Volume 334, number 2, 210–214 FEBS 13244 November 1993

In vitro characterization of Ro 46-2005, a novel synthetic non-peptide endothelin antagonist of ET_A and ET_B receptors

Volker Breu*, Bernd-Michael Löffler, Martine Clozel

Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd., Grenzacherstrasse 124, CH-4002 Basel, Switzerland

Received 21 September 1993

Ro 46-2005 is a new synthetic non-peptide endothelin (ET) receptor antagonist. In binding experiments, Ro 46-2005 proved to be equipotent (IC_{50} 200–500 nM) for inhibition of [^{125}I]ET-1 binding on the two known ET receptor subtypes (ET_A and ET_B). Scatchard analysis was consistent with a competitive binding mode. Ro 46-2005 also inhibited the functional consequences of ET-1 stimulation: the ET-1-induced release of arachidonic acid from rat mesangial cells was inhibited with an IC_{50} of 1.8 μM.

Endothelin: Endothelin antagonist; Endothelin receptor; Arachidonic acid release

1. INTRODUCTION

Endothelin (ET), a 21 amino acid peptide, containing two disulfide bridges, is the most potent vasoconstrictor known to date [1]. It has been detected in man in the form of three different isopeptides, named ET-1, ET-2 and ET-3 [2]. Increased ET levels have been described in diseases accompanied by vasoconstriction or inflammation, e.g. subarachnoidal hemorrhage [3,4], congestive heart failure [5,6], angina pectoris [7], ulcerative colitis [8] and renal failure [9]. Additionally, ET might play a key role in chronic diseases like atherosclerosis [10] and hypertension [11]. Two G-protein-coupled ET receptors with seven transmembrane spanning domains have been described, cloned from human tissue and defined as ET_A [12] and ET_B receptors [13]. Whereas ET_A receptors are selective for ET-1 and ET-2 over ET-3, ET_B receptors bind all three isopeptides with similar potency. ET_A receptors, mainly located on smooth muscle cells, were originally thought to be responsible for vasoconstriction, while ET_B receptors, located on endothelial cells, were believed to mediate vasodilation. However, ET_B receptors which mediate vasoconstriction have recently also been described [14,15]. This new receptor subtype is not blocked by the peptidic ET_A selective antagonists, BQ 123 [16] and FR 139317 [17]. The role of the ET receptor subtypes in the pathology of the above mentioned diseases is unknown. However, taking into account that both ET_A and ET_B receptors may be involved in vasoconstriction it might be an advantage to block both receptor subtypes with an unselective receptor antagonist. Our goal was to study whether Ro 46-2005, a novel non-peptide ET antagonist [18], would fulfill this requirement. We therefore performed competition and saturation binding studies on tissues or cells containing either ET_A or ET_B receptors. Moreover, we analyzed the effect of Ro 46-2005 on the release of arachidonic acid, one of the second messengers involved in the action of ET [19,20].

*Corresponding author. Fax: (41) (61) 688 2438.

This work has been presented in part at the Third Endothelin Conference, Houston, TX, February 1993.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{125}I]ET-1, [^{125}I]ET-2 and [^{125}I]ET-3 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 obtained from Sigma, Bio-Science Products AG (Emmenbrücke, Switzerland). Human placentas were obtained from a local maternity hospital and porcine cerebellum was obtained from the local slaughter house. All tissues were stored at −80°C. Culture reagents were from Gibco Laboratories (Paisley, Scotland, UK).

2.2. *Cell culture*

Rat aortic endothelial cells, rat mesangial cells, human smooth muscle cells and baculovirus-infected insect cells (Sf9 cells) were cultured as described previously [21–23].

2.3. *Preparation of membranes*

Microsomal membranes from human placenta or porcine cerebellum were prepared as described earlier [24]. Briefly, the tissue was homogenized in 5 mM Tris buffer, pH 7.4, containing 1 mM $MgCl_2$ and 250 mM sucrose with a polytron (Kinematica Ltd., Switzerland) and subsequently with a potter homogenizer (Vetter Ltd., Germany). After centrifugation at 3,000 × g for 15 min at 4°C, the supernatant was centrifuged again at 72,000 × g for 40 min. The resulting pellet was finally suspended in 2.5 ml 75 mM Tris buffer (pH 7.4) containing 25 mM $MgCl_2$ and 250 mM sucrose. Baculovirus infected insect cells

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expressing either recombinant ET_A or recombinant ET_B receptor cloned from human placenta [25,26] were broken by three freeze/thawing cycles in hypotonic Tris buffer (5 mM, pH 7.4, 1 mM $MgCl_2$), homogenized and centrifuged at 72,000 × *g* for 15 min. The pellet was washed twice with 75 mM Tris buffer, pH 7.4, containing 25 mM $MgCl_2$ and 250 mM sucrose, resuspended in the same buffer and stored in aliquots at −80°C.

