjacent to half-cystine residues, the fourth position was that of Lys^{9}. The side chains of these amino acids obviously interfere with the disulfide bond be-

ing formed. Verification of this assumption would require the knowledge of the three-dimensional structure of both ET isomers in solution: inversion of the configuration of the corresponding amino acid in modeling experiments could then reveal which configuration is sterically more hindered. Unfortunately, such structure is available for the naturally occurring, *i.e.* 1,4 isomer only. It has been made clear with the aid of this model that Val^{12} interferes with the 1–15 disulfide bond and that Lys^{9} interferes with the α-helical arrangement of the 11-15 segment.

The following identical results were obtained with both series of scanning experiments. The isomer with a higher retention time on HPLC is the one with the higher relative binding and agonistic affinity and is also obtained in higher yield (thermodynamically dominant). The only isomer escaping an explanation is the one with D-Met^{7} (the isomer with a lower retention time has been isolated in a larger yield), showing a high affinity for the receptor and a low contracting activity.

The changes of configuration of the individual amino acid residues led to derivatives with a lower activity than that of the Ala derivative in both series of experiments. A three- to five-fold increase in binding affinity of some analogs has led to the conclusion that the side chains of two polar amino acids (Lys^{9} and His^{16}) are not involved in ionic interactions with the receptor. Moreover, the D-Lys^{9} analog shows a higher activity in the contraction test than the Ala^{9} analog, the finding which seems to indicate interactions occurring also outside the receptor pocket.

6.1.2. Disulfide Bonds

An important aspect necessary for the understanding of protein structure is the dynamics of the folding of its molecule. Primary interest has been focused on the isolation of folding intermediates and on studies of factors affecting their stability. The isolation of such intermediates is troublesome, however, peptides rich in disulfide bonds offer such a possibility.

ET is an attractive and biologically acceptable model for such analyses. Its four cysteine residues permit the formation of three disulfide isomers[70] (Fig. 3). The positions of these four cysteine residues, however, limit the disulfide formation to two isomers only (1,3 and 1,4 isomers). The 1,4-ET isomer is the naturally occurring one with paired 1,4 (Cys^{1}-Cys^{15}) and 2,3 (Cys^{3}-Cys^{11}) half-cystines (numbered from the N-terminus). The 1,3-ET isomer with 1,3 (Cys^{1}-Cys^{11}) and 2,4 (Cys^{3}-Cys^{15}) half-cystines paired has the same disulfide arrangement as apamin whose structure in solution is completely different. The 1,4-ET isomer has been shown by NMR studies to pos-

sess α-helical structure of the Lys[9]-Cys[15] segment and a bend of the Ser[4]-Asp[8] segment[73]. This conformation is retained also in the reduced structure of the peptide and is known as the C-conformer. The 1,3-ET isomer is the crossed form of the 1,4 isomer and needs an additional reversed bend for the formation of the 1-4 disulfide[74].

The effect of protecting groups and of the oxidizing agents was examined in experiments in which the ET analog was linked through its disulfide bond directly to the resin[75]. The 1,4 isomer is preferentially formed. When the Trt group is selectively removed by 3% TFA in DCM and subsequent oxidation with the di-*tert*-butyl azodicarboxylate is carried out the 1,3 isomer and 1,4 isomer is obtained, the ratio being 3 : 1, respectively. By contrast the S-conformer is preferentially formed in solution. The formation of one isomer only is also supported by the incorporation of penicillamine (Pen) into the molecule of the peptide[76]. After the oxidation of ET analogs – [Pen[1,11],Nle[7]]ET-1 and [Pen[3,15],Nle[7]]ET-1, the 1,4 isomer is formed at a ratio exceeding 20 : 1.

6.1.3. Physico-Chemical Studies on Endothelin Conformation

Several conformation studies have been performed in an effort to understand the fundamental principles of receptor recognition and activation by the peptide. The conformation of ET-1 was subject of ^{1}H NMR studies in DMSO (refs[77–81]), aqueous acetonitrile[82], aqueous ethane-1,2-diol[83], and in dilute acetic acid[84,85]. Also studies carried out by CD measurements[78,81,86], fluorescence measurements, Raman spectroscopy, and X-ray diffraction measurements[87–89] were described. Other compounds also falling into the group of ETs and SRTXs, such as *e.g.* ET-3 (refs[90–92]), SRTX-6b (refs[93,94]), [Nle[7]]ET-1 (ref.[95]), human proendothelin-1 (refs[96,97]), and an ET antagonist cyclo(D-Asp-Pro-D-Val-Leu-D-Trp) designated BQ-123 (ref.[98]) have been also investigated. In spite of the large number of studies performed, it still remains to be shown which conformation aspects are essential and what is their role in receptor activation/inhibition and recognition.

^{1}H NMR studies on ETs and SRTXs, no matter whether carried out in predominantly aqueous or organic solvents, generally show the presence of a dextrorotary α-helical structure between[73,81] Lys[9] and Cys[15]. By contrast, Munro *et al.*[80] were able to detect α-helical structure in the region between Leu[6] and Cys[11] and an obviously non-helical structure in the region between Cys[11] and Cys[15]. The existence of α-helical structure in predominantly aqueous media has been reported in regions Glu[10]-Cys[15], Lys[9]-Cys[15], and Lys[9]-His[16] (refs[82–84,92,99]). It was reported in a recent ^{1}H NMR

and CD study on conformation of human ET-2 that solution structure forms an α-helix over residues Lys^9-Leu^{17}, and that residues Ser^5-Asp^8 might form a β-turn type I. The authors suggested the theory that any difference in affinity to corresponding receptor between ET-1 and ET-2 can be attributed to the character and local position of their side chains, rather than to a differently folded backbone[100].

The existence of α-helical structure documented by a negative band at 223 nm and a positive band at 192 nm has been confirmed in most CD studies. The 223 nm band, known to indicate α-helix induction or stabilization, was more marked in aqueous TFE (ref.[78]).

On the basis of CD measurements of ETs and SRTXs carried out by Tamaoki *et al.*[87] these peptides can be classified as falling into three groups: (i) ET-1, ET-2, and VIC; (ii) SRTXs; (iii) ET-3. ET-3 seems more flexible and with a minor tendency to form α-helical structure in TFE and is more sensitive to changes in pH than other peptides of this group. The 223 nm band was most marked in SRTXs. Each member of the group can assume a slightly different conformation and solvent dependent variations can be envisaged.

The cysteine residues in apamin are located in the same positions as in ETs and SRTXs yet disulfide pairing is different, namely Cys^1-Cys^{11} and Cys^3-Cys^{15}. Nevertheless, the existence of α-helical structure in the region between amino acid residues in positions 9 and 17 has been demonstrated by NMR studies[101] of this peptide. Kobayashi *et al.* suggested[102] that the arrangement of half-cystine residues in the motive Cys-XXX-Cys through Cys-X-Cys is a structural element which may be correlated to the biological activity of neurotoxins. These results are in agreement with the structure–activity studies on ET-1 showing that many amino acids sensitive to substitution (such as Asp^8, Glu^{10}, Val^{12}, Tyr^{13}, Phe^{14}) are located in the region with α-helical structure[61].

