In vitro characterisation of Ro 46-8443, the first non-peptide antagonist selective for the endothelin ET_B receptor

Volker Breu*, Martine Clozel, Kaspar Burri, Georges Hirth, Werner Neidhart, Henri Ramuz

Pharma Division, Preclinical Research, c/o F.Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4070 Basel, Switzerland

Received 6 February 1996

Abstract We describe here Ro 46-8443, the first non-peptide endothelin ET_B receptor selective antagonist. It displays up to 2000-fold selectivity for ET_B receptors both in terms of binding inhibitory potency and functional inhibition. The observed parallel rightward shift of concentration-response curves with different antagonist concentrations is consistent with a competitive binding mode. Since Ro 46-8443 selectively inhibits ET_B receptor mediated responses, it is a valuable tool for clarifying the role of ET_B receptors in pathology.

Key words: Endothelin; Endothelin antagonist; Endothelin ET_B receptor; Endothelin ET_A receptor

1. Introduction

Endothelins (ET-1, ET-2 and ET-3) are a family of 21-amino acid peptides, which contain two disulfide bridges between positions 1–15 and 3–11 and six highly conserved amino acid residues at the carboxyl terminus [1]. ET-1 represents the most potent vasoconstrictor known to date. It binds on at least two subtypes of G-protein coupled receptors with seven transmembrane spanning domains, named ET_A [2] and ET_B receptor [3]. ET_A receptors selectively bind ET-1 and ET-2, whereas ET_B receptors display similar potency for all three isopeptides. The profound vasoconstriction is mediated both by ET_A and ET_B receptors located on smooth muscle cells [4–6]. In addition, ET_B receptors located on endothelial cells are able to mediate vasodilatation through release of endothelium derived relaxing factor or prostacyclin [7]. Interestingly, certain pathological situations have been associated with increased density of ET_B receptors [8–11], but the lack of ET_B receptor selective antagonists has hampered further elucidation of the role of ET_B receptors in pathology.

Peptidic agonists with selectivity for ET_B receptors, like [Ala^{1,3,11,15}]ET-1 [12], sarafotoxin S6c [13], BQ 3020 [14] and IRL 1620 [15] and recently also peptidic ET_B receptor selective antagonists, e.g. IRL 1038 [16], BQ 788 [17] and RES 701 [18] have been described. Among these antagonists, BQ 788 reveals only a low selectivity for ET_B on human ET receptors, IRL 1038 lacks chemical stability [19] and the synthetic form of RES 701 is much less potent than the naturally occurring peptide [20]. Yet, for studying the role of ET_B receptors in pathology, chemically stable and selective ET_B receptor antagonists are essential. The present paper describes Ro 46-8443, the first non-peptide ET_B receptor selective antagonist.

*Corresponding author. Fax: (41) (61) 688 2438.
E-mail: volker.breu@roche.com

2. Materials and methods

2.1. Materials

[^{125}I]ET-1 and [^{125}I]sarafotoxin S6c were obtained from Anawa (Wangen, Switzerland). ET-1, ET-2, ET-3 were purchased from Novabiochem AG (Läufelfingen, Switzerland). [^{3}H][Arachidonic acid^{5,6,8,9,11,12,14,15}] was from Amersham (Rahn AG, Zürich, Switzerland). Phosphoramidon was purchased from Sigma, Bio-Science Products AG (Emmenbrücke, Switzerland). Ro 46-8443 and BQ 788 were synthesized at Hoffmann-La Roche AG (Basel, Switzerland). As reference compounds, RES 701-1, RES 701-3 and IRL 1038 were purchased from Alexis Corporation (Läufelfingen, Switzerland). In addition, IRL 1038 was also bought from Neosystem (Strasbourg, France). Human placentas were obtained from a local maternity hospital and porcine trachea was obtained from the local slaughterhouse. All tissues were stored at −80°C. Culture reagents were purchased from Gibco Laboratories (Paisley, Scotland).

