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The Discovery of Nonpeptide Endothelin Receptor Antagonists. Progression towards Bosentan

Werner Neidhart*, Volker Breu*, Daniel Bur, Kaspar Burri, Martine Clozel, Georges Hirth, Marcel Müller, Hans P. Wessel, and **Henri Ramuz***

Abstract. Since its discovery, endothelin-1 has attracted considerable scientific interest for its extremely potent and long-lasting vasoconstrictor effect and its binding to G-protein-coupled receptors. The endothelins appear to be part of a functional regulatory system in the circulation and strong evidence has accumulated for their involvement in clinical disorders associated with vasoconstriction (*e.g.* renal failure, congestive heart failure).

In a program aimed at identifying nonpeptide ET receptor antagonists, a distinct class of substituted arylsulfonamido pyrimidines was discovered from a chemical substance library. Lead optimization led to orally active antagonists of ET_A and ET_B receptors possessing mixed or receptor-subtype-selective profiles in the low nanomolar range. From these compounds, the mixed antagonist bosentan was selected for development; it shows efficacy in several pathophysiological models of local and systemic vasoconstriction and promising clinical results in patients suffering from congestive heart failure.

Chemical modifications in this structural class in combination with X-ray crystal data analysis for key compounds led to more in-depth understanding of antagonist-receptor interaction. Structural determinants of bosentan binding to the ET_A receptor were defined on the molecular level by site-directed mutagenesis experiments. This led to a 3D model of the antagonist binding domain which proved valuable to rationalize structure-activity relationships.

1. Introduction

An endothelium-derived constricting factor had been described in 1985 by *Hickey* [1] which we started to purify from human endothelial cells in 1987.

Yanagisawa first achieved the purification and cloning of this constricting factor (1988) and gave it the name endothelin [2]. The endothelins (ETs) consist of a family of vasoconstrictive and mitogenic peptides produced by endothelial cells of which ET-1 represents the most potent vasoconstrictor known to date. Elevated plasma levels of ET-1 have been observed in a variety of cardiovascular, pulmonary, renal, and gastrointestinal diseases characterized by abnormal vascular tone and/or proliferation [3].

After the structural elucidation of endothelin, we initiated a preclinical drug discovery program and described first specific binding of ET-1 on human vascular smooth muscle cells [4]. Drug discovery was aimed at developing nonpeptide ET antagonists as tools to elucidate the pathophysiological role of these endothelium-derived peptides.

The endothelins have inspired intensive research activities in clinical institutes and pharmaceutical companies as documented by the publication of more than 7000 scientific papers since their discovery [5].

1.1. *Endothelin Structural Features*

Endothelins (ET-1, ET-2, and ET-3) are made of 21 amino acids and are bicyclic owing to the presence of two disulfide bridges linking the cysteines of position 1 to 15 and 3 to 11 (*Fig. 1*). They are further characterized by a variable loop region and, at the carboxy terminus, by six highly conserved amino-acid residues which constitute an important determinant for peptide-receptor binding. These structural features are shared with a class of snake venom toxins, the sarafotoxins, isolated from *Atractaspis engaddensis* [6]. In a X-ray crystal structure of ET-1 recently obtained, the loop region is defined by an irregular helix with the C-terminal hexapeptide pointing away from the bicyclic core and showing a helical conformation [7]. In contrast, an aqueous solution conformation of ET-3 derived by NMR spectroscopy is characterized by a close association of the C-terminal hexapeptide with the bicyclic core, driven by hydrophobic clustering of the amino-acid residues 14, 19, 20, and 21 [8]. The topology of this cluster is thought to determine biological activity to some extent and was used for molecular modeling studies.

In the organism, endothelins are synthesized from physiologically inactive 39 amino acid precursors, named big endothelins, by endothelin-converting enzymes (ECE), a recently discovered family of zinc-endopeptidases. Two enzymes, differentiated by cellular localization and pH optima, have been cloned [9][10].

1.2. *Endothelin Receptors*

Endothelins elicit their biological effects on binding to a family of membrane-associated G-protein-coupled receptors with seven transmembrane-spanning segments (*Fig. 2*). The ET_A receptor subtype predominates in vascular smooth muscle cells and is defined by its higher affinity for ET-1 and ET-2 than ET-3. Its stimulation activates a cascade of events leading to vasoconstrictive and proliferative responses.