A reversed bend in the Ser^5-Asp^8 segment of ET-1 and human proendothelin has been described in several studies[83,84] yet contradicted in others. A reversed bend between Asp^4 and Leu^7 has been reported to exist in VIC (ref.[92]). This result shows that even though many positions are modified in the external ring of VIC compared to ET-1, the former takes up a very similar conformation, the finding which is in agreement also with the results of CD studies.

The most controversial point is the conformation of the C-terminal linear hexapeptide of ET-1. Saudek *et al.*[77,78] reported a structure in which the hexapeptide is bent back toward the cycle formation whereas no such interaction between the C-terminus and the main peptide body has been ob-

served by other authors[73,80,82]. The cleavage of ET-1 by proteolytic enzymes proceeds in two steps: scission of the external ring between Ser^5 and Leu^6 and subsequent cleavage of the C-terminus at the Asp^{18}-Ile^{19} bond[103]. This can be explained by the compact structure of ET-1 in which the C-terminus is not available for the proteolytic enzymes unless the external ring is opened.

^{1}H NMR studies on human proendothelin[96,97] have focused on the elucidation of structural features at the site of cleavage of the Trp^{21}–Val^{22} bond. A high probability of a β-bend at the cleavage site was suggested by the Chou–Fassman prediction of secondary structure, the finding which is in agreement with theories regarding these conformation features as component parts of the signaling system for endoproteases. Whereas a defined structure in the ET-1 part was found in these studies, a series of conformations in dynamic equilibrium and without a preference for tertiary structure was characteristic of residues 17 to 34.

The present development of small cyclic antagonists with relatively restricted conformations and more defined structures, such as cyclo(D-Asp-Pro-D-Val-Leu-D-Trp) – BQ-123 (refs[98,154]), may pave the way to the understanding of features necessary for recognition and inhibition. From molecular modeling of this cyclopeptide, Satoh and Barlow[104] were able to conclude that its conformation is close to that of Leu^6-Met^7-Asp^8 in ET-1. These authors postulate that two sites with donor hydrogen bond, two nonspecific hydrophobic sites, and a third site with a positive charge are necessary for the recognition of the ET_A receptor. Structure–activity studies have pointed to the important role of the tertiary structure of ET-1 in the binding to the ET_A receptor (especially of the disulfide loop) and of aspartic acid in position 8 (in L-configuration). Also the necessity of Trp^{21} and of its C-terminal carboxyl function for the recognition of the receptor were clearly documented. Even though BQ-123 lacks the C-terminus, the side chain of D-Asp adjacent to the indole ring exerts such function. Another linear antagonist with the same C-terminus Leu^{17}-Trp^{21} as ET-1, designed PD 142893, shows a high affinity for ET_A receptors, the fact indicating that the recognition of this receptor need not necessarily be correlated with the tertiary structure formed by two disulfides[61].

The comparison of BQ-123 with ET-1 should be carried out prudently since the former has been defined as a noncompetitive antagonist[105] and may thus react with other sites than the natural sites of ligand binding.

A difference in ET_A receptor antagonistic properties of the cyclo(D-Leu-D-Val-Pro-D-Asp-Trp) (IPI 147) and cyclo(D-Trp-D-Asp-Acp-D-Val-Leu) (IPI 725) have been explained from ^{1}H NMR and computation studies[106]. It

was suggested that these cyclopentapeptides differ in their space structure. The IPI 147 contains a type II β-turn with a hydrogen bond between N–H of D-Val and C=O of D-Asp; IPI 725, on the other hand, contains two turns, the type II β-turn with a hydrogen bonds between N–H of D-Asp and C=O of D-Val, as well as β-turn with a hydrogen bond formed between D-Val N–H group and D-Asp C=O group. Although both β-turns contain the same residues, their orders are reversed. Therefore IPI 147, which is only a weak antagonist, appears to be more flexible than the strong ET_A antagonist IPI 725.

Recent studies in this field have been devoted to the conformation of ET in solution and to fluorescence measurement[107] of the conformation dynamics of the C-terminus of ET-1 and of some of its analogs. A new mimetic analog of the C-terminal ET-1 hexapeptide with a disulfide bridge was designed and synthesized to be an antagonist for the ET_B receptor mediating ETs effects of the bronchial smooth muscle constriction[108]. CD measurement over a broad range of pH tempted suggestion that the C-terminal hexapeptide of ET-1 may undergo a conformation change on binding to the receptor *via* an interaction with the His imidazole ring. This suggestion is consistent with the results from Cody *et al.*[109] showing that only stereoisomers of His were tolerated at this position.

More recently, ^{1}H NMR spectroscopy was utilized to elucidate the change in the binding activity of the IRL 1620 {Suc[Glu^9,$Ala^{11,15}$]ET-1(8-21)}, a strong ET_B receptor agonist[161], and some of its analogs, with replacement of the Asp^{18} residue by Gly in the corresponding linear derivatives. These peptides also consist of 14 amino acid residues; however, this replacement resulted in 10- and 12-fold increases in the affinity to the ET_A receptor. Therefore, the conformation of these series with dipalmitoyl phosphatidylcholine vesicles was elucidated[110]. The space structures for the ET_A selective analogs investigated are characterized by a β-turn for the C-terminal Ile^{19}-Ile^{20}-Trp^{21} part and α-helix in the N-terminal region. The replacement of the Asp^{18} by the Gly residue allows these ET_B selective analogs to take a "folding back conformation" of the C-terminal region to the helical face, which can cause an increase in affinity to the ET_A receptor. A solution structure of another ET_B receptor selective agonist [Cys(Acm)^{1,15},Ala^3,Leu^7,Aib^{11}]ET-1, was determined by molecular modeling based on ^{1}H NMR data[111]. It was concluded that this linear analog folds into α-helix in a water–methanol (1 : 1) solvent mixture and that the removal of the disulfide bridges in this case interferes neither with the ET-1 secondary structural features nor with the ET_B receptor binding activity.

6.2. Amino Acid Residue Modifications

The research on the structure–activity relationships of ETs and SRTXs has led to the conclusion that the replacement of certain amino acid residues by others results in dramatic changes in the biological potency of the peptide. Unsubstituted N- and C-termini are absolutely essential for its activity: its amides and esters are almost inactive[112,113]. A decrease in biological activity is also brought about by extension or alkylation of both the N- or C-terminus, removal of the carboxyterminal Trp[21] (decreases the activity to 1/1 000 of the original value, probably by decreasing the ability of the peptide to bind to the receptor), shortening the length of the peptide chain from the C-terminus (ET-1(1-18) has zero activity[112–115]) and manipulation with disulfide bonds (an analog of the ET-1 with closed 3–11 bond and with Ala in positions 1 and 15 is 200 times less active than ET-1, the monocyclic analog with Ala in positions 3 and 11 retains 10% of the activity of ET-1)[71,116]. Cleavage of the Cys^3–Cys^{11} bond and protection as a Cys(Acm) or, alternatively, reduction of both disulfides with subsequent carboxamidomethylation give rise to a weak partial agonist (with respect to ET-1) judging by the constriction test with porcine aorta[115]. Enzymatic cleavage at the carboxy-terminal Ser^5 by bovine endopeptidase gives rise to the monocyclic peptide with biological activity comparable to that of the intact peptide[103]. Pelton *et al.*[117] reported that the two analogs, in which Cys of either one of the disulfide bonds has been replaced by Ala thus giving rise to a monocyclic analog, bind with a high binding activity to rat cerebral membrane and that these analogs are full agonists when tested with rat aorta[118]. Hence the decrease in biological activity is rather due to the character of the protecting groups than to the reduction of disulfide bonds and the disulfide bond is not a necessary prerequisite for the activation and recognition of the receptor. A comparison of both monocyclic analogs has revealed that a higher activity is retained by the analog with the external disulfide bond intact[71,112]. The deaminodicarba analog [$Ala^{3,11}$]ET-1 is completely inactive[112].

