

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/23246604>

Pharmacology of Macitentan, an Orally Active Tissue-Targeting Dual Endothelin Receptor Antagonist

Article in Journal of Pharmacology and Experimental Therapeutics · October 2008

DOI: 10.1124/jpet.108.142976 · Source: PubMed

CITATIONS

322

READS

984

14 authors, including:



Marc Iglarz

Idorsia Pharmaceuticals Ltd.

91 PUBLICATIONS 3,714 CITATIONS

SEE PROFILE



John Gatfield

Idorsia PharmaceutucialsLtd,

78 PUBLICATIONS 3,368 CITATIONS

SEE PROFILE



Keith Morrison

Actelion Pharmaceuticals Ltd

18 PUBLICATIONS 948 CITATIONS

SEE PROFILE



Alexander Treiber

Actelion Pharmaceuticals Ltd

78 PUBLICATIONS 2,635 CITATIONS

SEE PROFILE

Pharmacology of Macitentan, an Orally Active Tissue-Targeting Dual Endothelin Receptor Antagonist

Marc Iglarz, Christoph Binkert, Keith Morrison, Walter Fischli, John Gatfield, Alexander Treiber, Thomas Weller, Martin H. Bolli, Christoph Boss, Stephan Buchmann, Bruno Capeleto, Patrick Hess, Changbin Qiu, and Martine Clozel

Drug Discovery Department, Actelion Pharmaceuticals Ltd., Allschwil, Switzerland

Received July 2, 2008; accepted September 8, 2008

ABSTRACT

Macitentan, also called Actelion-1 or ACT-064992 [*N*-[5-(4-bromophenyl)-6-(2-(5-bromopyrimidin-2-yloxy)ethoxy)pyrimidin-4-yl]-*N'*-propylaminosulfonamide], is a new dual ET_A/ET_B endothelin (ET) receptor antagonist designed for tissue targeting. Selection of macitentan was based on inhibitory potency on both ET receptors and optimization of physicochemical properties to achieve high affinity for lipophilic milieu. In vivo, macitentan is metabolized into a major and pharmacologically active metabolite, ACT-132577. Macitentan and its metabolite antagonized the specific binding of ET-1 on membranes of cells overexpressing ET_A and ET_B receptors and blunted ET-1-induced calcium mobilization in various natural cell lines, with inhibitory constants within the nanomolar range. In functional assays, macitentan and ACT-132577 inhibited

ET-1-induced contractions in isolated endothelium-denuded rat aorta (ET_A receptors) and sarafotoxin S6c-induced contractions in isolated rat trachea (ET_B receptors). In rats with pulmonary hypertension, macitentan prevented both the increase of pulmonary pressure and the right ventricle hypertrophy, and it markedly improved survival. In diabetic rats, chronic administration of macitentan decreased blood pressure and proteinuria and prevented end-organ damage (renal vascular hypertrophy and structural injury). In conclusion, macitentan, by its tissue-targeting properties and dual antagonism of ET receptors, protects against end-organ damage in diabetes and improves survival in pulmonary hypertensive rats. This profile makes macitentan a new agent to treat cardiovascular disorders associated with chronic tissue ET system activation.

An increasing level of evidence shows that the endothelin (ET) system, ET-1 and its two receptors, is a tissular system and that ET-1 does not act as a circulating hormone but as a tissular—paracrine or autocrine—factor. By tissular mechanisms beyond vasoconstriction, ET-1 can change tissue structure and induce fibrosis (Shi-Wen et al., 2001), cell proliferation (Yang et al., 1999), and inflammation (Hochoer et al., 2000). Of the ET-1 produced by endothelial cells, 80% is secreted basolaterally toward the vessel wall and hence the tissue, whereas only 20% is released apically into the bloodstream (Wagner et al., 1992). Accordingly, endothelium-restricted ET-1 overexpression in mice leads to vascular hypertrophy without blood pressure increase (Amiri et al.,

2004). Infusion of big ET-1, which per se is inactive and requires enzymatic processing to ET-1 to exert activity, induces peripheral and coronary vasoconstriction without any significant elevations in plasma ET-1 (Teerlink et al., 1995). ET-1 overexpression leads to renal and lung fibrosis and chronic inflammation without systemic effects on blood pressure (Hochoer et al., 1997, 2000), and cardiac-specific ET-1 overexpression in mice induces dilated cardiomyopathy, cardiac inflammation, and heart failure (Yang et al., 2004). Tissular ET-1 acts via binding to two G protein-coupled receptors located on a large variety of cell types such as endothelial cells and macrophages (ET_B only) (Haug et al., 2001), vascular smooth muscle cells (Haug et al., 2001), fibroblasts (Shi-Wen et al., 2001), cardiomyocytes (Serner et al., 2000), adrenal cells (Rossi et al., 1994), and stellate cells (ET_A and ET_B) (Yokomori et al., 2001).

In certain pathological situations, there is increased expression of both tissue ET-1 and ET receptors. This up-regulation

This research is entirely supported by Actelion Pharmaceuticals Ltd., and all authors are employees of Actelion Pharmaceuticals Ltd.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

doi:10.1124/jpet.108.142976.

ABBREVIATIONS: ET, endothelin; ACT-064992, *N*-[5-(4-bromophenyl)-6-(2-(5-bromopyrimidin-2-yloxy)ethoxy)pyrimidin-4-yl]-*N'*-propylaminosulfonamide; FLIPR, fluorometric imaging plate reader; DOCA, deoxycorticosterone acetate; Veh, vehicle; MCT, monocrotaline; MPAP, mean pulmonary arterial pressure; RV, right ventricle; LV, left ventricle; S, septum; STZ, streptozotocin; VEGF, vascular endothelial growth factor; SB209670, (+)-(1*S*,2*R*,3*S*)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid; ACT-132577, [5-(4-bromophenyl)-6-(2-(5-bromopyrimidin-2-yloxy)ethoxy)-pyrimidin-4-yl]-sulfamide.