The ET_B receptor subtype is characterized by its similar binding affinities to all three isopeptides. Its role is at present less

**Correspondence*:
Drs. W. Neidhart, V. Breu, H. Ramuz
Pharma Division, Preclinical Research
F. Hoffmann-La Roche Ltd.
Grenzacherstrasse 124
CH–4070 Basel

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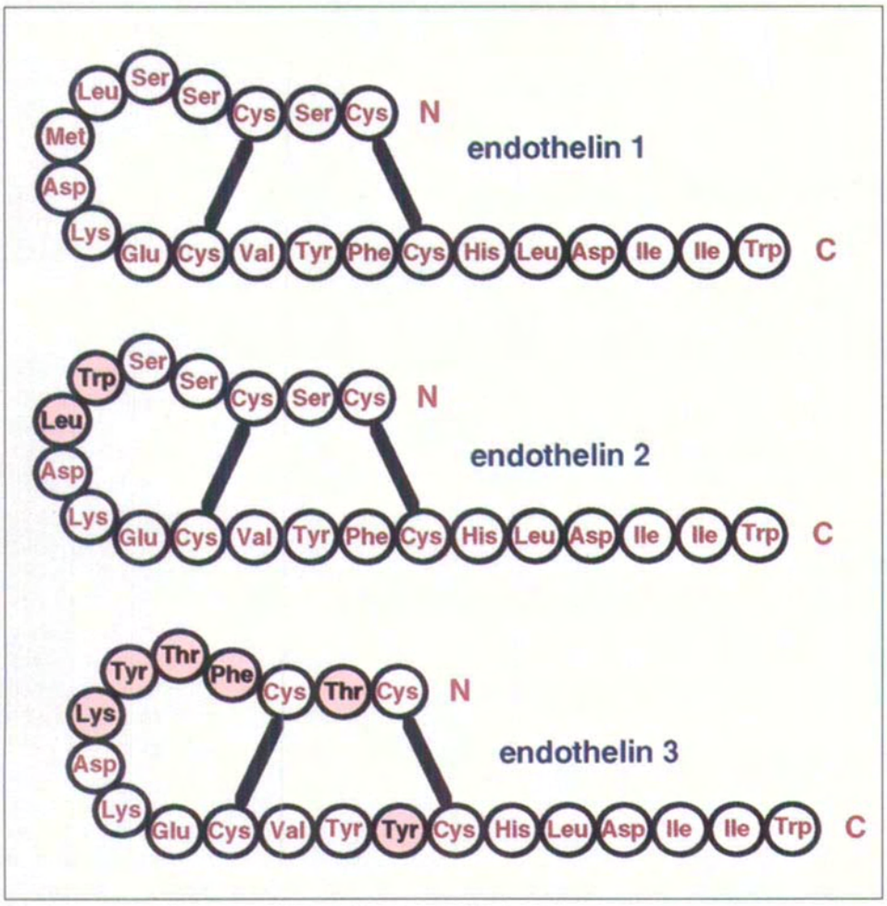

Fig. 1. *Amino-acid sequences of the endothelin peptide family*

clearly understood. It is present on endothelial cells where its activation mediates vasodilatation *via* the release of nitric oxide and prostacyclin. However, ET_B receptors present on vascular smooth muscle cells can also mediate constriction [11][12]. Since only one ET_B receptor gene has been found so far, these opposing effects mediated by ET_B receptors may be attributed to tissue-dependent differences in the signalling cascade.

ET_B receptors seem to play an important role in certain pathological situations. Thus, 'constricting' ET_B receptors are upregulated in dog basilar arteries after subarachnoidal hemorrhage [13], in atherosclerotic human coronary arteries [14], and also in hypertensive rats, together with dilating ET_B receptors [15][16].

These observations suggest that, in certain pathophysiological situations, a mixed ET_A-ET_B receptor blockade might have therapeutic advantage over ET_A-receptor-selective blockade.

2. Nonpeptide Antagonists

The lead discovery program was directed to identify *nonpeptide* low-molecular-weight endothelin antagonists. An alternative peptidomimetic approach based