2.2. Chemical synthesis of Ro 46-8443

(*R*)-4-*tert*-Butyl-*N*-[6-(2,3-dihydroxy-propoxy)-5-(2-methoxy-phenoxy)-2-(4-methoxy-phenyl)-pyrimidin-4-yl]-benzenesulfonamide was prepared by treatment of 4,6-dichloro-5-(2-methoxy-phenoxy)-2-(4-methoxy-phenyl)-pyrimidine with *p*-*tert*-butylbenzolsulfonamide potassium salt in acetonitrile to give 4-*tert*-butyl-*N*-[6-chloro-5-(2-methoxy-phenoxy)-2-(4-methoxy-phenyl)-pyrimidin-4-yl]-benzenesulfonamide which was further reacted with (*S*)-1,2-di-*O*-isopropylideneglycerol and sodium hydride in dimethyl sulfoxide to give (*S*)-4-*tert*-butyl-*N*-[6-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-5-(2-methoxy-phenoxy)-2-(4-methoxy-phenyl)-pyrimidin-4-yl]-benzenesulfonamide. Subsequently deprotection was achieved with 1 N aqueous HCl in dioxane to give Ro 46-8443.

2.3. Cell culture

Rat mesangial cells, baculovirus infected insect cells (Sf9 cells), CHO cells and human vascular smooth muscle cells, obtained from umbilical veins, were cultured as described previously [21].

2.4. Preparation of membranes

Microsomal membranes were prepared as described earlier [22] from human placenta, porcine trachea, rat trachea, baculovirus infected insect cells expressing recombinant human ET_A receptor and CHO cells expressing recombinant ET_B receptor cloned from human placenta [23,24], and stored in aliquots at −80°C. Protein was determined according to the method of Lowry [25].

2.5. Competition binding assays

Competition binding assays on ET_A receptors were performed on membrane preparations of baculovirus infected insect cells expressing human recombinant ET_A receptor and on attached human smooth muscle cells or rat mesangial cells using [^{125}I]ET-1 as radiolabelled ligand. Binding assays on ET_B receptors were also performed in the presence of [^{125}I]ET-1 using membranes of CHO cells expressing human recombinant ET_B receptor or membranes of human placenta. Membranes of porcine or rat trachea were used for binding studies on ET_B receptors mediating constriction. Since rat and porcine trachea contain both ET_A and ET_B receptors, the ET_B selective agonist [^{125}I]sarafotoxin S6c was used as labelled ligand in these experiments.

Binding assays on membranes were performed in 250 μl 50 mM Tris buffer (pH 7.4, 25 mM $MnCl_2$, 1 mM EDTA, 0.5% (w/v) BSA) containing 0.1–35 μg protein, 32 pM ^{125}I-labelled ligand and increasing amounts of unlabelled antagonists. After incubation for 2–3 h at 22°C, bound and free ligand were separated by filtration.

PII S0014-5793(96)00213-X

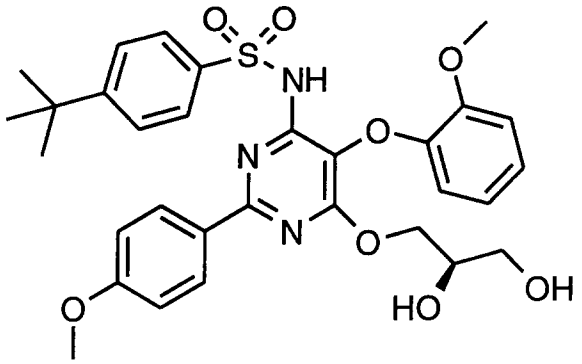

Fig. 1. Structure of Ro 46-8443, (*R*)-4-*tert*-butyl-*N*-[6-(2,3-dihydroxy-propoxy)-5-(2-methoxy-phenoxy)-2-(4-methoxy-phenyl)-pyrimidin-4-yl]-benzene-sulfonamide.