explains why the ET system plays a role in pathology more than in physiology and why ET receptor antagonists have such little effects in physiology and marked effects in pathology (Breu et al., 1998). Together with the increased expression of ET receptors, there is often a change in the ET_A/ET_B ratio (Kakoki et al., 1999). In these pathological situations, both ET_A and ET_B receptors mediate the detrimental actions of ET-1, and dual blockade of both receptors may be necessary. Indeed, a cross-talk between ET_A and ET_B receptors has been evidenced in many cell types, such that the other receptor subtype can substitute for the blockade of a single receptor only. Heterodimerization of ET_A and ET_B receptors is likely to contribute to this cross-talk (Grgan et al., 2004). Accordingly, blocking of both ET_A and ET_B receptors can have exquisite effects to inhibit vasoconstriction to ET-1 (Sauvageau et al., 2007), collagen synthesis (Shi-Wen et al., 2001), smooth muscle cell proliferation (Porter et al., 1998), inflammation (Muller et al., 2000), and bronchoconstriction (Clozel and Gray, 1995). Survival of mice with dilated cardiomyopathy caused by cardiac overexpression of ET-1 and of rats with coronary ligation is prolonged by a mixed ET_A and ET_B receptor antagonist but not by a selective ET_A receptor antagonist (Mulder et al., 1997, 2002; Yang et al., 2004). Survival of rats with monocrotaline-induced pulmonary hypertension is also prolonged by a dual ET_A/ET_B receptor antagonist but not by a selective ET_A receptor antagonist (Jasmin et al., 2001).

Therefore, our goal was to identify a potent dual ET receptor antagonist with tissue-targeting properties. To do so, we focused during the discovery process on the identification of molecules targeting both ET_A and ET_B receptors and possessing a high partition coefficient as indicator of strong affinity for the tissues. Macitentan, also called Actelion-1 or ACT-064992 (Fig. 1), resulted from this tailored screening and indeed shows potential as a disease-modifying agent in

different animal models. In vivo, a major metabolite, ACT-132577, is formed that contributes to its pharmacological activity. Here we describe the general pharmacology of macitentan, its pharmacokinetics, and its ability to protect against end-organ damage and improve survival in animal models.

Materials and Methods

Physicochemical Characterization and Pharmacokinetics of Macitentan

Ionization Constant: pK_a . The ionization constant was determined with a multiwavelength spectrophotometric method, using the GLpKa/D-PAS instrument from Sirius Analytical (Lakewood, NJ) as described previously (Allen et al., 1998).

Distribution Coefficient: Log D. The distribution of macitentan between *n*-octanol and aqueous phosphate buffer, pH 7.4, was determined in duplicates based on the shake flask method (Lund, 1994). In brief, macitentan was dissolved at a concentration of 0.2% in the organic phase (*n*-octanol), which was further mixed with the aqueous phase (67 mM phosphate buffer, pH 7.4). After 15-min shaking, the two phases were separated by centrifugation, and macitentan concentrations in each phase were measured by high-performance liquid chromatography. D is given by the direct quotient of the macitentan concentration in the organic and aqueous phases. Log D determination was similarly performed for bosentan and ambrisentan (a dual ET_A/ET_B and a selective ET_A receptor antagonist, respectively).

Pharmacokinetics

Single- and multiple-dose pharmacokinetic studies with macitentan were performed in male Wistar rats. For the single-dose pharmacokinetic study, macitentan was administered intravenously at a dose of 0.5 mg/kg ($n = 4$). For the multiple-dose pharmacokinetic study, macitentan was administered orally at dose of 10 mg/kg daily for 26 weeks. Macitentan and ACT-132577 were quantified in plasma using liquid chromatography coupled to mass spectrometry after plasma protein precipitation.

In Vitro Binding and Functional Assays

Binding Assay. The cDNAs for human ET_A and ET_B receptors were cloned, sequenced, and stably overexpressed in Chinese hamster ovary cells. Membranes were prepared from these cells as described previously (Clozel et al., 1999). The competition binding assay was performed in 200 μ l of 50 mM Tris/HCl buffer, pH 7.4, including 25 mM $MnCl_2$, 1 mM EDTA, 0.5% (w/v) bovine serum albumin (fraction V), and 50 μ g/ml 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefablock; Sigma-Aldrich, St. Louis, MO) in polypropylene microtiter plates. Membranes containing 0.5 μ g of protein (ET_A) or 0.2 μ g of protein (ET_B) were incubated for 2 h at room temperature with 16 pM ^{125}I -ET-1 (8000 cpm) and increasing concentrations of unlabeled test compounds. Maximal and minimal binding was determined in samples without and with 100 nM unlabeled ET-1 (Bachem, Bubendorf, Switzerland), respectively. After 2 h of incubation, the membranes were filtered onto filter plates containing GF/C filters (Unifilterplates; Canberra Packard S.A., Zürich, Switzerland). To each well, 50 μ l of scintillation cocktail was added (MicroScint 20; Canberra Packard S.A.), and the filter plates counted in a microplate counter (TopCount; Canberra Packard S.A.). Test compounds were dissolved, diluted, and added to the assay in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the assay was 2.5%, which was found not to interfere with the binding. The IC_{50} value was calculated as the concentration of antagonist inhibiting 50% of the specific binding of ET-1.

Inhibition of Intracellular Calcium Mobilization. Primary human pulmonary arterial smooth muscle cells (Lonza Verviers

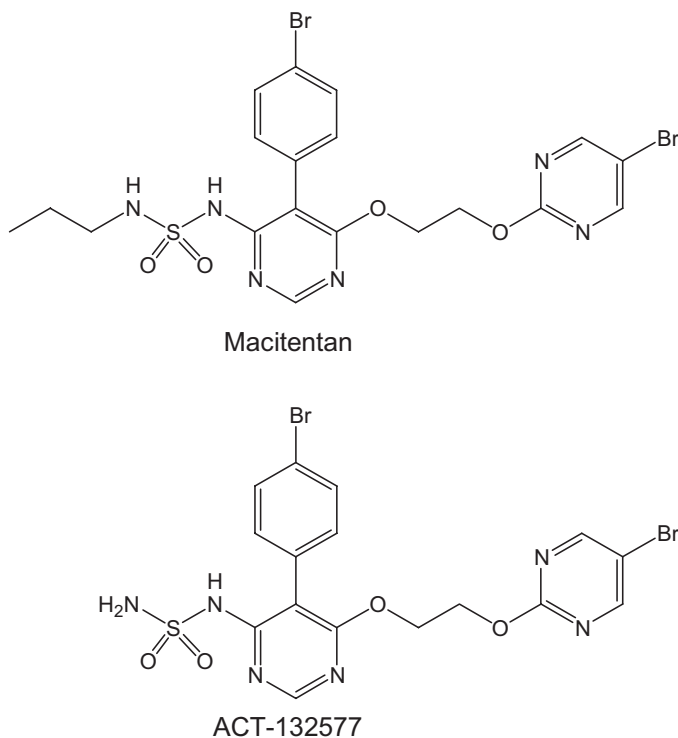


Fig. 1. Chemical structures of macitentan and its major metabolite ACT-132577.