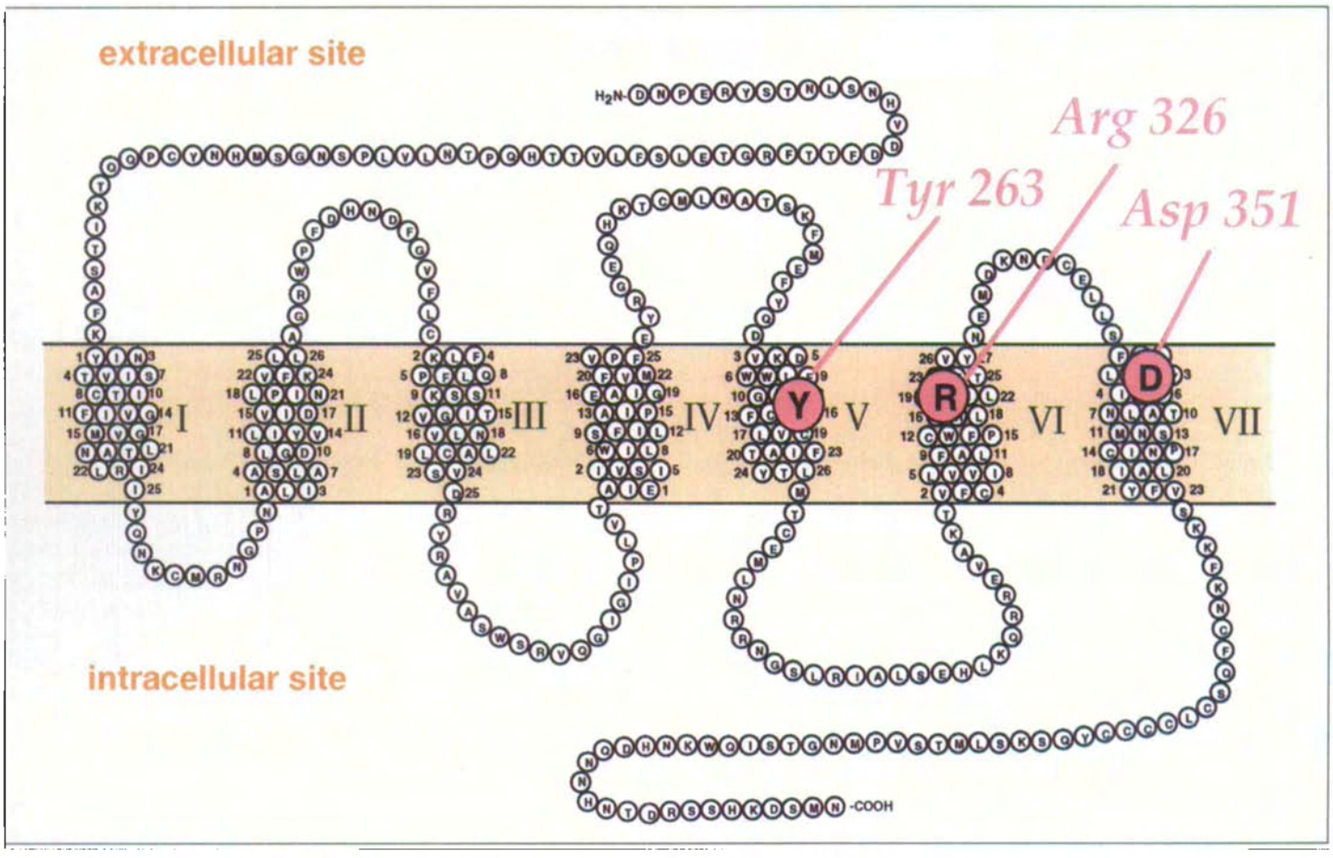

Fig. 2. *Diagram of the human endothelin-A receptor with the putative binding site for the nonpeptide antagonist bosentan*

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on the endothelins as scaffolds for the synthesis of antagonists was not considered as appropriate due to the inherent metabolic instability of peptides and peptidomimetics which limit their usefulness as drugs.

2.1. *Lead Discovery Approach*

As it is in the current state of understanding not yet possible to design *de novo* nonpeptide molecules with high affinity for macromolecular receptors, a chemical file screening approach was used as summarized in *Fig. 3*. An organic-compound library consisting of about 100000 molecules was screened as mixtures of 10. A high-flux radio-ligand-binding assay was used with endothelin-receptor-enriched membranes prepared from human placenta, which contains mainly ET receptors of the B-type and, subsequently, with recombinant human ET_A receptor expressed in Sf9 or CHO cells. Hits were then evaluated for binding and functional potency, selectivity, and structural consistency. The critical issues of *in vivo* activity and oral bioavailability were already addressed at this stage, using different pathophysiological models - the knowledge in pharmacology and physiology was thereby progressing in parallel with synthetic chemistry in this new area of endothelin blockade [17].

Lead optimization was then comprising computer-assisted molecular modeling based on X-ray crystal data of the lead molecules in comparison to low-energy conformations obtained for the endothelin peptides, as mentioned above, and a 3D model of receptor-ligand interaction deduced from site-directed mutagenesis experiments.