Binding assays with whole attached cells were performed in 500 μl Dulbecco's Eagle medium (DMEM) containing 2 mg/ml BSA and 25 mM Hepes. After incubation (2 h, 22°C) in the presence of 35 pM [^{125}I]ET-1 and increasing concentrations of Ro 46-8443, the cells were extensively washed and finally solubilized in 1% (w/v) SDS, 0.5 M NaOH, 100 mM EDTA.

Each assay was performed three times in triplicate and non-specific binding was assessed in the presence of 100 nM unlabelled ET-1. Specific binding was defined as the difference between total binding and non-specific binding. IC_{50} values were determined after logit/log transformation of the binding data.

2.6. Measurement of arachidonic acid release

[^{3}H]Arachidonic acid release was measured by a modification of the method of Felder et al. [26]. Confluent CHO cells grown in 24 well plates, expressing recombinant human ET_B receptor, were incubated for 24 h with [^{3}H]arachidonic acid (1 μCi/well), washed three times with DMEM supplemented with 2 mg/ml bovine serum albumin, 25 mM Hepes, pH 7.4 and incubated in this medium for 30 min at 37°C. After an additional washing step the reaction was started by replacing the buffer with fresh medium containing varying amounts of sarafotoxin S6c and Ro 46-8443. All incubation and washing steps were performed in the presence of phosphoramidon (100 μM). After incubation, the supernatant from each well was removed and centrifuged (10 min, 1100 rpm) to sediment detached cells. Finally, [^{3}H]arachidonic acid release into the incubation medium was quantified by liquid scintillation counting. Three experiments were performed in triplicate. Data are presented as mean ± standard error of the mean (S.E.M.).

2.7. Functional inhibition of contraction

2.7.1. Isolated rat aortic rings. In isolated rat aortic rings denuded of their endothelium, contractions induced by ET-1 are mediated through ET_A receptors. Male 14–16 week old Wistar-Kyoto rats were anesthetized with Inactin (sodium thiobutabarbital, 100 mg/kg intraperitoneally), the thoracic aorta was removed and cut into 5 mm rings. The endothelium was removed by gentle rubbing of the intimal surface, and each ring was suspended in a 10 ml isolated organ chamber containing gassed (95% O_2/5% CO_2) and warmed (37°C) Krebs-Henseleit solution of the following composition, in mM: NaCl 115, KCl 4.7, $MgSO_4$ 1.2, $KHPO_4$ 1.5, $NaHCO_3$ 25, $CaCl_2$ 2.5 and glucose 10. Isometric force was recorded. The rings were stretched to a resting force of 3 g. After a 60 min equilibration period, the rings were contracted using norepinephrine (10^{-7} M). Endothelium denudation was assessed by the absence of relaxation to acetylcholine (10^{-5} M). The rings were then washed and stretched if necessary until a stable baseline force was obtained. The rings were incubated with various concentrations (3×10^{-6} to 1×10^{-4} M) of Ro 46-8443. After 10 min, cumulative doses of ET-1 were added, the interval between doses was determined by the time required for the force generated to reach a plateau.

2.7.2. Isolated rat tracheal rings. In rat tracheal rings denuded of epithelium, the contractions induced by sarafotoxin S6C are mediated through smooth muscle ET_B receptors. Male rats were anesthetized, the trachea was removed and cut into 5 mm rings. The epithelium was removed by gentle rubbing of the luminal surface, and each ring was suspended in a 10 ml isolated organ chamber containing gassed and warmed Krebs-Henseleit solution as described above. The rings were stretched to a resting force of 2 g. After a 60 min equilibration period, the rings were contracted using potassium chloride (50 mM) and washed afterwards. After a 10 min incubation with Ro 46-8443 (3×10^{-7} to 1×10^{-5} M), cumulative doses of sarafotoxin S6C were added, the interval between doses was determined by the time required for the force generated to reach a plateau.