SPRL, Verviers, Belgium) were grown in the supplied medium for at least 7 days before trypsinization and plating into gelatin-coated 384-well fluorometric imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA) plates at a density of 10,000 cells/well. The rat aortic smooth muscle cell line A10 was grown in Dulbecco's modified Eagle's medium/20% fetal bovine serum/penicillin/streptomycin and harvested by trypsinization. Seeding onto gelatin-coated 384-well FLIPR plates occurred at a density of 10,000 cells/well. 3T3 Swiss fibroblasts were grown in Dulbecco's modified Eagle's medium/10% fetal bovine serum/penicillin/streptomycin, trypsinized, and seeded at 10,000 cells/well into gelatin-coated 384-well FLIPR plates.

Cell staining was performed according to standard protocol. In brief, after cell seeding, cells were grown overnight, the medium was discarded, and cells were incubated with 25 μ l of dye buffer (3 μ M fluo4-acetoxymethyl ester, 0.0375% NaHCO₃, 20 mM HEPES, and 5 mM probenecid in Hanks' balanced salt solution, pH 7.4). After 60-min incubation at 37°C, cells were washed three times with 50 μ l of wash buffer (0.0375% NaHCO₃, 20 mM HEPES, 2.5 mM probenecid, and 0.1% bovine serum albumin in HBSS, pH 7.4), supplemented with 50 μ l of this buffer, and equilibrated for at least 30 min. In the FLIPR, the antagonists were tested versus 10 nM ET-1, at concentrations ranging from 10 pM to 10 μ M, and the IC₅₀ value was calculated.

Isolated Rat Aortic Rings. Male 10 to 12-week-old Wistar rats were euthanized with CO₂, and the descending thoracic aorta was removed and cut into 3-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% O₂, 5% CO₂ and warmed (37°C) Krebs-Henseleit solution of the following composition: 115 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.5 mM KHPO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂, and 10 mM glucose. 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 phenylephrine (1 μ M). Endothelium denudation was assessed by the absence of relaxation to acetylcholine (10 μ M). The rings were then washed and stretched if necessary until a stable baseline force was obtained. The rings were incubated with various concentrations of macitentan (0.1–1 μ M) or ACT-132577 (0.3–3 μ M). After 30 min, cumulative concentrations of ET-1 were added, and the interval between concentrations was determined by the time required for the force generated to reach a plateau.

Isolated Rat Tracheal Strips. Male 10 to 12-week-old Wistar rats were euthanized with CO₂, and the trachea was removed and cut into 5-mm rings. The tracheal strips were prepared, the epithelium was removed by gentle rubbing of the luminal surface, and each strip was suspended in a 10-ml isolated organ chamber containing gassed and warmed Krebs-Henseleit solution as described above. The strips were stretched to a resting force of 1 g. After a 60-min equilibration period, the strips were contracted using potassium chloride (50 mM). The strips were then washed and stretched if necessary until a stable baseline force was obtained. After a 30-min incubation with macitentan (3–30 μ M) or ACT-132577 (10–70 μ M), cumulative concentrations of sarafotoxin S6c were added. The interval between concentrations was determined by the time required for the force generated to reach a plateau.

Analysis and Calculations. For in vitro cell tests, the IC₅₀ value represents the geometric mean (Brinkmann et al., 2002) of at least three independent experiments. For isolated organ tests, the maximal 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 (EC₅₀) was calculated. The pA₂ value (negative logarithm of the molar concentration of antagonist that causes a 2-fold parallel shift to the right of the agonist concentration-response curve), and apparent pK_B values were used as indices of functional inhibitory potency.

In Vivo Pharmacology

Animals. Normotensive male Wistar rats were delivered from Biotechnology and Animal Breeding Division (RCC Ltd., Füllinsdorf, Switzerland) and the Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). All animals were housed in climate-controlled conditions with a 12-h light/dark cycles in accordance with local guidelines (Basel-Landschaft cantonal veterinary office or Animal Care and Use Committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences). All rats were maintained under identical conditions and had free access to normal pelleted rat chow and drinking water.

Effects on ET-1 Plasma Concentrations. Sublingual blood samples for ET-1 plasma measurements were collected under 2.5% isoflurane anesthesia from male Wistar rats before and 6 h after macitentan (0.1–30 mg/kg), bosentan (1–300 mg/kg), or vehicle administration by gavage ($n = 4$ /group). ET-1 concentration was measured by chemiluminescent immunoassay (Quantiglo; R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany).

Acute Effects on Blood Pressure in Conscious Hypertensive Rats. After an acclimatization period of at least 7 days, male Wistar rats were anesthetized (2.5% isoflurane), the right kidney was removed by a flank incision, and a deoxycorticosterone acetate (DOCA; 40-mg) pellet was subcutaneously implanted. Drinking water consisted of a 1% saline solution. Five weeks after starting salt administration, a telemetry system was implanted. The rats were anesthetized with a mixture of 90 mg/kg ketamine-HCl (Ketavet; Parke-Davis, Berlin, Germany) and 10 mg/kg xylazine (Rompun; Bayer AG, Leverkusen, Germany) administered intraperitoneally. The pressure transmitter was implanted into the peritoneal cavity under aseptic conditions and a sensing catheter (model TA11PA-C40; Data Sciences International, St. Paul, MN) was placed in the descending aorta below the renal arteries pointing upstream. The transmitter was sutured to the abdominal musculature, and the skin was closed.

Blood pressure data were collected continuously using the Dataquest ART Gold acquisition system, version 3.01 (Data Sciences International, St. Paul, MN). A receiver platform (RPC-1; Data Sciences International) connected the radio signal to digitized input that was sent to a dedicated personal computer (OptiPlex, GX270; Dell, Round Rock, TX). Blood pressure signals were sampled at 500 Hz. Arterial pressures were calibrated by using an input from an ambient-pressure reference (APR-1; Data Sciences International).