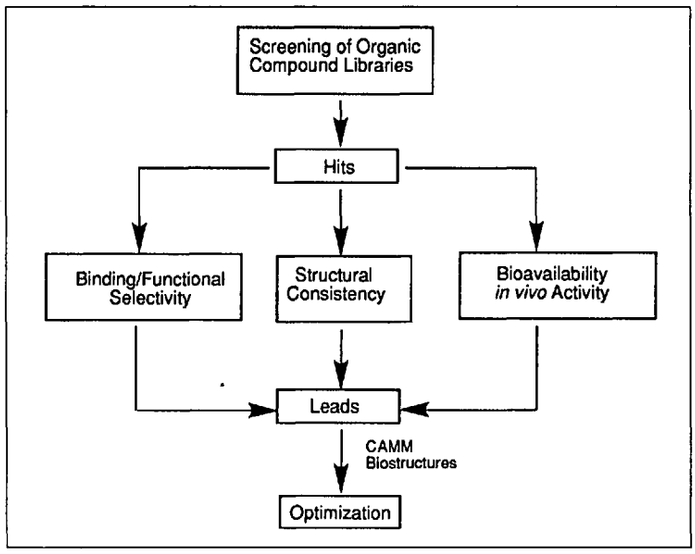
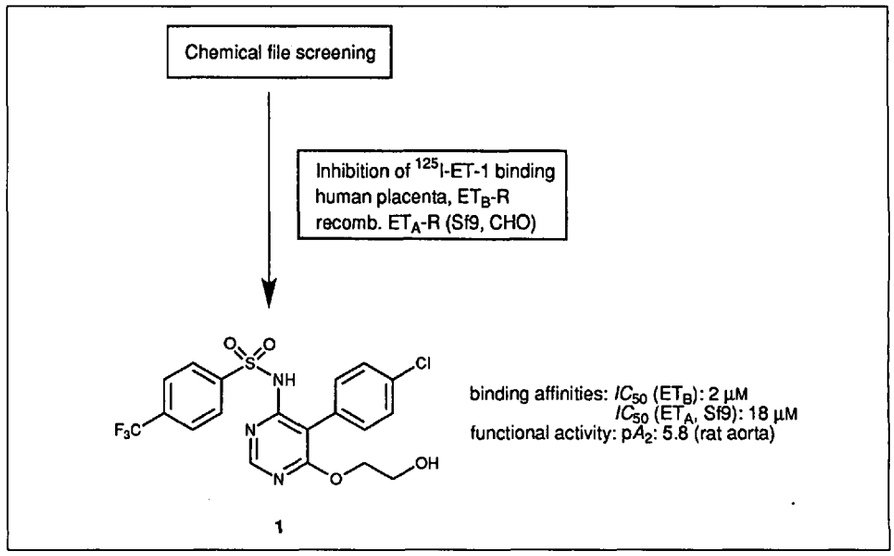


Fig. 3. *Lead finding strategy*

Scheme 1. *Discovery of a Class of Substituted Arylsulfonamido Pyrimidines*



2.2. *Results*

The result of this effort was the discovery of a class of substituted benzenesulfonamido pyrimidines with micromolar affinities for ET receptors [18], of which **1** was further in-depth evaluated as lead structure (*Scheme 1*). Its binding characteristics were assessed as 2 μM and 18 μM (IC_{50} values) for ET_B (human placenta) and ET_A receptors (recombinant human ET_A receptors expressed in baculovirus-infected Sf9 insect cells), respectively. *In vitro* functional activity was examined on vascular preparations carrying ET_A receptors. In isolated rat aortic rings denuded of their endothelium (ET_A receptors), **1** had no agonistic effect but antagonized the contraction induced by ET-1 in a concentration-dependent manner. Analysis of the data [19] yielded a pA_2 value (negative logarithm of the molar concentration causing a twofold parallel shift to the right of the concentration-response curve) of 5.8. *In vivo*, in a rat model of renal ischemia, **1** antagonized ischemia-induced renal vasoconstriction dose-dependently when injected in the renal artery.

The molecule had been synthesized as part of a research project on potential antidiabetic agents, it was orally bioavailable, itself devoid of hypoglycaemic activity and therefore suitable as a lead structure for chemical optimization.

2.3. *Lead Optimization*

The objective of the chemical program was to improve *in vitro* and *in vivo* activity of **1** by performing broad structural modifications at the various sites of the molecule. Considerable efforts in synthetic chemistry were directed to provide mixed antagonists for both receptors, ET_A and ET_B, as drug candidates suitable for clinical evaluation.

Progression to Bosentan

The progression of the optimization program is summarized in *Scheme 2* and is expressed in terms of binding affinities (IC_{50} values on recombinant ET_A receptors expressed in baculovirus-infected Sf9 cells) and *in vitro* functional activities (pA_2 values, rat aortic rings). An important step towards the identification of bosentan was the implementation of an electron-rich 2-methoxyphenoxy substituent in the posititon 5 of the central pyrimidine template giving rise to **2** with 12-fold improved affinity over **1**. Further introduction of a phenyl group into the position 2 of the pyrimidine ring yielded considerably improved antagonists with respect to *in vitro* functional activity as shown for **3** (pA_2 6.9), probably due to increased lipophilicity of the molecule. From the compounds with various aromatic and heteroaromatic substituent at this site, the deriv-

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ative **4** incorporating a 2'-pyrimidinyl substituent showed the best affinity and functional activity profile (pA_2 7.4, IC_{50} values of 0.08 μM and 0.008 μM for recombinant ET_A-R expressed in Sf9 and CHO cells; 0.15 μM for ET_B-R). The compound was orally bioavailable and subsequently selected for development as bosentan due to its favorable *in vivo* activity profile [20].