Functional differences between individual peptides of the ETs family can be ascribed to differences in their N-terminal amino acid sequences. As a rule, the amino acid replacements in this N-terminal part affect the biological activity of both ETs and SRTXs (ref.[43]). The amino acid sequence in the 2–7 region is very important for the binding to the receptor, Asp^8 and Glu^{10} are essential for the vasoconstrictor function and their replacement by Asn and Gln results in almost inactive analogs[119].

The amino acids in positions 4, 5, 6, 7, and 9 can be replaced with the subsequent small decrease in activity only[112]. Multiple substitution of these tolerant sites with Ala leads to analogs with EC_{50} 36 nM in contraction tests with arteries of rodents[71]. It seems that the negatively charged side chains of amino acids occupying positions 8 and 10 are very important unlike the positively charged groups of Lys in position 9. Both [Ala^9]ET-1 and [Leu^9]ET-1 are 100% agonists. Even the substitution of Lys with a negatively charged group, such as in [Glu^9]ET-1, gives rise to a full agonist[120].

The amino acid residues which occupy positions in the ring formed by Cys^3-Cys^{11} are tolerant to configuration inversion with the exception of [D-Asp^8]ET-1 which displays merely 0.6% of the activity of ET-1; [D-Lys^9]ET-1 is even four times more active than ET-1 (ref.[72]).

Val^{12} is conserved in VIC and also in the endothelin group and thus the presence of a neutral and hydrophobic residue is obviously required in this position. The less hydrophobic Ala^{12} analog is less potent than ET-1, yet Leu^{12} is adequately active. The existence of α-helical structure comprising residues in the positions 9–15 has been stressed in almost all conformation studies. Insertion of Pro into this sequence may interfere with this structure; in spite of that [Pro^{12}]ET-1 shows a high binding activity in certain tissues. However, its activity tested in functional studies is slightly decreased[121]. It thus appears that the α-helical structure is not a critical signal for receptor recognition and activation.

The substitution of Tyr^{13} with Ala afforded an analog with the activity similar to that of ET-3, *i.e.* a weak constrictor at low concentrations yet a full agonist at higher concentration values. The replacement of Phe^{14} by Ala results in the complete loss of vasoconstricting activity[72,122]. The substitution of positions 15 and 14 with D-amino acids decreases the activity below 10% as well as the substitution of Ser^2 which together with Tyr and Phe belongs to the ring between the two bridges.

As to the substitution of position 16, the [Phe^{16}]ET-1 is more potent than ET-1, as regards both binding and contracting activity, and is ten times more selective for the ET_A receptor[121]. The substitution of positions 17 and 19 radically decreases the activity of the resulting analog[122] as demonstrated also by Watanabe[119] who substituted Gly for Leu^{17}. Also the effect of substitution in position 18 on the behavior of the peptide was treated by Kikuchi *et al.*[123]. They found the insertion of amino acid residues with a hydrophobic side chain (Nva, Val, Ile, Leu, Phe, γ-MeLeu) to this position to cause a large decrease in contractile activity in porcine coronary artery. Moreover, the corresponding ET-1 analogs and also that with the Glu^{18} substitution exhibited antagonistic activity.

All amino acids in the C-terminal hexapeptide are sensitive to the inversion of configuration and their substitution with D-isomers produces analogs possessing merely 5% of the activity of ET-1 (ref.[72]).

Structure–activity studies on the ET fragments revealed that the carboxyterminal hexapeptide part of the ET-1(16-21), a full agonist when examined with guinea pig bronchus[124], inhibits in larger doses the binding of iodine-labeled ET-1 in guinea pig tracheal epithelium and smooth muscles[125]. The same activity has been observed with the corresponding carboxyterminal amide, expected to be less potent, and a similar activity has also been reported for the inhibition of ET-1 by acetylendothelin examined in rat cerebral tissue[126]. The weak affinity of ET-1(16-21) to ET receptors has led to speculations that the terminal hexapeptide does not act *via* ET receptors[127]. Additional structure–activity studies[128] with guinea pig bronchus revealed behavioral features similar to those of ET-1. ET-1(16-21) amide and D-Trp-containing analogs were less active and the activity was rapidly decreased when the sequence was shortened. Additional testing of various analogs of this hexapeptide demonstrated that antagonism is displayed merely by analogs with a D-amino acid in position 16 (refs[129,130]). ET-1(1-15) amide, ET-1(1-20) and the C-terminal hexapeptide[114] inhibit iodine-labeled ET-1 in renal arteries of hamster.

D-Trp analog of ET-1(16-21) induces a slow and long-term vasoconstriction, whereas a much faster vasoconstriction kinetics is observed with derivatives with shorter C-terminus. It has been concluded from these results that it is especially the C-terminal Trp which is essential for extremely long-term vasoconstriction activity characteristic of ET (ref.[115]). Position 21 has been scanned by Koshi *et al.*[131] The biological activity of all derivatives prepared by the replacement of Trp by other amino acids was measured on rat aorta strips and their binding activity was examined with rat cerebral membrane. The vasoconstriction activity of [Tyr^{21}]ET-1 and [Phe^{21}]ET-1 was by one order of magnitude lower than that of ET-1, the remaining analogs showing activities lower by two and more orders. None of the analogs tested behaved as an antagonist. The results of this study show that the existence of the aromatic side chain in position 21 is essential for vasoconstriction activity and for the binding to the receptor.

The linear [$Ala^{1,3,11,15}$]ET-1 analog ([4Ala]ET-1 in what follows)[117] is 1 700 times more selective for ET_B receptors than for ET_A receptors[132]. The analogs with shorter chains, [4Ala]ET-1(6-20), [4Ala]ET-1(8-21), and *N*-Ac-[4Ala]ET-1(10-21) still retain a high affinity to ET_B receptors unlike analogs [4Ala]ET-1(6-20) and [4Ala]ET-1(1-21) which show a significantly decreased affinity to these receptors. These data demonstrate that the Glu^{10}-Trp^{21} sequence without

disulfide bonds is sufficient for the binding to the ET_B receptor. The *N*-Ac-[4Ala]ET-1(10-21) analog is the shortest ET_B agonist and D-amino acid and Ala scanning studies of this fragment provided evidence showing that positions 14, 17, 20, and 21 are critical for the ET_B agonism.