Two weeks or more after implantation of the telemetry system, oral administration of macitentan (0.3–30 mg/kg p.o.) or bosentan (0.3–300 mg/kg p.o.) was performed by single gavage as a suspension of gelatin (7.5%) in six to nine rats. Vehicle (gelatin solution, 7.5%) was also tested. Data were collected every 5 min. Hourly means of blood pressure were calculated. Each rat served as its own control, with the blood pressure data of the last 24 h before treatment taken as reference. Efficacy was measured by both the maximal reduction in blood pressure and the measurement of the area between curves. Area between curves was calculated using the hourly means of blood pressure between data collected 24 h before administration of the compound and the data collected during the treatment period. Mean arterial blood pressure was expressed in mm Hg.

Chronic Effects in Pulmonary Hypertensive Rats. Male Wistar rats were randomly assigned into experimental groups, and normal rat chow (Veh), macitentan (0.3–100 mg/kg/day in food admix; $n = 14$ –15/dose) or bosentan (10–300 mg/kg/day in food admix; $n = 14$ –15/dose) were initiated immediately after subcutaneous monocrotaline (MCT) injection (60 mg/kg; Sigma-Aldrich) and continued for a duration of 4 weeks. As a control group, age-matched rats received an equal volume of saline.

In a first study, different doses of macitentan and bosentan were administered chronically for 4 weeks to determine their respective effects on mean pulmonary arterial pressure (MPAP) and right ventricular hypertrophy in MCT-treated rats. At the end of the treat-

ment phase, the rats were anesthetized by intraperitoneal injection of 100 mg/kg thiobutabarbital-sodium (Inactin; Byk-Gulden, Konstanz, Germany) and placed on a thermostatically controlled heating table to maintain body temperature at 36–38°C. A tracheotomy tube was put in place, and a catheter was inserted in the pulmonary artery via the right jugular vein for measurement of MPAP (Clozel et al., 2006). Measurements were recorded for 15 min using a PowerLab data acquisition system (IOX 1.7.0 Data acquisition; Emka Technologies, Paris, France). At the end of the recording, rats were sacrificed. The heart was removed and weighed, and the ratio of organ weight to body weight was calculated. The right ventricle (RV) and the left ventricle plus septum (LV + S) were separated and weighed; the ratio RV/LV + S was used as an index of right ventricular hypertrophy.

In a second study, a maximally effective dose of macitentan was selected to evaluate its effect on survival. Male Wistar rats were randomly assigned into three experimental groups, and treatment with either normal rat powdered chow (Veh; $n = 30$) or macitentan (30 mg/kg/day in food admix; $n = 30$) was started immediately after MCT injection. A group of 10 rats injected with saline (instead of MCT) and treated with normal rat powdered chow was used as control. Rats were inspected for death twice a day on weekdays and daily on weekends and holidays. Per protocol the survival study would be terminated after a mortality of 50% in the untreated MCT-rats was achieved. Accordingly, the duration of the survival study was 42 days.

Chronic Effects in Diabetic Rats. After a 1-week adaptation period, all male Wistar rats were anesthetized with a mixture of Ketamine-Rompun (50 mg/kg; 5 mg/kg i.p.), and the right kidney was removed to hasten the development of diabetic nephropathy. Two weeks later, the rats were made diabetic by intravenous injection of streptozotocin (STZ; Sigma Aldrich) (35 mg/kg body weight).

The rats were randomly assigned into experimental groups, and treatment of macitentan (30 mg/kg/day in food admix; $n = 14$ /group) was initiated immediately after STZ injection for a duration of 24 weeks. The rats were placed in individual metabolic cages for 24-h urine collection every 4 weeks. Total 24-h urinary protein was measured using the Bradford assay (Bio-Rad, Hercules, CA), and bovine serum albumin was used to generate the standard curve.

After 24 weeks of treatment, a renal clearance experiment was conducted as described previously (Ding et al., 2003). In brief, a catheter was inserted into the left femoral vein for infusion of synthetic plasma, inulin, and *p*-aminohippurate in anesthetized animals. A polyethylene cannula was placed in the left femoral artery and connected to a pressure transducer (MLT1050 precision BP transducer; ADInstruments Ltd., Hastings, UK) for recording of arterial blood pressure and periodic sampling of blood. During surgery, the rat received an intravenous infusion of 1 ml of synthetic plasma and a 0.5-ml bolus of 0.9% NaCl containing 40 mg/ml inulin (Sigma-Aldrich) and *p*-aminohippurate (0.5%; Merck Research Labs, West Point, PA). Subsequently, a continuous intravenous infusion of 0.9% NaCl containing the same concentrations of inulin and *p*-aminohippurate at a rate of 40 μ l/min was initiated. After a 45-min equilibration period, renal clearance experiments were begun in which two consecutive 20-min urine collections were performed, with midpoint arterial blood samples.

At the end of the study, the left kidney was obtained and cut longitudinally. One half was placed in 10% buffered formalin and embedded in paraffin. Sections of 5 μ m in thickness were stained with hematoxylin and eosin and examined by light microscopy.

The investigators who were blinded as to the experimental group assessed the severity of the morphological changes, i.e., the presence of glomerulosclerosis, tubulointerstitial lesions, and vascular lesions in the renal cortex. Each type of lesion was graded semiquantitatively as described previously (Ding et al., 2003). In brief, to assess glomerulosclerosis, 50 glomeruli in each kidney were observed at 400 $\times$ magnification and graded according to the following criteria: 0, normal; 1+, slight glomerular damage such as a mild increase in the

mesangial matrix and/or hyalinosis with focal adhesion, involving <25% of the glomerulus; 2+, sclerosis of 25–50%; 3+, sclerosis of 50–75%; and 4+, sclerosis of >75% of the glomerulus. A glomerular damage index was calculated by averaging the grades assigned to all glomeruli. The tubulointerstitial lesions were assigned an injury grade (0–3): grade 0, normal; 1, lesions involving <25%; 2, lesions involving 25–50%; and 3, lesions involving >50% of the field. A score for tubulointerstitial lesions for each kidney was obtained by averaging the grades given to all fields. Vascular lesions in each kidney were attributed grades of severity from 0 to 4: grade 0, normal vessel; 1, mild vascular wall thickening; 2, moderate thickening; 3, severe thickening (onion skin pattern); and 4, fibrinoid necrosis. The vascular lesion score was obtained using the same procedure as described above.