Synthesis

The preparation of bosentan was following a classical approach of pyrimidine synthesis [21]. Accordingly (*Scheme 3*), 2-amidinopyrimidine was condensed with (2-methoxyphenoxy)malonic acid diethyl ester in methanol with sodium methylate as a base to yield the 2-substituted tetrahydropyrimidine-4,6-dione **5** in 87% yield. Subsequent conversion to the 4,6-dichloro derivative **6** was achieved on treatment with $POCl_3/PCl_5$ at reflux temperature. The [4-(*tert*-butyl)phenyl]sulfonamide group was introduced as potassium salt in DMSO to provide **7** in 86% yield. Final treatment with sodium glycolate in ethylene glycol at 100° gave bosentan in 89% yield.

Physicochemical and Structural Characteristics

Due to its arylsulfonamide functional group, bosentan is a weak acid with a pK_a of 5.5 which contributes significantly to its aqueous solubility of 0.3% (at pH 7.4). Lipophilicity values (log*P* 3.1, log*D* 1.3) are in the adequate range for orally bioavailable drugs.

A X-ray crystal structure of **4** (obtained from a methanolic solution) is depicted in *Fig. 4* revealing some important conformational characteristics: the sulfonamide functional group is *syn*-oriented with a dihedral angle of 62°; the two pyrimidine rings are coplanar forming an extended aromatic plate; the 5-(2-methoxyphenoxy) substituent is forced by 4,6-disubstitution into a plane perpendicular to that formed by the two pyrimidine residues.

These structural characteristics have been observed for several derivatives of this class, analyzed by X-ray crystallography. It implies that the respective orientation of the two aromatic domains, the [4-(*tert*-butyl)phenyl]sulfonamide group and the 5-(2-methoxyphenoxy) substituent, in relation to the central aromatic plate, constitute key features for antagonist-receptor interaction. A further critical element for binding affinity is provided by the sulfonamide functional group as shown below.

Structure-Activity Relationships

Since the discovery of **1** as lead structure, some 1400 compounds have been synthesized and characterized up to the identification of bosentan. This effort has revealed some important structure-activity relationships in this class of compounds (*Fig. 5*):

i) The acidic proton of the sulfonamide group is mandatory for high-affinity binding. Removal and replacement by a methyl group destroys affinity as shown for the bosentan analog **8** (IC_{50} on ET_A-R > 100 μM).

ii) The arylsulfonamide group requires a lipophilic electron-donating substituent in *para*-position to obtain antagonists in the low nanomolar range, as revealed by **9**, which lacks the *tert*-butyl group at this site.

iii) The 5-(2-methoxyphenoxy) substituent seems to be engaged in hydrophobic aromatic interaction with receptor components. The nature of this substituent is size-limited and critical with respect to substitution. Hydrophilic groups at this site largely diminish affinity (as shown for **10** with over 300-fold diminished binding affinity in comparison to bosentan).

iv) Removal of the hydroxyethyl moiety incorporated in bosentan and other compounds of this class led to a dramatic drop of affinity (by a factor greater than 1600) as shown for **11**. This may be attributed to reduction of hydrogen-bonding interactions to the receptor as well as to changes of electronic properties of the central pyrimidine nucleus. Exchange of the latter by a more lipophilic benzene template resulted into 16-fold drop of binding affinity in comparison to that of bosentan (**12**, *Fig. 5*).

Receptor Subtype Selectivity

Substitution at the 4-position of the central pyrimidine ring constitutes an important element to control receptor subtype selectivity, which can be further modulated by variations of the three other groups attached to the central pyrimidine template (*Fig. 6*). Thus, compounds incorporating a more polar (*R*)-glyceryl function at this site generally give antagonists with a selectivity for the ET_B receptor of a factor greater than 100, as shown for **13**.

In contrast, implementation of a 2-(methylsulfinyl)ethoxy substituent, which is devoid of hydrogen-bond-donating properties, gives rise to antagonists showing a preference for ET_A receptors (**14**, *Fig. 6*). These findings are in accordance with local hydrophilicity differences at receptor sites defined by the substituent in position 4 of the central pyrimidine ring.