2.7.3. Analysis and calculations. The maximum force was defined as the force generated with the highest concentration yielding a maximal effect, and from this the ET-1 or sarafotoxin S6C concentration yielding a half-maximal effect (EC_{50}) was calculated. The pA_2 value (negative logarithm of the molar concentration of antagonist which causes a twofold parallel shift to the right of the agonist concentration-response curve), as an index of potency, was determined for each individual curve by the equation $pA_2 = \log(\text{concentration ratio} - 1) - \log[B]$, where concentration ratio is the ratio of EC_{50} values with/without antagonist and [B] the concentration of antagonist. Regression analysis of the plot log (concentration ratio−1) against log [B] (Schild plot) allowed to confirm the competitive nature of the antagonist by assessing its slope [27].

3. Results

On ET_A receptor preparations, Ro 46-8443, a pyrimidyl sulfonamide (Fig. 1), displayed a binding potency in the μM range. It competed for the binding of [^{125}I]ET-1 on cultured human vascular smooth muscle cells, rat mesangial cells and membranes of baculovirus infected insect cells expressing human recombinant ET_A receptor resulting in monophasic competition binding curves with average IC_{50} values of 2.2, 0.9 and 6.8 μM (Fig. 2), respectively.

Consistently higher potency was observed for ET_B receptor preparations. Ro 46-8443 competed for the binding of [^{125}I]ET-1 on membranes of CHO cells carrying recombinant human ET_B receptor and microsomal membranes from human placenta with monophasic binding curves resulting in IC_{50} values of 69 nM and 34 nM, respectively (Fig. 2). Membranes of porcine or rat trachea, a tissue which responds by constriction when stimulated with ET_B selective agonists like

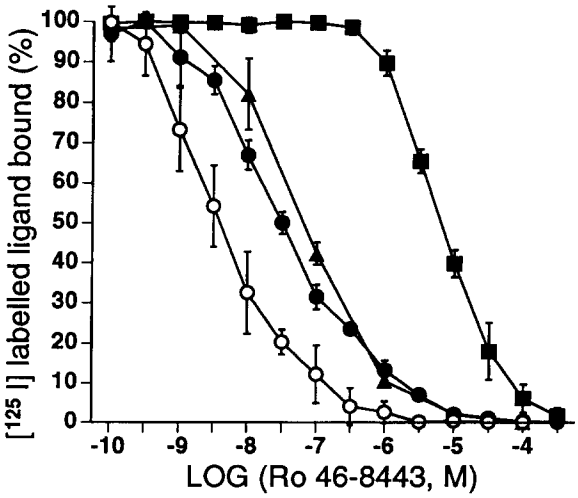

Fig. 2. Competition binding curves with Ro 46-8443: using either [^{125}I]ET-1 and (■) membranes of baculovirus infected insect cells expressing human ET_A receptor, (▲) membranes of CHO cells expressing human ET_B receptor and (●) membranes of human placenta carrying mainly ET_B receptor, or [^{125}I]sarafotoxin S6c and (○) membranes of porcine trachea. Values are means ± standard deviation of at least three independent experiments performed in triplicates.