The selectivity of ET-1(8-21) analogs for ET_B decreased as a result of the replacement of Asp^8 by Ala to a value sixty times lower and increased on the contrary after the replacement of Lys^9 by Ala or Glu (ref.[116]).

Efforts to prepare a compound specifically binding to one type of receptor only have led to the synthesis of the Suc[Glu^9,$Ala^{11,15}$]ET-1(8-21) analog, IRL 1620, with a 120 times higher selectivity for ET_B than for ET_A. This analog is even more selective for ET_B receptors than [4Ala]ET-1 (ref.[133]) and was also described as a selective radio ligand[134].

The ET_B agonism of SRTX-6c was derived from experiments with amino acid replacements in positions 9 and 13 which in ET-1, ET-2, ET-3, and SRTX-6b are occupied by Lys and Tyr, respectively, whereas in SRTX-6c by Gln and Asn. A series of linear analogs with alterations in positions 9 and 13 was prepared to verify this theory. All the analogs described showed a weak affinity for ET_A and a sequentially dependent affinity to ET_B. The results verified the view that one disulfide at least must be retained so that the binding to the ET_A receptor may occur and that for a high selectivity, both the N-terminus and a linear C-terminus are needed. The C-terminal ligand only is required for the selectivity to the ET_B receptor. Position 13 affects the affinity to ET_B more than position 9. Analogs with a shorter chain and Tyr in position 13 showed a surprisingly higher binding affinity to ET_B receptors than Asn-containing analogs. A series of shorter analogs with the naturally not occurring 11-15 ring showed a weak affinity to both types of receptors[135,136].

6.3. Endothelin as a Model for Peptide Synthesis Methodology

The structure of ET is a rare combination of the amino acid sequence relatively difficult from the viewpoint of synthesis and of two disulfide bonds. The first chemical synthesis of ET was reported by Japanese authors[137] in 1988. They were able to prepare all disulfide isomers of human ET-1 and to compare them with the naturally occurring peptide by HPLC.

The region selective linkage of the disulfides was made possible by Cys(MeBzl) protection in positions 1-15 (analog A), 1-11 (analog B), 1-15 (analog C), and by Cys(Acm) protection in positions 3-11 (analog A), 3-15 (analog B), and 1-3 (analog C). Analog A (disulfides 1-15, 3-11) was found to be identical with naturally occurring ET-1. They also prepared a peptide

protected by MeBzl groups only which they subjected to simultaneous deprotection followed by random oxidation. The two main peaks in the HPLC elution profile were found to correspond to analogs of type A and B, their ratio being 3 : 1, a negligible quantity of analog C was present. The behavior of analog A in HPLC in various systems was identical with that of naturally occurring ET and this led the authors to conclude that this type of disulfide bond also exists in the natural ET. The analogs prepared were also tested for contraction activity. Analog A gave almost the same response as naturally occurring ET whereas the remaining two analogs gave the same reaction when applied in 150 times higher doses.

A method using deprotection of Cys(Acm) by heavy metal salts (such as mercuric acetate) was published[138] in 1989. The advantages of this method were summarized by the authors in four points:

a) Selective deprotection; Cys is deprotected in the first step whereas the remaining protecting groups are unaffected and the peptide is still bound to the support.

b) The reagents and heavy metal salts are easily removed by washing with DMF.

c) Unlike with other methods, no loss of material during precipitation of heavy metal salts.

d) Selective activation of Cys by the mixed disulfide method.

Nomizu *et al.*[139] synthesized several analogs of human and porcine ET with prolonged peptide chain (22-39) on C-terminus for the purpose of comparison of their biological activities. They employed a two-step procedure for deprotection using TMSiBr/TFA in step 1 and HF or TMSOTf-thioanisole/TFA in step 2. They observed that the peptides cleaved off the resin by the two-step method using strong acids show a higher purity than products from the low-high HF procedure, with the exception of the analog of pig ET-1(39) from which a side product is formed as a result of the cleavage of the His^{16}–Leu^{17} bond.

Koshi[131] closed the disulfide bonds by atmospheric oxygen in 0.2 M solution of ammonium trifluoroacetate containing 5 M guanidinium chloride. The oxidation was allowed to proceed for two days.

A study whose authors synthesized ET-1 and its analogs using partial protection of Met in the form of Met(O), Trp(For), Cys(Acm) and examined the effect of the protection on the biological activity of the analogs, was reported[140] in 1993. They also described in their study an improvement of some synthetic procedures consisting in the oxidation with I_2 in 80% AcOH and removal of the For group with piperidine. The authors are convinced

that such oxidation gives better results than with I_2 in MeOH and that the use of piperidine does not lead to any degradation of the peptide.

Another synthetic procedure was based on the use of TMSiCl for regioselective closure of the disulfide bond[141]. The disulfide bond between Cys^1 and Cys^{15}, protected by Acm group, was closed by the classical procedure, *i.e.* with I_2 in MeOH whereas Cys^3 and Cys^{11}, protected by tBu group, were linked within 15 min in the presence of methyltrichlorosilane and diphenyl sulfoxide. This method needs a protection of Trp by the formyl group since iodination of the unprotected indole ring is preferred to the formation of the S–S bond. The authors also point out that the use of the Fmoc strategy during the subsequent Trp formylation is more advantageous than the classical Boc strategy.

Closing of the disulfide bond by oxidation directly on polymerous support[75] was made possible by a combination of protection of four Cys residues by Acm and Trt groups. The Acm group was removed by $Hg(AcO)_2$–2-mercaptoethanol mixture. The di-*tert*-butyl azodicarboxylate was used as a mild oxidizing agent. The second disulfide bond was closed by I_2, isothiocyanate or thallium(III) acetate.

ETs have been also prepared in a multisynthesizer. Modified polyethylene rods with an acid-labile linker were used as support. Twenty seven peptides (such as oxytocin, vasopressin, somatostatin, cholecystokinin, bradykinin, and others) were synthesized simultaneously with ETs. One of the ETs synthesized was natural ET-1, another peptide obtained had cleaved disulfide bonds with Cys protected by Acm groups. The purity of the crude products was 58.4 and 69.2%, respectively[142].

The method of fragment condensation was chosen for the preparation of ET-1 *via* its dicarba analog[112] for the sake of comparison with the stepwise synthesis[137]. A similar approach was used in the synthesis of the 1-deaminomonocarba analogs of ET-1 (ref.[143]). Two routes have been chosen for their synthesis. The first one used a fragment condensation strategy at which the aminoterminal 1-deamino-15-carba-pentadecapeptide was prepared on acid-labile Rink resin, split off from the resin by solvent mixture AcOH–DCM–TFE and after cyclization and allyl ester group removal with $[Pd(Ph_3P)_4]$ and morpholine in the same solvent mixture was finally coupled to the carboxy-terminal hexapeptide bound to Wang resin. After cleavage of the analog from the resin with TFA in DCM the inner disulfide bridge was closed by air oxidation at pH 8.5 in DMSO with ammonium trifluoroacetate buffer. In the second route the same peptide and its 1-carba analog were prepared by stepwise assembling of individual amino acids from the C-terminus on a Merrifield type resin[144]. The carba bridge replac-

ing the outer disulfide bond was closed on the resin and, after simultaneous removal of the side-chain protecting groups and splitting the peptide from the resin the inner disulfide bond was closed by air oxidation in aqueous DMSO at pH maintained at 8 with aqueous ammonia.