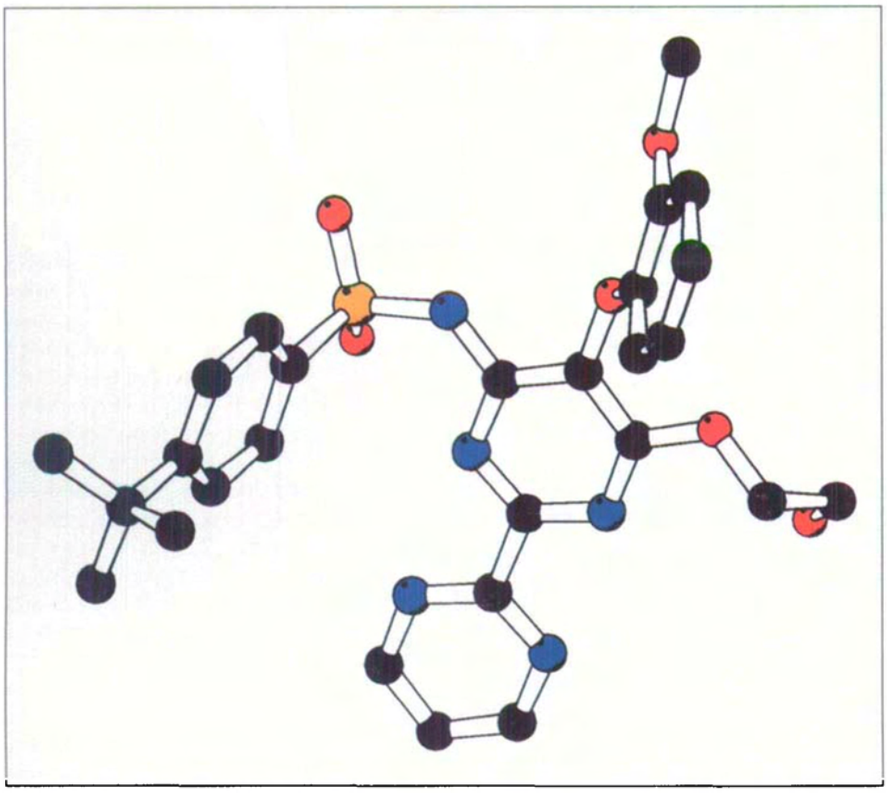

Fig. 4. *X-ray crystal structure of bosentan*

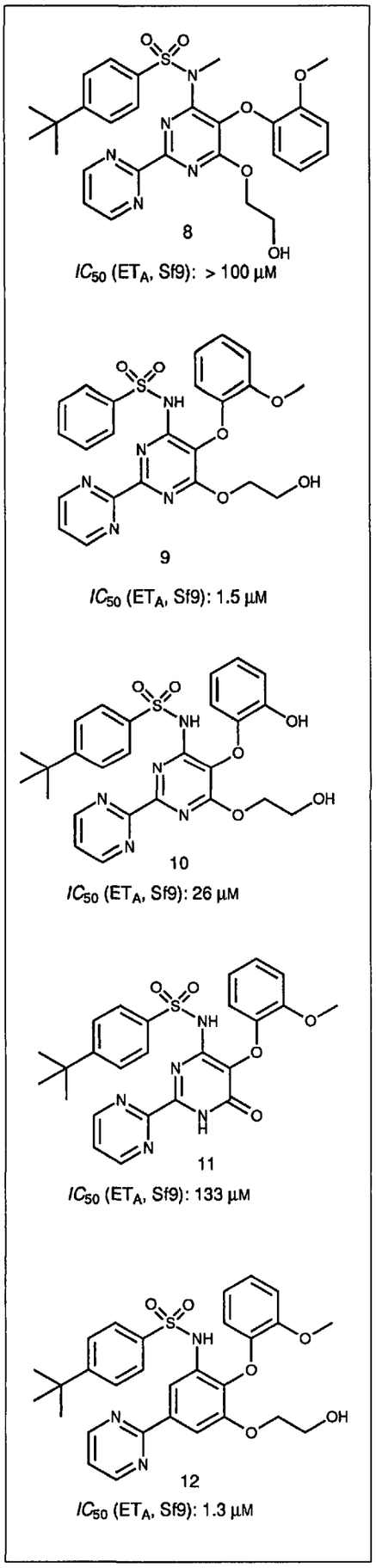

Fig. 5. *Structure-activity relationships*

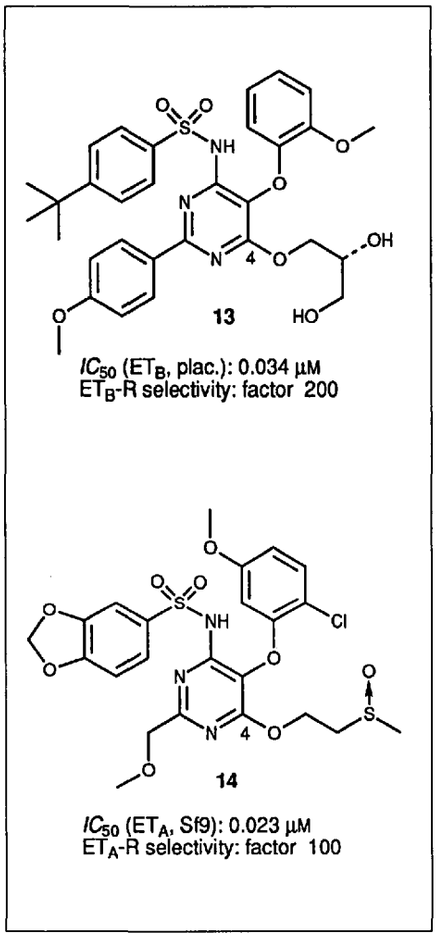

Fig. 6. *Receptor-subtype-selective antagonists*

3. Bosentan-Binding Determinants on the ET_A Receptor

In order to study the specific interaction between bosentan and the ET_A receptor on a molecular level, 18 amino-acid residues pointing to the inside of the receptor according to a three-dimensional model were selected and mutated by oligonucleotide-directed mutagenesis experiments. The mutant genes were expressed in COS-1 cells and bosentan binding was determined in a radioaffinity assay with ^{3}H-labelled bosentan.

This led to the identification of Tyr263, Arg326, and Asp351 (*Fig. 2*) as critical determinants for high-affinity bosentan-receptor binding [22]. All three amino-acid residues are unique for ET receptors and conserved among the two subtypes which is in accordance with high receptor specificity observed for bosentan and its mixed profile of activity. According to a 3D model of the bosentan-receptor interaction, the basic side chain of Arg326 provides a crucial binding contact of *Coulombic* nature to the acidic sulfonamide functional group.

4. Pharmacological Activity

The affinity of bosentan for the ET receptors was determined in different tests for each receptor subtype [20]. Overall, the compound is more potent on ET_A than on ET_B receptors by a factor between 2 to 20 (*Scheme 2*). Binding affinity for ET_A receptors expressed in Chinese hamster ovary cells (CHO) is 10-fold higher than for receptors expressed in baculovirus-infected Sf9 cells, probably due to incomplete glycosylation of the receptors in the latter expression system. On human smooth muscle cells (HSMC), bosentan binds with a K_i of 5 nM (*Scheme 2*).