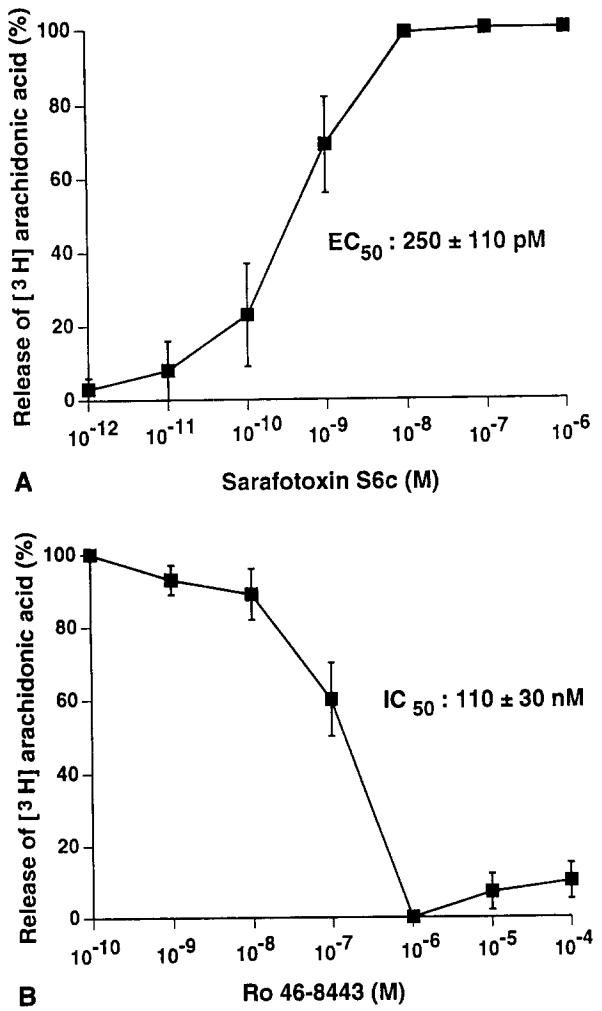

Fig. 3. (A) Sarafotoxin S6c mediated release of [^{3}H]arachidonic acid from CHO cells expressing recombinant human ET_B receptor during 30 min incubation. (B) Inhibition of the sarafotoxin S6c (0.1 nM) mediated release of [^{3}H]arachidonic acid from CHO cells expressing recombinant human ET_B receptor by Ro 46-8443. Represented is the mean ± S.E.M. of three independent experiments performed in triplicate.

sarafotoxin S6c, were used to assess the binding inhibitory potency of Ro 46-8443 on 'constricting' ET_B receptors. In these binding assays, Ro 46-8443 competed for the ET_B selective agonist [^{125}I]sarafotoxin S6c with IC_{50} values of 4.9 (Fig. 2) and 3.2 nM, respectively. Thus, Ro 46-8443 displays 300–2000-fold selectivity for these constricting ET_B receptors over the above mentioned ET_A receptor preparations. In order to profile Ro 46-8443 against previously described peptidic ET antagonists, the potencies of RES 701-1, RES 701-3, BQ 788 and finally IRL 1038 from two different purchasers were assessed on recombinant human ET_A receptor expressed on baculovirus infected insect cells and ET_B receptor on membranes from human placenta. In these assays, both IRL 1038 batches did not show any potency up to 10 μM, BQ 788 displayed IC_{50} values of 3.8 μM on ET_A receptor and 47 nM on ET_B receptor and RES 701-1 or RES 701-3 showed IC_{50} values of 120 or 45 μM on ET_A receptor and 17 or 11 μM on ET_B receptor.

In order to characterize the functional inhibition of ET_B receptors, we studied the ET receptor mediated release of arachidonic acid from CHO cells carrying recombinant human ET_B receptor. Upon stimulation with sarafotoxin S6c, we obtained a time and concentration dependent release of [^{3}H]arachidonic acid with an apparent EC_{50} value of 250 ± 110 pM (Fig. 3A), which reached a plateau after 20 min. The maximal response represented a 15-fold stimulation over the basal release of [^{3}H]arachidonic acid. Ro 46-8443 dose dependently inhibited this response with an apparent IC_{50} value of 110 ± 30 nM (Fig. 3B).

The potency of Ro 46-8443 to inhibit the sarafotoxin S6c mediated constriction of rat tracheal rings was also assessed as a measure of functional inhibitory potency on ET_B receptors. Ro 46-8443 caused a dose dependent parallel rightwards shift of the dose response curves resulting in a pA_2 value of 7.1 ± 0.2 (Fig. 4). The ET-1 induced constriction of rat aortic rings, mediated via ET_A receptors, was inhibited with a pA_2 value of 5.7 ± 0.06.