Measurement of retinal VEGF content, as a surrogate for microvascular permeability, assessed the severity of diabetic retinopathy (Cukiernik et al., 2004). At the end of the 24-week study, the right eye was dissected, the eyeball was opened through the corneoscleral portion, and a 2 $\times$ 2-mm area of retina above the optic nerve was cut, homogenized in phosphate buffer, and centrifuged at 12,000 rpm. The concentration of VEGF in the supernatant was measured by enzyme-linked immunosorbent assay kit (Shanghai Senxiong Bio-Tech Ltd., Shanghai, China).

Expression of Results

Results are expressed as mean $\pm$ S.E.M. Analysis of variance for repeated measures and Dunnett's test were used to assess the effect of macitentan on plasma ET-1 concentrations. For other in vivo experiments, statistical analyses were performed by analysis of variance in STATISTICA (StatSoft, Berikon, Switzerland) and Student-Newman-Keuls procedure for multiple comparisons. Survival analysis was performed with a log-rank test.

Compounds

¹²⁵I-Labeled ET-1 tracer was obtained from ANAWA Trading S.A. (Wangen, Switzerland). ET-1 and sarafotoxin S6c were obtained from Bachem. Test compounds (macitentan, ACT-132577, bosentan, and ambrisentan) were synthesized by Actelion Pharmaceuticals Ltd. (Allschwil, Switzerland).

Results

Physicochemical Characterization and Pharmacokinetics. A distribution coefficient (log D) favoring partition to lipids and a high pK_a value reflecting a high nonionized fraction were used as markers of high affinity for lipidic milieu and tissue targeting. Macitentan exhibits a pK_a value of 6.2 and consequently has a relatively high percentage of nonionized form in an aqueous environment at physiological pH 7.4 compared with other endothelin receptor antagonists (Table 1). In addition, it shows a distribution of 800 to 1 between octanol and aqueous buffer and thus a good distribution to lipids and tissues. In comparison, bosentan also has a preferred affinity for the lipophilic phase as shown by a distribution of 20 to 1, whereas ambrisentan has more affinity for the aqueous milieu than for lipids. Overall, macitentan

TABLE 1
Physicochemical parameters of macitentan, bosentan, and ambrisentan

	Distribution Coefficient D (<i>n</i> -Octanol/Aqueous Buffer)	Log D	pK_a	Nonionized Form at pH 7.4 %
Macitentan	800:1	2.9	6.2	6
Bosentan	20:1	1.3	5.1	1
Ambrisentan	1:2.5	−0.4	3.5	0.01

TABLE 2

Pharmacokinetic parameters of macitentan and ACT-132577 in male rats after single and multiple administration of macitentan

	Single Dose			Multiple Dose (26 wk)		
	Dose i.v.	Half-Life	V_d	Dose p.o.	C_{max}	AUC_{0-24}
	mg/kg	h	l/kg	mg/kg	$\mu\text{g/ml}$ (μM)	$\mu\text{g} \cdot \text{h/ml}$
Macitentan	0.5	2	1	10	1.5 (2.6)	9.1
ACT-132577		8.4	1		4.3 (7.9)	41

 V_d , volume of distribution; AUC_{0-24} , area under the curve (0 to 24 h).

presents a 40- and 2000-fold increased affinity for the lipid phase versus bosentan and ambrisentan, respectively.

After intravenous administration, macitentan has a terminal half-life of 2 h in the rat and a volume of distribution largely exceeding plasma volume (Table 2). Macitentan is metabolized to a major metabolite, ACT-132577, the only circulating metabolite in the rat, also a dual ET receptor antagonist (Fig. 1; Table 2). ACT-132577 also has a volume of distribution greater than the plasma volume and a longer half-life than its parent compound in the rat (Table 2). Multiple oral dosing of macitentan in rat at pharmacologically active dose (10 mg/kg) leads to exposure levels of ACT-132577 [area under the curve (0–24 h)] 4 to 5-fold higher than those of the parent compound, reaching plasma concentrations (C_{max}) for the two molecules above the IC_{50} value measured in vitro (see below), indicating contribution of this metabolite to the pharmacological activity observed following chronic administration of macitentan.

In Vitro Receptor Selectivity and Functional Inhibitory Potency. Affinity of macitentan and its major metabolite ACT-132577 for the ET receptors was assessed in microsomal membranes of Chinese hamster ovary cells stably overexpressing human ET_A and ET_B receptors. Macitentan inhibited binding of ^{125}I -ET-1 to recombinant ET_A receptors, with a mean IC_{50} value of 0.5 ± 0.2 nM ($n = 17$). The mean IC_{50} value for ET_B receptors was 391 ± 182 nM ($n = 17$). For ACT-132577, the mean IC_{50} values for ET_A and ET_B receptors were 3.4 ± 0.4 nM ($n = 4$) and 987 ± 185 nM ($n = 9$), respectively.

In a functional in vitro assay, macitentan and ACT-132577 achieved full inhibition of intracellular calcium increase induced by ET-1 on nonrecombinant cells (primary human pulmonary smooth muscle cells and rat aortic smooth muscle cell line A10 and mouse fibroblast cell line 3T3) (Table 3). Macitentan has a potency of approximately $IC_{50} = 1$ nM, and the metabolite IC_{50} value was approximately 10-fold higher compared with the parent compound.

Inhibition of ET-1-induced contraction was assessed in rat isolated tissue preparations. Macitentan and ACT-132577 induced a parallel shift to the right in the concentration-response curve for ET-1-mediated contraction of isolated rat

aorta denuded of endothelium (ET_A receptor-mediated) and for sarafotoxin S6c-mediated contraction of rat trachea denuded of epithelium (ET_B receptor-mediated). There was no significant change in the maximum response to ET-1 or sarafotoxin S6c. Schild analyses yielded slopes that were not significantly different from unity, consistent with macitentan being a competitive antagonist at ET receptors. A similar profile was also found for ACT-132577, although the highest concentration of the compound tested caused a small but significant reduction in the maximum contraction of trachea to sarafotoxin S6c. The apparent pK_B value was therefore calculated for ACT-132577 at ET_B receptors in rat trachea. In these functional assays, both macitentan and ACT-132577 behave as dual receptor antagonists. ACT-132577 is approximately 5-fold less potent than macitentan on ET_A receptors and presents an ET_A/ET_B inhibitory potency ratio of 16, versus 50 for its parent compound. The functional inhibitory potencies (pA_2 and pK_B values) are reported in Table 4.