The mixed binding profile also translates into a mixed functional antagonism. Bosentan potently inhibits the ET_A-receptor-mediated constriction of rat aortic rings (pA_2 7.4), the ET_B-receptor-mediated relaxation of precontracted rabbit superior mesenteric arteries (pA_2 6.7) and the ET_B-receptor-mediated constriction of rat tracheal rings (pA_2 of 6.0).

The compound has been used to study the therapeutic potential of mixed ET receptor antagonists in experimental pathological models of local and systemic vasoconstriction. It showed efficacy in several animal models, both after acute treatment (ischemic- and glycerol-induced acute renal failure, cerebral vasospasm after subarachnoidal hemorrhage) and in chronic situations after oral administration (certain models of systemic hypertension, pulmonary hypertension, chronic heart failure).

Beside its hemodynamic effects, bosentan was also effective in preventing cardiac hypertrophy and fibrosis in DOCA-salt-hypertensive rats, right ventricular and pulmonary vascular remodeling in chronically hypoxic rats. The clinical results using bosentan in congestive heart-failure patients have confirmed the therapeutic potential of ET receptor blockade [23].

5. Conclusion

Based on a lead structure derived from chemical file screening, a novel class of nonpeptide endothelin receptor antagonists was developed with subtype-selective or mixed profiles. From these compounds, **4** is presently investigated in clinical trials as bosentan. It is characterized by its mixed antagonism on both ET_A and ET_B receptors with binding affinities in the nanomolar range. The orally bioavailable compound is active *in vivo* in several pathophysiological models of endothelin-related vasospasms and shows promising