Protein was determined according to the method of Lowry [27] using bovine serum albumin (BSA) as a standard.

2.4. *Binding assays*

2.4.1. Competition binding

Competition binding assays were performed either on membrane preparations (human placenta, porcine cerebellum, baculovirus-infected insect cells) or on whole attached cells (rat mesangial cells, human smooth muscle cells, rat endothelial cells).

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 5–35 μg protein, 32 pM ^{125}I-labelled ET and increasing amounts of unlabelled ligands. In binding assays with [^{125}I]ET-3 on ET_A receptor a tracer concentration of 213 pM was used. After incubation for 2 h at 22°C, bound and free ligand were separated by filtration.

Binding assays with whole attached cells were performed in 500 μl Dulbecco's modified Eagle's 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 46-2005, 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.

2.4.2. Saturation binding

Saturation binding experiments were performed either with membranes of baculovirus-infected insect cells carrying recombinant human ET_A receptor, (5 μg protein/well), or with membranes from human placenta (35 μg protein/well), containing mainly ET_B receptors. The membranes were incubated for 3 h with increasing amounts of radiolabelled [^{125}I]ET-1 (6.4–660 pM) in the presence of varying concentrations of Ro 46-2005. The assays were performed in triplicate and non-specific binding was assessed in the presence of 100 nM unlabelled ET-1. K_D and B_{max} values were calculated by linear regression analysis of Scatchard plots.

2.5. *Measurement of arachidonic acid release*

[^{3}H]Arachidonic acid release was measured by a modification of the method of Felder et al. [28]. Confluent rat mesangial cells grown in 24 well plates (400,000 cells/well) 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 ET-1 and Ro 46-2005. All incubation and washing steps were performed both in the presence and in the absence of phosphoramidon (100 μM). After incubation (0–30 min), the supernatant from each well was removed and centrifuged (10 min, 1,100 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 ± S.E.M.

3. RESULTS

Ro 46-2005 competed for the binding of [^{125}I]ET-1 on cells carrying ET_A receptor, i.e. human smooth muscle cells and rat mesangial cells, with IC_{50} values of 220 ± 60 nM and 430 ± 140 nM, respectively (Fig. 1A). Its potency for binding to ET_B receptors was similar on membranes of human placenta (IC_{50} 160 ± 77 nM) and porcine cerebellum (IC_{50} 227 ± 92 nM), but differed on rat endothelial cells (IC_{50} 1,140 ± 340 nM) (Fig. 1B). We could demonstrate that human placental membranes contain ≥ 90% ET_B receptors (data not shown). To assess the potency of Ro 46-2005 on pure receptor subtypes, we used recombinant ET_A ($recET_A$) and ET_B ($recET_B$) receptors. These recombinant receptors showed the characteristic pattern of potency for ET-1 and ET-3, $recET_A$ receptor being selective for ET-1 (IC_{50} 0.290 ± 0.075 nM) compared to ET-3 (IC_{50} 150 ± 70 nM) and $recET_B$ receptor being about equipotent for ET-1 (IC_{50} 0.44 ± 0.28 nM) and ET-3 (IC_{50} 0.11 ± 0.01 nM). The ET_A-selective peptide antagonists, BQ 123 [16] and FR 139317 [17], potently competed for [^{125}I] ET-1 binding to the $recET_A$ receptor with IC_{50} values of 63 ± 16 nM and 13 ± 4 nM, respectively.