Random oxidation with subsequent identification of individual conformers by enzymatic cleavage or by testing of the binding to the receptor were utilized for the synthesis of human big ET (refs[112,139,145]). The separation of the isomers, however, was troublesome and required HPLC in isocratic mode. Moreover the replacement of some amino acids led to significant changes in the ratio of both isomers as demonstrated by Ala scans.

Ramage and Steward[146] compared methods used for the closure of the disulfide bond from the viewpoint of product purity and successful outcome of the reaction. They abandoned the protection of Cys by the tBu-group combined with acid cleavage with 2-mercaptoethanol and subsequent oxidation with potassium ferricyanide. The authors do not recommend this procedure for the synthesis of ET-1 either. The chemical advantages of this group are lessened by its hydrophobic character which is conveyed to the peptide. The synthesis of ET-1 carried out with the use of tBu protection of Cys leads to an S-protected peptide insoluble in any solvent suitable for gel filtration. They carried out their further experiments with Cys(Trt). A certain drawback of this protection was a slow condensation of trityl derivatives and a great heterogeneity of the crude product. The authors used therefore Cys(Acm) and obtained the tetra-Acm peptide in 80% yield after cleavage from the Wang resin by means of reagents mixture TFA–anisol–EDT and subsequent gel filtration. Oxidation with I_2 in AcOH proceeded slowly and a number of side products were formed.

The problems of using I_2 oxidation in the presence of Met and Trp have been also treated[147]. The deprotection by Hg^{2+} ions can be often incomplete, especially in sequences with several Cys residues. The authors therefore used Bu_3P to remove S(tBu) protection and the first disulfide bridge closed by oxidation with potassium ferricyanide. However, after closing the second disulfide bridge by treatment of the monocyclic bis-S(Acm)peptide with iodine in 80% acetic acid the [β-3-oxindolylalanine[21]]ET-1 was identified as the product due to oxidation of unprotected Trp[21]. They found the Ac-Trp-OH to be an excellent additive which suppressed the destruction of this C-terminal Trp.

Another oxidation procedure tested[148] has used glutathione in guanidinium chloride solution. The reaction proceeded smoothly with complete transition of the peptide into solution. The combination of Cys protection with Acm and the subsequent mixed disulfide oxidation is re-

garded by the authors as the most suitable method of formation of the disulfide bond. The region-selective disulfide closure appears somewhat problematic in this case and so far no compromise satisfying the necessity of a hydrophilic group protecting Cys, deprotection under mild conditions and suitable scavenger selection has been found.

The course of oxidation of the Cys residues can be examined, *e.g.* by the Ellman reagent[149] or by HPLC which is rather difficult in the case of some ET analogs because of the low solubility of the compounds. The correctness of the linkage can be verified by enzymatic cleavage and by the analysis of the synthesized fragments. A problem arises when the disulfides in certain analogs are close to each other since the enzyme need not necessarily to cleave the peptide to unambiguous fragments and a prolongation of the reaction time may lead to an attack on a minority fragment already produced. An alternative solution of this problem consisting in a partial reduction leading to a peptide with one cleaved disulfide bond is complicated by thiol disulfide interchange[150].

Gray[151] was able to develop a method of partial reduction at pH 3 in which this interchange is minimized. The products are subsequently separated at pH 2 without significant disulfide interchange and the free SH groups are then alkylated. Original pairing can be assayed in a sequencer. Some disulfide interchange may occur during the alkylation, yet the side products are easily identificable.

Structure alterations of the ETs and SRTXs molecules have led to the synthesis of over three hundreds analogs with modified agonistic and antagonistic vasoconstrictor activity. In a majority of the agonists, the rigid cyclic structure with both disulfide bridges between Cys residues was maintained and the changes in the activity were performed mainly due to modifications in the side chains of the other amino acid residues. On the other hand, shortening and reduction of the amino terminal cyclic part of ETs molecules has led to antagonists of ET receptors.

As to the synthetic strategy, both Boc/Bzl and Fmoc/tBu amino acid protection systems were utilized; the sulfhydryl groups were also protected with Acm and Trt groups. Major attention was paid to disulfide bonds closure. Utilizing the “natural folding” of the ETs molecules, some authors have made no differentiation between the outer and inner Cys residues protection and obtained ET peptides with the correct, biologically active 1,4-arrangement of the cyclic part in satisfactory yields. However, for unambiguous formation of the double disulfide structure of ETs, the regioselective procedure seems to be a better, higher yields affording, approach than spontaneous random oxidation of fully reduced ET.

6.4. Endothelin Receptor Mapping Using of Antagonists

Efficient and selective antagonists of ET receptors are suitable tool for a detailed studying of ET receptor pharmacology. For that reason, the antagonists of both ET_A and ET_B receptors have been prepared having either peptide or non peptide structures. The peptide antagonists proposed on the basis of rational design and optimization of prospective compounds as found in scanning programs have been synthesized. Making use of their structure–activity studies Spinella *et al.*[152] prepared "full peptide sequence" antagonists [Dpa^1,Asp^{15}]ET-1, with the external disulfide bond (1–15) replaced by the amide bond.

The choice of the external ring for modification was substantiated by the results of studies showing that this bond is more important for ET to retain its biological activity than the bond between Cys^3 and Cys^{11} and that it may play a bigger role in ET activity than that of a mere structure element. To preserve the primary amino group, which is also essential for intact biological activity, 2,3-diaminopropanoic acid was placed in position 1. Another methylene group was inserted between the N-terminus and the α-carbonyl and the number of atoms of the bridge was decreased from 4 to 3. This analog was found to be an efficient antagonist when assayed in experiments with ET-induced pulmonary vasoconstriction. The analog shows IC_{50} = 2 nM (IC_{50}(ET) = 0.05 nM) in competitive binding studies performed with rat pulmonary arteries. Even though the latter analog cannot inhibit ET-3-induced vasoconstriction, its receptor activity has been proved.

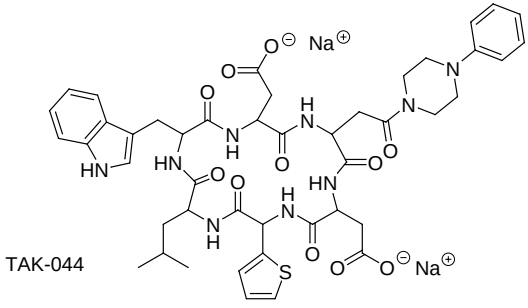
The antagonism observed with [Dpa^1,Asp^{15}]ET-1 is in contrast with the results obtained by Nakajima[112] in 1989 with an analog containing also the Cys^1–Cys^{15} disulfide bond replaced by the amide bond. However, this peptide was inactive, obviously because the second disulfide bond was eliminated by the replacement of the half-cystine residues by Ala and because the N-terminal α-amino group was absent.