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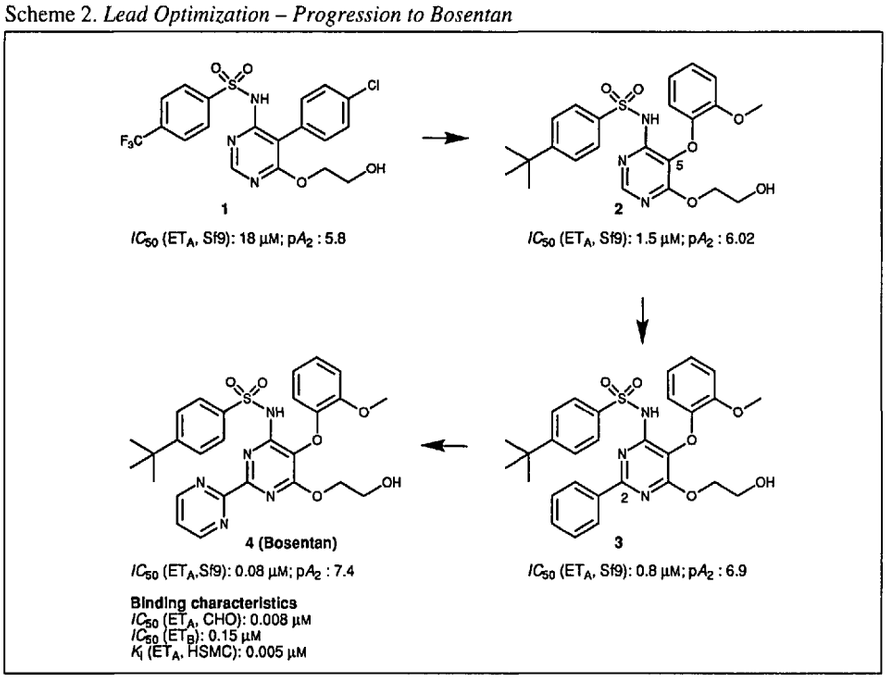
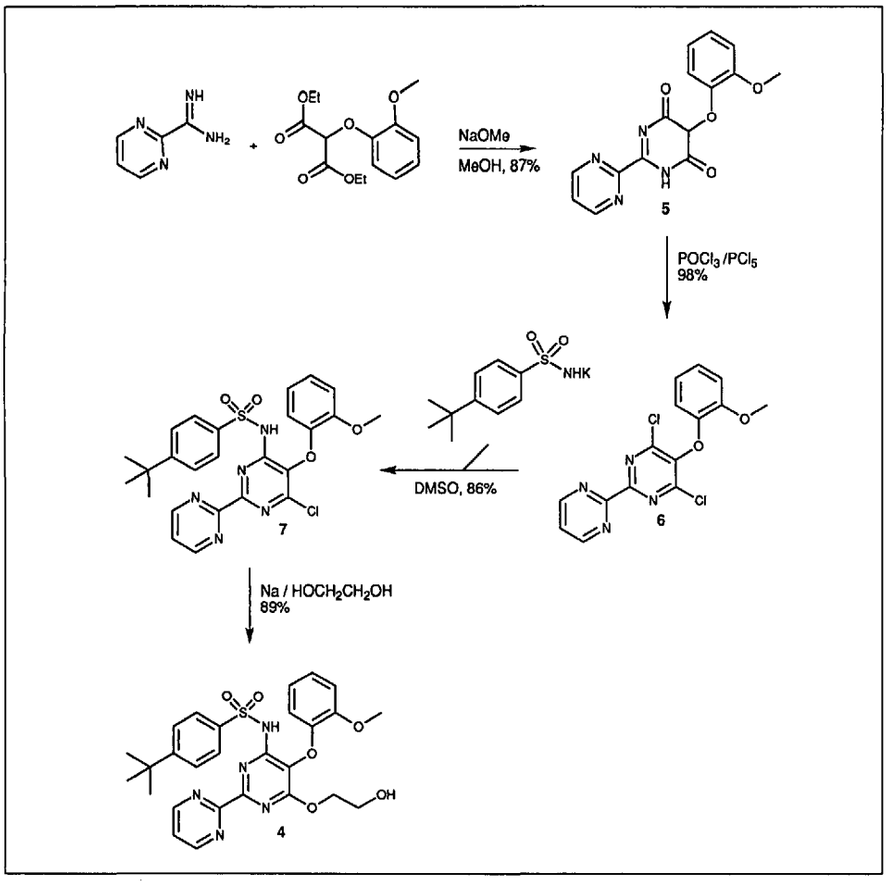
Scheme 2. *Lead Optimization – Progression to Bosentan*



Scheme 3. *Synthesis of Bosentan*



effects in patients suffering of congestive heart failure.

On the molecular level of receptor-antagonist interaction, structural determinants of bosentan binding were defined for the ET_A receptor by site-directed mutagenesis experiments. This led in combination with X-ray crystal data analysis obtained for selected antagonists to a 3D model of the antagonist binding domain. This model has valuable properties for the rationalization of the results obtained from chemical modifications and as a guide for the chemical optimization program.

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