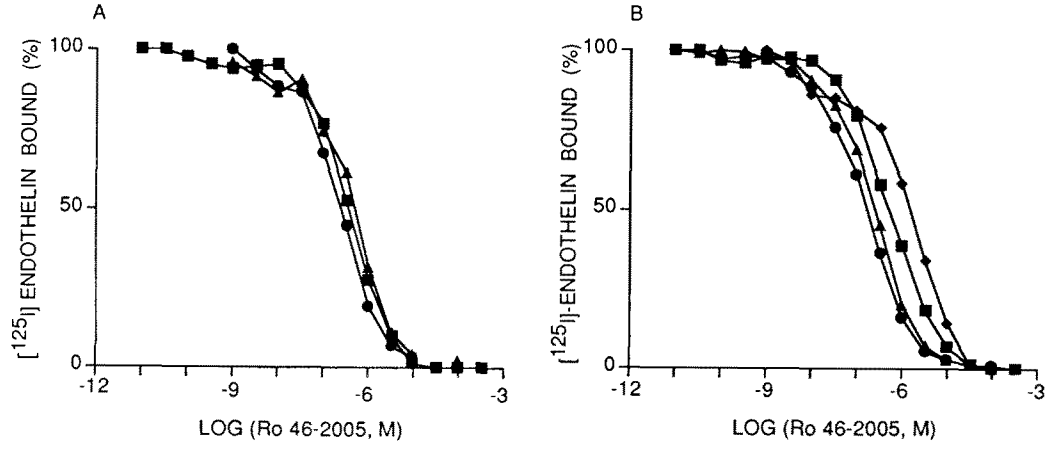

Fig. 1. Competition binding curves with [^{125}I]ET-1 and Ro 46-2005. Values are means of three independent experiments. (A) Human smooth muscle cells (●), rat mesangial cells (▲) and recombinant ET_A receptor (■). (B) Rat endothelial cells (◆), human placenta (●), porcine cerebellum (▲) and recombinant ET_B receptor (■).

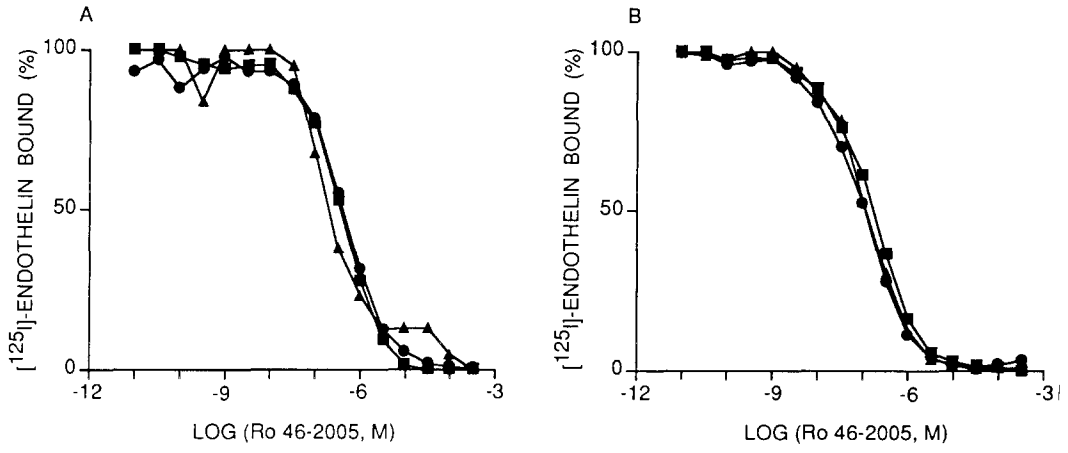

Fig. 2. Competition binding curves with different ^{125}I-labelled ET peptides, [^{125}I]ET-1 (32 pM, ■), [^{125}I]ET-2 (32 pM, ●), [^{125}I]ET-3 (213 pM, ▲) and unlabelled Ro 46-2005. Values are means of three independent experiments. (A) Recombinant ET_A receptor. (B) Human placenta (≥ 90% ET_B; ≤ 10% ET_A).

However, neither ligand competed for [^{125}I]ET-1 binding on the $recET_B$ receptor up to a concentration of 100 μM. In contrast, the ET_B-selective ligand, sarafotoxin S6c [29], did not bind to the $recET_A$ receptor, but had a potency of 0.120 ± 0.01 nM on the $recET_B$ receptor. Ro 46-2005 displayed a potency (IC_{50}) of 360 ± 160 nM on $recET_A$ receptors (Fig. 1A) and 530 ± 150 nM on $recET_B$ receptors (Fig. 1B).