A small cyclic pentapeptide, cyclo(D-Glu-Ala-allo-D-Ile-Leu-D-Trp), designated BE-18257B, was isolated from the fermentation medium of *Streptomyces mysakiensis* and was found by Ihara *et al.*[153] to be a relatively efficient antagonist. This peptide was specific for ET_A receptors in competitive binding studies and inhibited vasoconstriction when tested on isolated hamster ileum arteries even though it was lacking any vasoconstriction activity at concentrations of 100 μmol/l. Structure–activity studies[154] on this peptide stimulated the development of cyclo(D-Asp-Pro-D-Val-Leu-D-Trp) designated BQ-123 which binds to the ET_A receptor with IC_{50} = 22 nM and acts as an-

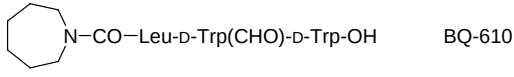
tagonist of ET-induced vasoconstriction tested on isolated porcine artery strips.

Activity studies have shown that cyclopentapeptides cyclo(D-Leu-D-Val-Pro-D-Asp-Trp) (IPI 147) and cyclo(D-Trp-D-Asp-Acp-D-Val-Leu) (IPI 725), exhibit ET_A antagonistic activities from a weak to strong due to their different space structures and flexibility[106].

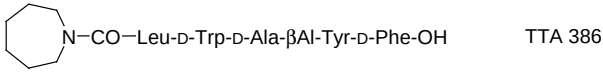
The cyclic hexapeptide[155] TAK-044 (Takeda, Osaka) exhibits antagonistic activity against the ET_A receptor. Subsequent structure–activity studies have led to the development of the potent ET_A receptor selective linear tripeptide BQ-610 and the hexapeptide TTA 386 antagonists[156–159].



TAK-044



BQ-610



TTA 386

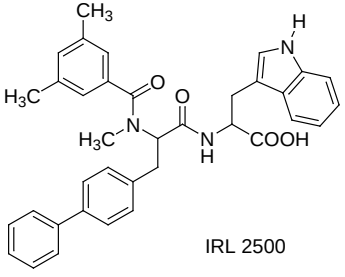
For the development of selective ET_B antagonists the findings that ET_B receptors bind amino acid residues common to all ETs were utilized. Thus the compound IRL 1038 which is a shortened ET-1 analog [Cys^{11},Cys^{15}]-ET(11-21) was synthesized, being 100 times more selective for ET_B than for ET_A (ref.[160]). However, further modifications resulted in ET_B receptor selective agonists IRL 1620 and BQ-3020 (refs[157,161]). Another linear peptides exhibiting a selective antagonistic action on ET_B receptor, IRL 2500, IRL 2659, IRL 2796, and BQ-788, were finally designed and synthesized[157,162,163]. The introduction of D-Trp analogs of BQ-123 and BQ-788 with C-2 substituents resulted in potent ET receptor antagonists with various ET_A/ET_B subtype selectivity. Compounds with 2-cyano-D-Trp were ET_B receptor-selective

antagonists, those with 2-halo- and 2-methyl-D-Trp exhibited a combined ET_A/ET_B antagonism[164].

A series of linear hexapeptide analogs[129] was prepared on the basis of the results gained in studies on the activity of the C-terminal hexapeptide of ET-1. Experiments with these analogs have shown that Ac-D-His-Leu-Asp-Ile-Ile-Trp-OH is a weak antagonist. Data on the behavior of the latter in bio-

Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp-OH
11 15 21
IRL 1620

H-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp-OH
11 15 21
BQ-3020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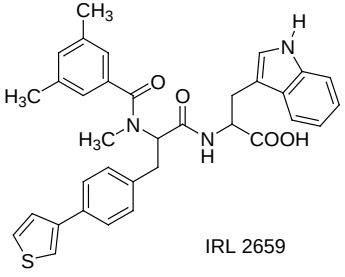
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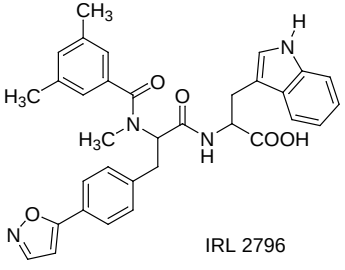
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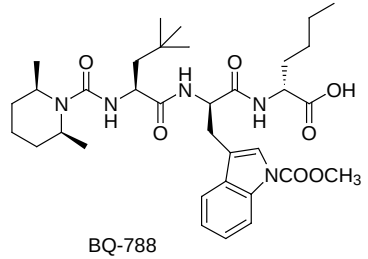

BQ-788

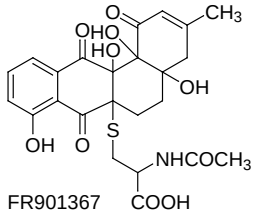
logical tests led to the synthesis of the Ac-D-Bhg-Leu-Asp-Ile-Ile-Trp(Me)-OH, which shows a good affinity for both receptor types and is an antagonist of ET-1 induced accumulation of inositol phosphate and of ET-1-stimulated

Ac-D-Bhg-Leu-Asp-Ile-Ile-Trp-OH PD 145065

vasoconstriction tested on pulmonary and thigh hamster arteries. Similarly, another linear hexapeptide Ac-D-Bhg-Leu-Asp-Ile-Ile-Trp-OH (PD145065) exhibits antagonistic activity against both ET_A and ET_B receptors[165]. A pro-

spective goal of the receptor antagonist studies was to optimize the features of this analog and to modify its structure so that an even smaller molecule may be obtained with increased activity and a higher ET_A/ET_B specificity which could be subsequently subjected to peptide mimetic modifications.

A chemical optimization of the natural non peptide antagonist FR901367 (ref.[166]), has led to the design of peptide FR139317, a potent ET_A receptor-specific antagonist[167]).



FR901367

FR139317

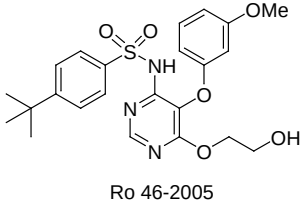
A series of cyclic depsipeptides, isolated from a *Microbispora* culture and observed to act as non selective antagonists[168], have also been prepared. Inhibitors worth interest are some lipopeptides isolated from a *Bacillus subtilis* culture. In experiments designed to identify the active unit in lipopeptides, some of their linear analogs were synthesized. It has been observed that the activity rests on a combination of a fatty acid chain with the C-terminal tripeptide unit Asp-D-Leu-Ile.

The small and potent selective peptide antagonists of ET receptors have proven to be extremely valuable tools for characterization of the ET receptors and for the determination of their tissue distribution. However, there are well-known limitations of the use of peptides *in vivo* for the treatment of chronic animal disease models and for clinical applications in human patients. Thus, much effort has been directed toward the discovery of non-peptidic ET receptor antagonists.