In all functional assays, Ro 46-8443 was devoid of any agonistic activity.

4. Discussion

Endothelin induced constriction may play a key role in a variety of diseases such as bronchial asthma, pulmonary hypertension, congestive heart failure, renal failure or subarachnoidal hemorrhage (for review, see [28]). It has been demonstrated that not only ET_A but also ET_B receptors can mediate vaso- or bronchoconstriction [4–6]. Furthermore, the importance of ET_B receptors in pathological situations may be reflected by enhanced receptor densities as recently described for the aorta of spontaneously hypertensive rats [9], the basilar artery of rabbits after subarachnoidal hemorrhage [11] and for the rabbit carotid artery after balloon denudation [29]. Most recently, an upregulation of ET_B receptors has also been demonstrated in human coronary arteries from atherosclerotic patients [10]. In order to further characterize the nature of these ET_B receptors and to clarify the role of ET_B receptors in pathology, ET_B receptor selective antagonists are an essential tool. Unfortunately, recently described ET_B receptor selective peptidic antagonists seem to be of limited value, either because the activity of the natural product differs from the chemically synthesized form, as it has been demonstrated for RES 701 or because of limited stability of the antagonist, as in

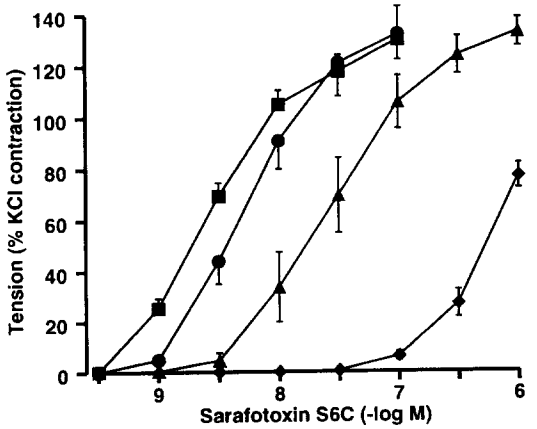

Fig. 4. Effect of Ro 46-8443 (●) 0.3 μM, (▲) 1 μM and (◆) 10 μM or its vehicle (DMSO, ■) on the concentration-response curves of sarafotoxin S6c mediated contraction in isolated rat trachea without epithelium.

the case of IRL 1038. This is in line with our observations that RES 701-1 as well as IRL 1038 from two different sources displayed only potency in the μM range and hardly any ET_B receptor selectivity in binding assays with human ET receptors. Similar observations from other laboratories resulted in withdrawal of IRL 1038 [19]. In our assay systems, also the ET_B receptor potency (IC_{50}: 48 nM) and selectivity (80-fold) of BQ 788 proved to be less favorable than previously published data describing an IC_{50} of 1.2 nM on ET_B receptors and a more than 1000-fold ET_B selectivity for human receptors [17]. Possible explanations for this discrepancy include the use of different assay systems or proteolytic degradation of the compound.

The issue of correct protein folding and stability is a well known drawback of peptidic ligands and provides a strong motivation for the development of non-peptide antagonists. This paper characterizes the in vitro properties of Ro 46-8443, which is to our knowledge the first non-peptide ET_B receptor selective antagonist.