Furthermore, Ro 46-2005 competed with a similar potency for binding of [^{125}I]ET-1, [^{125}I]ET-2 and [^{125}I]ET-3 on $recET_A$ (Fig. 2A), resulting in IC_{50} values of 360 ± 160 nM, 397 ± 53 and 212 ± 45 nM and on natural ET_B receptors (human placenta, Fig. 2B) giving IC_{50} values of 160 ± 77, 97 ± 5 and 113 ± 4 nM.

Saturation binding experiments were performed with the same receptor preparations using [^{125}I]ET-1 and varying concentrations of Ro 46-2005. On $recET_A$ receptors, the apparent K_D values for ET-1 increased in the presence of increasing concentrations of Ro 46-2005: 68 pM (control), 163 pM (0.65 μM Ro 46-2005), 198 pM (1.3 μM Ro 46-2005) and 477 pM (2.5 μM Ro 46-2005), the corresponding values for B_{max} remained unchanged at 3,000 ± 150 fmol/mg protein (Fig. 3A). A similar result was obtained with placental membranes (90% ET_B receptors) (Fig. 3B). In the presence of 0 nM (control), 0.1, 0.2 and 0.4 μM Ro 46-2005, the corresponding K_D values for ET-1 were 20, 29, 57 and 82 pM, respectively. Again, the apparent number of binding sites remained constant at 138 ± 7 fmol/mg. Thus, Ro 46-2005 displays a competitive mode of binding to both receptor subtypes.

We further analyzed whether the binding of Ro 46-2005 to the ET receptors was translated into a similar potency for inhibition of signal transduction. ET-1 induced a dose-dependent release of arachidonic acid from rat mesangial cells with an EC_{50} value of 4.4 nM. The maximal stimulation of arachidonic acid release was 870 ± 40% above baseline with 330 nM ET-1. This

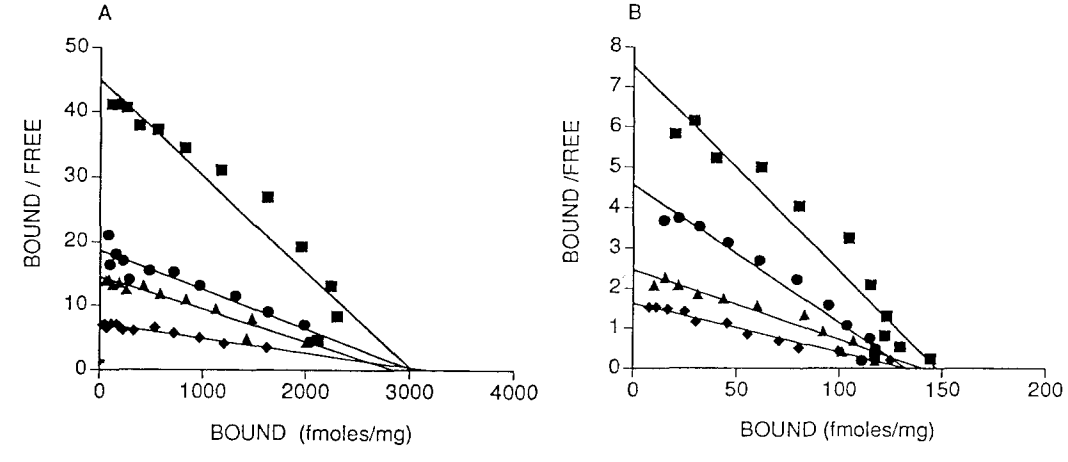

Fig. 3. Scatchard plots derived from saturation binding experiments with [^{125}I]ET-1 in the presence of varying concentrations of Ro 46-2005. Values are means of triplicate determinations. (A) Recombinant ET_A receptor; control (■) and in the presence of 0.65 μM (●), 1.3 μM (▲) or 2.6 μM (◆) Ro 46-2005. (B) Human placenta (ET_B); control (■) and in the presence of 0.1 μM (●), 0.2 μM (▲) or 0.4 μM (◆) Ro 46-2005.