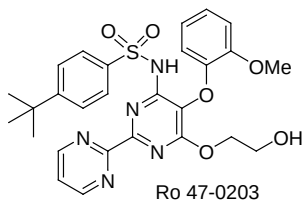
Some molecules of non-peptidic character have been identified by screening several thousands of compounds listed in libraries of natural and chemical products[169]. The antagonist FR901367 (ref.[166]), isolated from *Streptomyces sp.*, inhibits aorta contraction only when applied in high concentrations.

The screening for the compounds inhibiting the binding of the iodine-labeled ET-1 to human placental membrane was successful in development of non-peptidic ET-1 antagonists of low molecular weight and suitable for oral applications. The screening led to the choice of pyrimidinyl sulfonamides which were synthesized as part of an antidiabetic project and de-

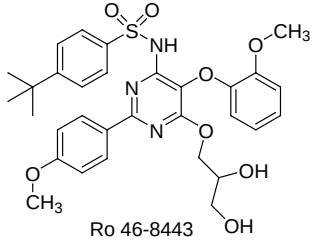
scribed as weak ET-1 inhibitors. In experiments with structural modification of these compounds the Ro 46-2005 was discovered[170]. This compound is a strong inhibitor lacking hypoglycemic activity. It completely inhibits the binding to human vascular smooth muscles *in vivo* (ET_A receptors) and to rat aorta endothelial cells (ET_B receptors). It shows no agonistic effect when assayed with isolated rat aorta rings, yet it is an antagonist of ET-1-induced contraction, the effect being dose-dependent. The antagonist is very selective for ET-1 since it does not affect contractions induced by angiotensin II, serotonine, noradrenaline, KCl, or prostaglandin. Probably the most interesting feature of this compound is the possibility of its oral application assayed on monkeys. The pathophysiological role of ET-1 and, moreover, the new concept of therapeutic blocking of ET receptors were shown after the application of the Ro 46-2005 in these experiments. Subsequent structure–activity studies led to synthesis of the anisyl derivative Ro 46-8443 and, above all pyrimidinyl derivative, Ro 47-0203, bosentan[171], a far more potent selective antagonist of pressor activity. Either oral or intravenous administration of this compound led to inhibition of both the initial transient depressor response on ET_B and the following prolonged pressor response on ET_A receptor induced by i.v. injected ET-1.



Ro 46-2005



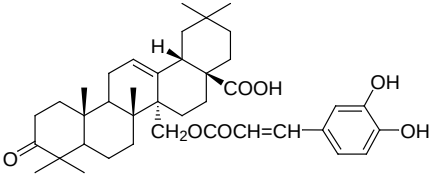
Ro 47-0203

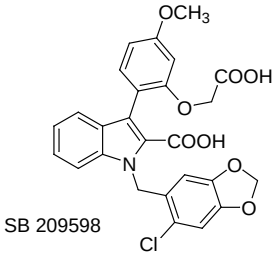


Ro 46-8443

Another compound designated 50-235 has been isolated by Fujimoto *et al.*[172] from the plant *Myrica cerifera* and shown to be a selective ET_A antagonist. Also diphenylindanes[173] like SB 209598 were introduced as nonpeptidic antagonists of ET receptors. Using ^1H NMR spectroscopy a three-

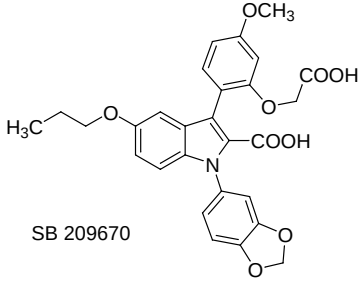
dimensional structure was compared with low-energy ^{1}H NMR-based conformations of ET-1 with the result that 1- and 3-phenyl groups of these antagonists are mimics of a combination of two of the aromatic side chains of Tyr^{13}, Phe^{14}, and Trp^{21} in ET-1.



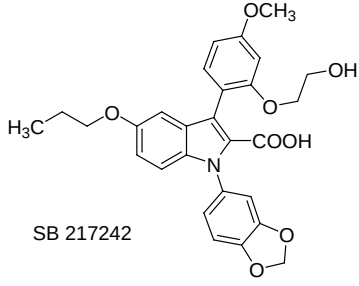


SB 209598

As a result of progress in peptido-mimetic simulation, an extremely potent non-peptidic ET receptor antagonists SB 209670 and SB 217242 have been synthesized and assayed[174]. Intraduodenal administration produced a dose dependent reduction of blood pressure in spontaneously hypertensive rats, which continued for 24–48 h. The antagonists also protected against ischemia-induced neuronal degeneration in a gerbil stroke model and attenuated neointima formation following rat carotid artery balloon angioplasty.



SB 209670

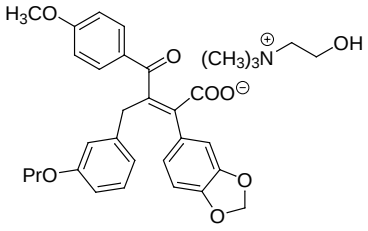


SB 217242

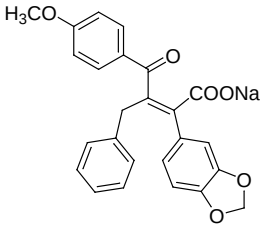
Other non-peptidic, orally active ETs antagonists PD 155080, PD 155719, PD 156707, specific for ET_A receptor and PD 160672 and PD 160 874 causing a nonselective inhibition of both the ET_A and ET_B receptors have been developed in Parke Davis[175]. The compounds, administered orally to rats, blocked ET-1-induced pressor responses.

A group of the nonselective sulfonamide antagonists L-749329, L-751281, and L-754142, specific for ET_A receptor was successful in prevention of acute renal insufficiency induced by aortic cross-clamping in dogs[176] and

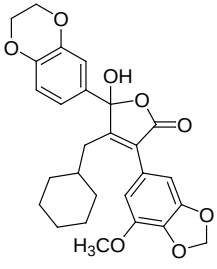
the T-0115 markedly suppressed monocrotaline-induced pulmonary hypertension in rats[177]. The J-104121 prevented ET-1-induced death in mice for up to 4 h following the i.v. application[178].



PD 155719



PD 155080



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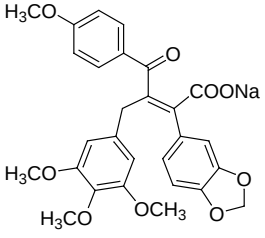

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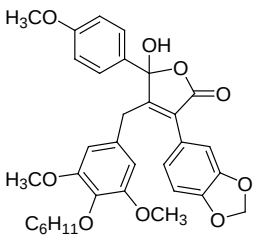
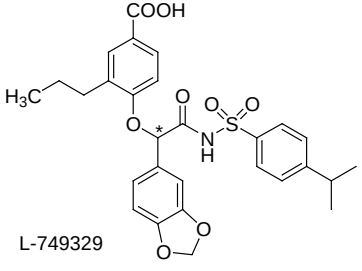
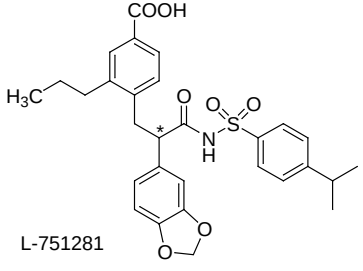