Ro 46-8443, a sulfonamide with central pyrimidine moiety, is structurally related to the recently described antagonists Ro 46-2005 [22,30] and bosentan [21], which bind to both ET_A and ET_B receptors with comparable potency. However, replacement of the second pyrimidine, found in the bosentan structure, by a *p*-methoxy-phenyl moiety and replacement of the hydroxy-ethoxy side chain by a glycerol residue resulted in an ET_B receptor selective antagonist. In competition binding assays with human ET receptors, Ro 46-8443 displayed an at least 100-fold selectivity for ET_B (IC_{50}: 34–69 nM) over ET_A receptors (IC_{50}: 6800 nM). This selectivity ratio reaches values of more than 2000, if the potency on constricting ET_B receptors, i.e. the potency on membranes of rat or porcine trachea, is considered. Unfortunately, so far no data with corresponding human constricting ET_B receptors are available. Efforts to measure sarafotoxin S6c mediated constriction in human atherosclerotic coronary arteries failed due to the occurrence of spontaneous phasic constrictions [10]. However, because similar binding potency and selectivity ratio was found between human and rat ET_A and ET_B receptors, Ro 46-8443 seems to display few if any species differences, suggesting a highly conserved binding site on the ET receptors. Establishing the differences of this interaction to the recently described binding site of bosentan [31] will be valuable for the future design of new ET_B receptor selective antagonists.

The observed binding potency of Ro 46-8443 on human ET_B receptors expressed in CHO cells translated well into the potency for functional inhibition of sarafotoxin S6c induced release of arachidonic acid. In addition, no agonistic activity of Ro 46-8443 was observed in the absence of sarafotoxin S6c suggesting that Ro 46-8443 binds to the inactive conformation of the ET_B receptor.

The observation that Ro 46-8443 also inhibited the sarafotoxin S6c mediated contraction of rat tracheal rings demonstrates its potency to inhibit constricting ET_B receptors and shows its capability for tissue penetration, enabling the compound to reach the smooth muscle cells in the media. Differences in the ability for tissue penetration and protein binding in rat aortic rings and rat tracheal rings may account for the somewhat lower ET_B receptor selectivity in these functional assays. The parallel rightwards shift of the concentration response curve by different Ro 46-8443 concentrations is in line with a competitive interaction with the ET_B receptor.

Overall, its non-peptidic nature, potency and ET_B receptor selectivity make Ro 46-8443 a unique tool which may make it possible to explore the role of ET_B receptors in different animal models such as hypertension [32].

Acknowledgements: We thank Mrs. B. Butscha, Mrs. L. Hilfiger, Mrs. M. Hug and Mr. B. Lack for their skilled technical assistance.