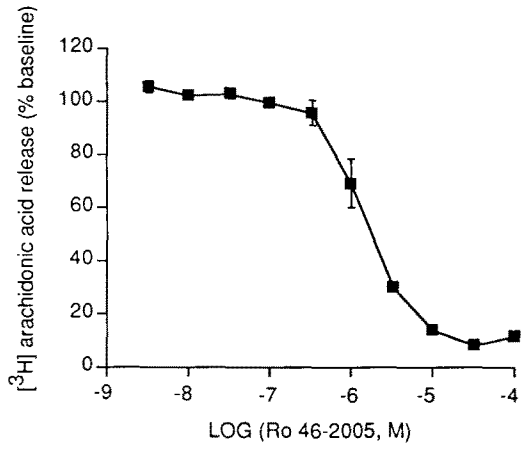

Fig. 4. Inhibition of the ET-1-mediated release of arachidonic acid by rat mesangial cells in the presence of Ro 46-2005. Represented is the mean ± S.E.M. of three independent experiments.

response could be increased to 1,400 ± 60% when all incubation steps were performed in the presence of 0.1 mM phosphoramidon. The EC_{50} value for ET-1 remained unchanged under these conditions. The ET-1 (10 nM)-stimulated release of arachidonic acid increased rapidly with time during the first 10 min and reached a plateau after about 30 min. In order to obtain a maximal response we thus performed our experiments with 10 nM ET-1 and an incubation time of 30 min in the presence of 0.1 mM phosphoramidon. Under these conditions Ro 46-2005 dose-dependently inhibited the release of arachidonic acid with an IC_{50} of 1.8 ± 0.3 μM (Fig. 4).

4. DISCUSSION

Endothelin (ET) mediates its effects via at least two different receptor subtypes, ET_A and ET_B. To inhibit its actions, it might be necessary to block these and possibly further existing receptor subtypes simultaneously. Ro 46-2005, a recently described synthetic non-peptide ET antagonist [18], was shown to prevent the cerebral vasoconstriction which follows subarachnoidal hemorrhage and the post-ischemic renal vasoconstriction in rats. In competition binding studies with [^{125}I]ET-1, [^{125}I]ET-2 and [^{125}I]ET-3, Ro 46-2005 is characterized by its non-selective interaction with ET_A and ET_B receptors. It binds to natural ET_A receptors containing tissue, i.e. human smooth muscle cells and rat mesangial cells, and also to recombinant human ET_A receptors with similar potencies. Comparable potencies are also observed for its binding to membranes containing natural ET_B receptors, i.e. porcine cerebellum and human placenta, and also for recombinant ET_B receptors. These data show that Ro 46-2005 exhibits little if any species-specific differences, suggesting that its binding sites are highly conserved. The observed potency of Ro 46-2005 to compete for [^{125}I]ET-1 binding on ET_B containing rat endothelial cells is about 5-fold lower than on membranes containing natural ET_B receptors. This may be due to different binding and buffer conditions.

Our results derived from saturation binding experiments in the presence of different concentrations of Ro 46-2005' indicate a competitive binding mode on both receptor subtypes. This conclusion confirms the observation that Ro 46-2005 induces parallel shifts to the right of the concentration–response curves for ET_A and ET_B receptors in isolated vessels without decrease in maximal response [18].

Another important question was whether the potency observed in these binding studies was translated into a similar potency for inhibition of signal transduction. The ET-1-mediated activation of phospholipase A_2, which leads to the release of arachidonic acid and its derivatives, has been described for different cells, like vascular smooth muscle cells [19], rat mesangial cells [20] and mouse astrocytes [30]. Our experiments were performed in the presence of phosphoramidon, an inhibitor of the ET-converting enzyme which cleaves ET from its precursor, big ET [31]. This results in an increase in the ET-1-mediated release of arachidonic acid by rat mesangial cells of 1.6 fold. We have recently shown that ET-converting enzyme inhibition by phosphoramidon can prevent desensitization of receptors by endogenously formed ET and increase the number of available sites, thereby increasing the maximal response to ET-1 without affecting its potency [21]. The potency of Ro 46-2005 for inhibiting arachidonic acid release is comparable to the potency found in binding studies, which confirms the receptor antagonistic effect of Ro 46-2005.

In summary, Ro 46-2005 is a competitive ET antagonist for both ET_A and ET_B receptors which should provide a useful tool to get further insight into the physiological actions of endothelins.

Acknowledgements: We would like to thank Dr. Y. Furuichi for providing plasmids with ET receptors which were cloned from human placenta, Drs. R. Gentz and E.J. Schlaeger for the expression and fermentation of recombinant endothelin receptors, and Dr. W. Fischli for critical reading of the manuscript. We further thank Mrs. B. Butscha, Mrs. L. Hilfiger and Mr. W. Löhrer for excellent technical assistance.

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