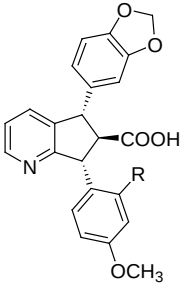
PD 160672



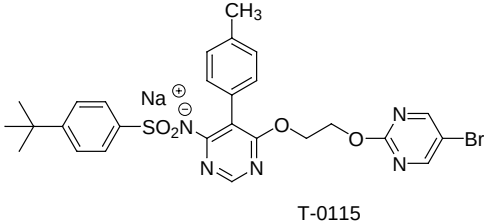
L-749329



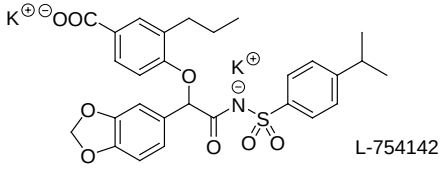
L-751281



J-104121

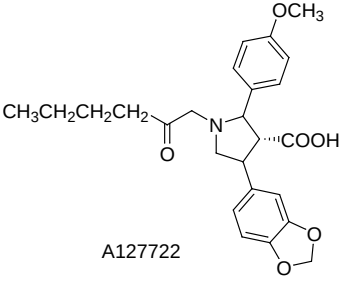


T-0115

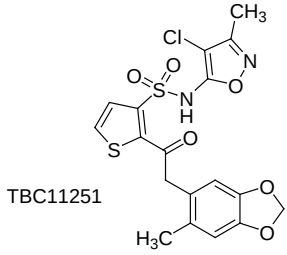


L-754142

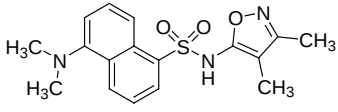
More recently, new orally active non peptide selective ET_A antagonists were described, *e.g.* BMS182874, TBC11251, and A127722 (refs[163,179–181]) which are currently under extensive investigation and are expected to be marketed in the near future.



A127722



TBC11251



BMS182874

These compounds, as well as potent and selective ET receptor antagonists yet to be discovered, will be a useful tool for discerning the therapeutic potential of future compounds designed to fight diseases resulting from overstimulation of ET receptors.

7. CONCLUSION

The physiological role of ET was unclear until 1988 when a group of Japanese scientists made enormous efforts to elucidate the character of this peptide. One of the explanations of the absence of integral information on ET is the fact that the compounds used for its investigation were ET agonists. It has been demonstrated that many systems are affected by ET acting on one of the two receptors cloned as yet or by a receptor of sequence so far unknown. In these experiments, however, the ET concentrations in blood or

tissues, were many times lower than pharmacological concentrations used to produce a functional response. The hypothesis assuming that local ET concentrations can be much higher than the concentrations in the entire tissue cannot be so far either confirmed or eliminated. A fact which is unambiguous at present is the senseless name of the peptide. ET is produced by a broad spectrum of tissue cultures and if many of them produce ET both *in vivo* and in cell culture, it is reasonable to assume that ET is present in the whole organism. Since ET has not been detected in storage particles of nerve endings and glands it is entirely clear that ET is neither a hormone nor a neurotransmitter in the common sense of these terms.

The specific features of ET, *i.e.* (i) high affinity to receptors – the strongest vasoconstrictor known at present, (ii) very slow dissociation from and association with receptor under usual conditions, and (iii) primary structure and conformation completely different from structures of other proteins and peptides found in mammals, which are unusual and unique, seem to support this theory. It can be stated that the ETs are a special class of vasoactive, long acting paracrine peptides. It follows from the properties of ET that it also plays a role in chronic diseases, such as hypertension. No doubt, all these facts will further stimulate studies on ET antagonists suitable for additional investigation of ET functions (*e.g.* it is necessary to find an antagonist capable of penetration into the central nervous system to map the role of ET in the brain) or of its medicinal applications, may it be a decrease in hazard of myocardial infarction, ischemic and cyclosporin-induced renal disorders, gastritis, and the decrease of hypertension.

The vasoconstrictor effects of endogenous ET-1 are blocked by selective ET_A receptor antagonists: however, vasodilator and natriuretic effects mediated by ET_B receptor are preserved. Contrary to the ET_A receptor the antagonists exhibited large benefits in a treatment of experimental models of cardiovascular diseases, the role of the ET_B receptor seems to be less clear. The last receptor can mediate both vasoconstriction and vasodilatation effects. However, results of early clinical studies on hypertension and chronic heart failure speak in favor of combined usage of $ET_{A/B}$ receptor antagonists[182].

Polyclonal antibody against to ET was found to inhibit *in vitro* the growth of cancer cells; yet this effect is probably highly specific and it is therefore unlikely that ET antagonists could play a role in cancer therapy. Even ET agonists could find application in medicine. A restoration of blood pressure after endotoxic shock is an example where long-acting ET agonists could be employed. A drop of the ET level in the central nervous system of patients suffering from depressions points to another area of possible use of ET

agonists. Similarly, on the condition of the validity of the vascular theory of migraine, an agonist could be useful for its treatment by contributing to a preferential contraction of the transition between arteries and veins. The effect of ET on the modulation of the cardiovascular system during the first minutes after birth indicates its potentials for the prevention of pulmonary hypertension in infants. A variety of methodologies and techniques in the interdisciplinary fields of peptide and general organic chemistry, biochemistry, molecular biology, cell biology, and pharmacology have greatly enhanced the understanding of the complexity of the ET system and no doubt will continue in providing the tools for future study.

SYMBOLS

Acm, acetamidomethyl; AcOH, acetic acid; Acp, 1-amino-1-cyclopropane carboxylic acid; ANF, atrial natriuretic factor; Bhg, 2-benzhydrylglycine; Boc, *tert*-butyloxycarbonyl; tBu, *tert*-butyl; CD, circular dichroism; cDNA, cyclic deoxyribonucleic acid; COS cells, SV40 virus transformed cells from kidney of African green monkey; DCM, dichloromethane; Dip, diphenylalanine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration; ECE, endothelin converting enzyme; EDRF, endothelin-derived relaxing factor; EDT, 1,2-ethanedithiol; ET, endothelin; Fmoc, fluorenylmethyloxycarbonyl; For, formyl; GMP, guanine monophosphate; IC_{50}, inhibitory concentration; MeBzl, methylbenzyl; MeOH, methanol; mRNA, messenger ribonucleic acid; NO, nitric oxide; Pen, 3,3-dimethylcysteine; Ph, phenyl; Pya, 3-(2-pyridinyl)-Ala; RIA, radioimmunoassay; SRTX, sarafotoxin; TFA, trifluoroacetic acid; $TFAONH_4$, trifluoroacetic ammonium salt; TFE, trifluoroethanol; TFMSA, trifluoromethanesulfonic acid; TMSiBr, trimethylsilyl bromide; TMSiCl, trimethylsilyl chloride; TMSOTf, trimethylsilyl trifluoromethanesulfonate; Trt, trityl; VIC, vasoactive intestinal contractor. The symbols of amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem*. **1984**, *138*, 9).

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