To assess its selectivity, macitentan was screened at a concentration of 10 μM in a panel of 63 radioligand binding assays. At this high concentration, macitentan did not inhibit by more than 50% the binding of any of the ligands tested (data not shown). Thus, macitentan is selective for the ET_A and ET_B receptors.

In Vivo Pharmacology Studies. Macitentan is best studied in vivo because this allows the study of the overall pharmacological effects of macitentan together with its metabolite. The dual ET_A/ET_B receptor antagonism of macitentan was tested in rats by measuring plasma ET-1 concentrations. When administered to normotensive rats, macitentan, like bosentan, increased plasma ET-1 concentration, confirming its dual blockade on both ET_A and ET_B receptors. This increase occurred at a 10-fold lower dose than with bosentan, indicating a more potent pharmacological activity in vivo (Fig. 2).

In hypertensive DOCA-salt rats, both macitentan and bosentan dose-dependently decreased mean arterial blood pressure. Neither macitentan nor bosentan had any effect on heart rate. Macitentan caused a decrease in mean arterial blood pressure, with a maximal effect of -26 ± 4 mm Hg at a dose of 10 mg/kg (ED_{50} , 1 mg/kg). For bosentan, the maximal reduction in mean arterial pressure was -20 ± 5 mm

TABLE 3

Inhibition of ET-1-induced intracellular Ca^{2+} increase in three different nonrecombinant cell types

	IC_{50}		
	HPASMC	RASMC	m3T3
		nM	
Macitentan	0.90	0.80	1.84
ACT-132577	14.0	5.80	10.9

HPASMC, primary human pulmonary arterial smooth muscle cells; RASMC, rat aortic smooth muscle cell line; m3T3, mouse fibroblast cell line.

TABLE 4

Inhibition (pA_2) of ET-1-induced contractions on isolated rat aortic rings without endothelium or S6c-induced contractions on isolated rat tracheal rings without epithelium in presence or in absence of endothelin receptor antagonist

	pA_2		ET_A/ET_B Selectivity Ratio
	Aorta (ET_A)	Trachea (ET_B)	
Macitentan	7.6	5.9	50/1
ACT-132577	6.7	5.5 ^a	16/1

^a pK_B .

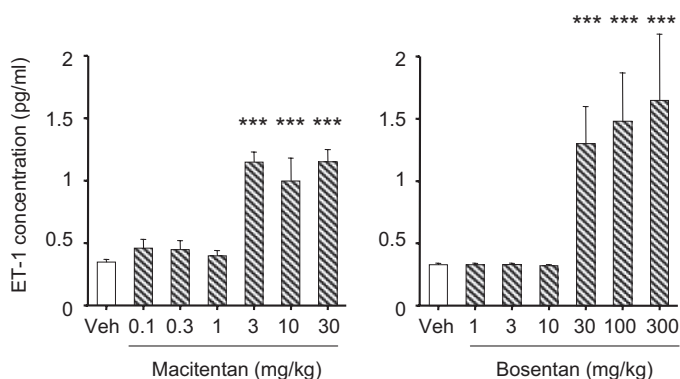


Fig. 2. ET-1 plasma concentrations in conscious Wistar rats 6 h after oral administration of macitentan and bosentan. Veh ($n = 4/\text{group}$). Data are presented as means $\pm$ S.E.M. ***, $p < 0.001$ compared with vehicle-treated rats.

Hg and was achieved at a 10 times higher dose of 100 mg/kg (ED_{50} , 10 mg/kg) (Fig. 3). Area between curves comparison indicated a better maximal efficacy of macitentan in terms of coverage of the blood pressure reduction (Fig. 3). At the maximal effective dose, the duration of the blood pressure response to macitentan was approximately 40 h, whereas with bosentan the response duration was 20 h (data not shown). Thus, in the DOCA-salt rat model of hypertension, macitentan is 10-fold more potent than bosentan and presents a higher efficacy as shown by the trends toward a stronger blood pressure reduction and a greater area between curves. Moreover, macitentan presents a 2-fold longer duration of action than bosentan.

Two chronic pathological models were used to study the effect of chronic macitentan treatment on hemodynamics and end-organ damage: the monocrotaline model of pulmonary hypertension and the streptozotocin model of diabetes. The monocrotaline rat model of pulmonary hypertension was used to test the effect of macitentan on cardiac hypertrophy and survival. Four-week oral administration of macitentan or bosentan dose-dependently prevented the development of pulmonary hypertension. It also prevented the development of right ventricle hypertrophy with a maximal efficacy obtained with a dose of 30 mg/kg/day for macitentan (Fig. 4). At this dose, no effect on systemic mean arterial blood pressure was observed (105.6 ± 3.1 versus 103.9 ± 4.6 mm Hg in treated and untreated monocrotaline rats, respectively). The maximal efficacious dose of bosentan was 300 mg/kg/day. Chronic oral administration of macitentan at 30 mg/kg/day

significantly improved the 42-day survival in monocrotaline rats (83 versus 50% survival in macitentan versus vehicle-treated rats; 66% reduction of mortality at 42 days; $p < 0.002$; Fig. 5).

We used the streptozotocin-induced diabetic rat model to study the impact of macitentan on end-organ damage, in particular renal damage, in diabetes. Untreated diabetic rats developed progressive nephropathy associated with renal structural damage, proteinuria (Fig. 6A), an increase of renal vascular resistance leading to decreased renal blood flow (Table 5), and increase of filtration fraction (0.35 ± 0.01 versus 0.20 ± 0.01 in control rats; $p < 0.05$). Twenty-four-week treatment with macitentan partially prevented the development of renal vasoconstriction and increased renal blood flow (Table 5). Macitentan increased glomerular filtration rate and decreased filtration fraction (0.31 ± 0.01 versus 0.35 ± 0.01 in diabetic rats; $p < 0.05$). Vascular and tubulointerstitial lesions and also glomerular damage were attenuated by chronic administration of macitentan (Table 5), and proteinuria was partially prevented (Fig. 6A). Macitentan slightly decreased systemic blood pressure (Table 5) and had no effect on body weight (274 ± 8 versus 271 ± 8 g in untreated diabetic rats). Three vehicle-treated rats died during the study versus one rat in the macitentan-treated group. At the retinal level, macitentan markedly prevented the increase in VEGF content observed in diabetic rats (Fig. 6B).