References

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Arai, H., Hori, S., Aramori, I, Ohkubo, H. and Nakanishi, S. (1990) Nature 348, 730–732.
[3] Sakurai, T., Yanagisawa, M, Takuwa, Y, Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Nature 348, 732–735.
[4] Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M. and Yano, M. (1992) Life Sci. 50, 247–255.
[5] Clozel, M., Gray, G.A., Breu, V., Löffler, B.-M. and Osterwalder, R. (1992) Biochem. Biophys. Res. Commun. 186, 867–873.
[6] Moreland, S., McMullen, D.M., Delaney, C.L., Lee, V.G. and Hunt, J.T. (1992) Biochem. Biophys. Res Commun. 184, 100–106.
[7] Takayanagi, R., Kitazumi, K., Takasaki, C. Ohnaka, K., Aimoto, S. Tasaka, K., Ohashi, M. and Nawata, H. (1991) FEBS Lett. 282, 103–106.
[8] Seo, B. and Lüscher, T.F. (1995) Hypertension 25, 501–506.
[9] Batra, V.K., McNeill, J.R., Xu, Y., Wilson, T.W. and Gopalakrishnan, V. (1993) Am. J. Physiol. 264, C479–C484.
[10] Dagassan, P.H., Breu, V., Clozel, M., Künzli, A., Vogt, P., Turina, M., Kiowski, W. and Clozel, J.-P. (1996) J. Cardiovasc. Pharmacol., in press.
[11] Roux, S., Löffler, B.-M., Gray, G.A., Sprecher, U., Clozel, M. and Clozel, J.P. (1995) Neurosurgery 37, 78–86.
[12] Saeki, T., Ihara, M., Fukuroda T., Yamagiwa, M. and Yano, M. (1991) Biochem. Biophys. Res. Commun. 179, 286–292.
[13] Williams, D.L. Jr., Jones, K.L., Pettibone, D.J., Lis, E.V. and Clineschmidt, B.V. (1991) Biochem. Biophys. Res. Commun. 175, 556–561.
[14] Ihara, M., Saeki, T., Fukuroda, T., Kimura, S., Ozaki, S., Patel A.C. and Yano M. (1992) Life Sci. 51, L47–L52.
[15] Takai, M., Umemura, I., Yamasaki, K., Watakabe, T., Fujitani, Y., Oda, K., Urade, Y., Inui, T., Yamamura, T. and Okade, T. (1992) Biochem. Biophys. Res. Commun. 184, 953–959.
[16] Urade, Y., Fujitani, Y., Oda, K., Watakabe T., Umemura, I., Takai, M., Okada, T., Sakata, K. and Karaki H. (1992) FEBS Lett. 311, 12–16.
[17] Ishikawa, T., Ihara, M., Noguchi, K., Mase, T., Mino, N., Saeki, T., Fukuroda, T., Fukami, T., Ozaki, S., Nagase, T., Nishikibe, M. and Yano, M. (1994) Proc. Natl. Acad. Sci. 91, 4892–4896.
[18] Tanaka, T. Tsukuda, E., Nozawa, M., Nonaka, H., Ohno, T., Kase, H., Yamada, K. and Matsuda, Y. (1994) Mol. Pharmacol. 45, 724–730.
[19] Urade, Y., Fujitani, Y., Oda, K., Watakabe, T., Umemura, I., Takai, M., Okada, T., Sakata, K. and Karaki, H. (1994) FEBS Lett. 342, 103.
[20] Katahira, R., Shibata, K., Yamasaki, M., Matsuda, Y. and Yoshida, M. (1995) Bioorg. Med. Chem. Lett. 5, 1595–1600.
[21] Clozel, M., Breu, V., Gray, G.,. Kalina, B, Löffler, B.-M., Burri, K., Cassal, J.-M., Hirth, G., Müller, M., Neidhart, W. and Ramuz, H. (1994) J. Pharmacol. Exp. Ther. 270, 228–235.
[22] Breu, V., Clozel, M. and Löffler, B.-M. (1993) FEBS Lett. 334, 210–214.
[23] Adachi, M., Yang, Y.-Y., Furuichi, Y. and Miyamoto, C. (1991) Biochem. Biophys. Res. Commun. 180, 1265–1272.
[24] Takasuka, T., Adachi, M., Miyamoto, C., Furuichi, Y. and Watanabe, T. (1992) J. Biochem. 112, 396–400.
[25] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[26] Felder, C.C., Kanterman, R.Y., Ma, A.L. and Axelrod, J. (1989) J. Biol. Chem. 264, 20356–20362.

[27] Arunlakshana, O. and Schild, H.O. (1959) Br. J. Pharmacol. Chemother. 14, 48–58.
[28] Filep, J.G. (1995) Drugs Today 31, 155–171.
[29] Azuma, H., Hamasaki, H., Niimi, Y., Terada, T. and Matsubara, O. (1994) Am. J. Physiol. 267, H2259–H2267.
[30] Clozel, M., Breu, V., Burri, K., Cassal, J.-M., Fischli, W., Gray, G.A., Hirth, G., Löffler, B.-M., Müller, M., Neidhart, W. and Ramuz, H. (1993) Nature 365, 759–761.
[31] Breu, V., Hashido, K., Broger, C., Miyamoto, C., Furuichi, Y., Hayes, A., Kalina, B., Löffler, B.-M. and Clozel, M. (1995) Eur. J. Biochem. 231, 266–270.
[32] Clozel, M. and Breu, V. (1996) FEBS Lett. 383, 42–45.