Discussion

Two goals have driven the selection of this new ET receptor antagonist: the optimization of its ability to target the tissue and its dual blockade of both ET_A and ET_B receptors. Tissue targeting was achieved by optimization of the molecule for its physicochemical characteristics favoring partitioning from the systemic aqueous compartment into the lipophilic tissue. This could be achieved first by increasing the pK_a value, resulting in an increased percentage of the nonionized form of the molecule in aqueous milieu at physiological pH. Only the nonionized form is able to cross cell membranes, and an increased concentration gradient of the nonionized molecule drives the net flux across lipophilic cell membranes. Furthermore, chemical modifications resulting in increased log D values also lead to increased affinity for the tissue because log D is considered to be an indicator of distribution between plasma and tissue.

Macitentan was also optimized for its potency and dual

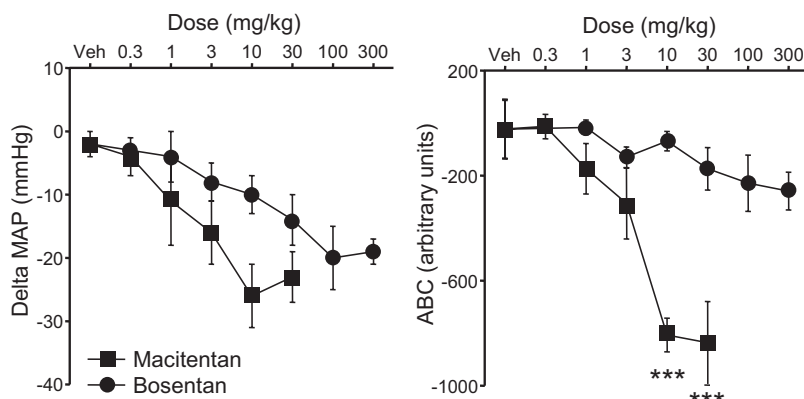


Fig. 3. Dose-response relationship on mean arterial blood pressure (left) and area between the curves (right) after single oral administration of macitentan or bosentan in conscious DOCA-salt hypertensive rats equipped with telemetry ($n = 6-9/\text{group}$). ABC, area between curves; MAP, mean arterial blood pressure. Data are presented as means $\pm$ S.E.M. ***, $p < 0.001$ compared with bosentan-treated rats.

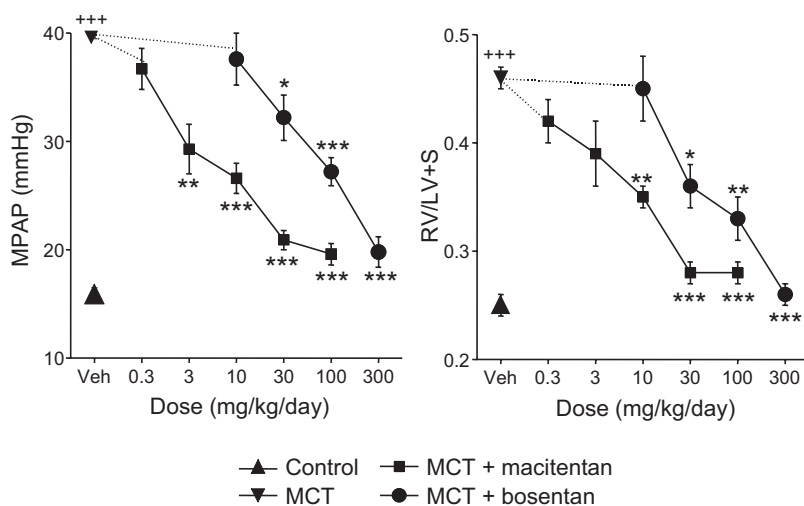


Fig. 4. Dose-response relationship for the effect of 4-week oral administration of macitentan or bosentan on mean pulmonary artery blood pressure and relative right ventricular weight in Wistar rats with monocrotaline-induced pulmonary hypertension ($n = 14-15$ per dose). Data are presented as means $\pm$ S.E.M. +, +, +, $p < 0.001$ compared with control rats. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with monocrotaline + vehicle.

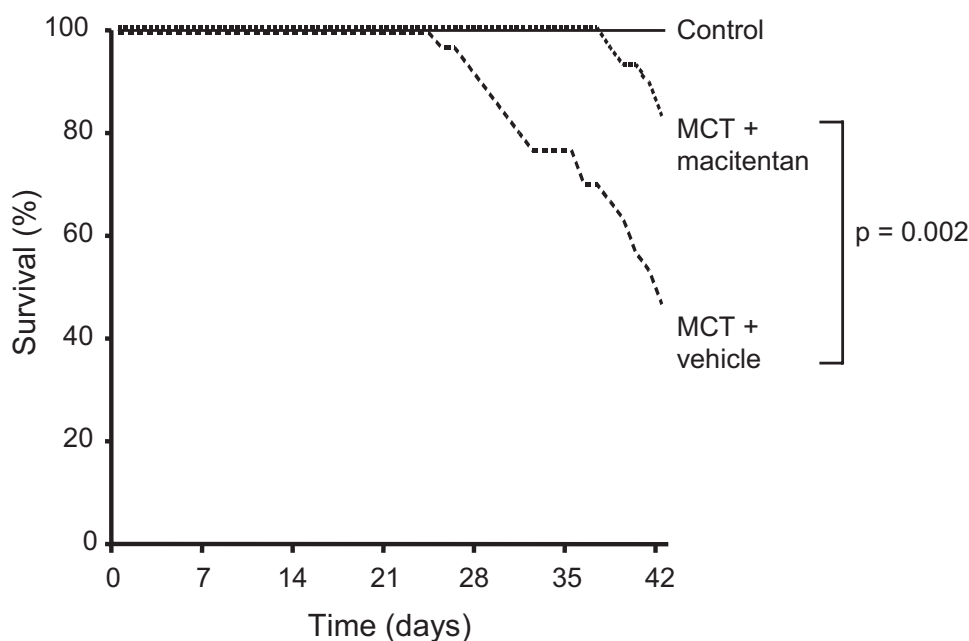


Fig. 5. Effect of oral administration of macitentan on survival of Wistar rats with monocrotaline-induced pulmonary hypertension [$n = 30$ /group, except control group ($n = 10$)].

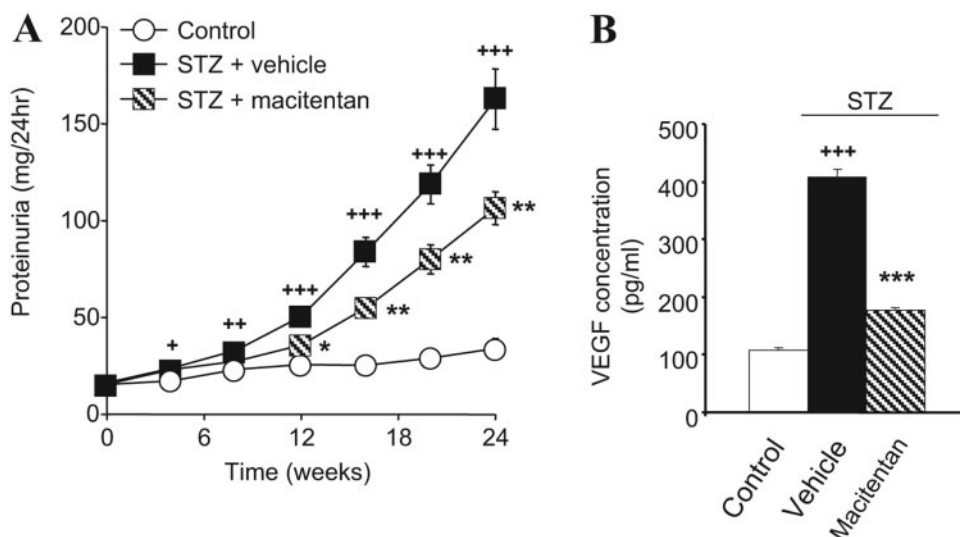


Fig. 6. Effect of chronic administration of macitentan on proteinuria (A) and retinal VEGF content (B) in streptozotocin-induced diabetic rats ($n = 14$ /group). Data are presented as means $\pm$ S.E.M. +, +, +, $p < 0.001$ compared with control rats. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ compared with streptozotocin rats + vehicle.

TABLE 5
Effect of chronic administration of macitentan on hemodynamics and renal morphometry in diabetic rats

	MAP	RBF	GFR	RVR	KW	GSA	GS (Index)	TIL (Score)	VL (Score)
	mm Hg	ml/min/100 g	ml/min/100 g	mm Hg/(ml/min)	g/100 g	$\mu\text{m}^2 \times 10^3$			
CTL	116 ± 2	2.89 ± 0.10	0.58 ± 0.03	4.3 ± 0.2	0.24 ± 0.02	9.8 ± 0.1	0.01 ± 0.01		0.11 ± 0.02
STZ + vehicle	107 ± 2**	1.86 ± 0.09***	0.64 ± 0.03	11.0 ± 0.7***	0.83 ± 0.01***	17.3 ± 0.1***	0.30 ± 0.03***	0.67 ± 0.07***	0.52 ± 0.06***
STZ + macitentan	99 ± 2†	2.40 ± 0.11†††	0.75 ± 0.03†	7.9 ± 0.3†††	0.79 ± 0.01	14.7 ± 0.1†††	0.14 ± 0.02†††	0.41 ± 0.04†††	0.25 ± 0.03†††

CTL, control; MAP, mean arterial blood pressure; RBF, renal blood flow; GFR, glomerular filtration rate; RVR, renal vascular resistance; KW, kidney weight; GSA, glomerular surface area; GS, glomerulosclerosis; TIL, tubulointerstitial lesions; VL, vascular lesions.

** $p < 0.01$, *** $p < 0.001$ compared with control rats.

† $p < 0.05$, ††† $p < 0.001$ compared with streptozotocin rats + vehicle.

blockade of ET_A and ET_B receptors since both receptors mediate the deleterious effects of ET-1 in pathology (Clozel and Gray, 1995; Cardillo et al., 1999; Shi-Wen et al., 2001; Sauvageau et al., 2007). In humans with hypertension, dual antagonism was superior to ET_A -selective antagonism in terms of maximal efficacy (Cardillo et al., 1999). This ranking of efficacy between selective and dual antagonism in pathology is inverse in physiology, where in healthy subjects, ET_A -selective antagonists induced greater vasodilation than dual (Verhaar et al., 1998). However, these observations may mean that selective antagonists give in physiology a nonselective vasodilation by stimulation of endothelial ET receptors, since their effect can be blocked by inhibition of nitric oxide synthase by N^ω -nitro-L-arginine methyl ester (Cardillo et al., 2000) but that in pathology the up-regulation of contractile smooth muscle cell ET_B receptors and the down-regulation of endothelial ET_B receptors makes dual antagonism superior in terms of maximal efficacy (Cardillo et al., 1999). In pathological situations, endothelial expression of ET_B receptors is decreased, whereas these receptors are up-regulated in the media (Kakoki et al., 1999). These observations have been confirmed functionally in humans: although ET_B -dependent vasodilation can be observed in healthy volunteers, this phenomenon is lost in pathological situations such as atherosclerosis (Böhm et al., 2002) and type II diabetes mellitus (Cardillo et al., 2002). In the present study, dual antagonism of both ET_A and ET_B receptors by macitentan was evidenced in vitro and in isolated organs and confirmed in vivo by the increase in ET-1 plasma concentrations. Such an increase can be observed only after administration of a dual but not an ET_A -selective receptor antagonist, at least acutely, and results from the blockade of ET_B -mediated clearance of ET-1 and from a possible spillover of ET-1 by prevention of its binding to tissue receptors (Löffler et al., 1993). The high affinity of macitentan for tissue ET receptors could explain the 10 times lower dose needed to achieve a similar increase of plasma ET-1 concentration compared with bosentan.

Pharmacokinetic experiments in rat show that macitentan has a long half-life (2 h) and is metabolized to ACT-132577. This major metabolite presents a longer half-life (2 days) and at pharmacologically active dose (10 mg/kg) of macitentan reaches a plasma concentration (7.9 μM) greater than that of macitentan (2.6 μM) and higher than inhibitory concentrations observed in natural cell lines, suggesting a significant contribution of the metabolite to the overall effect of macitentan. The plasma concentrations reached by the two compounds after macitentan administration are also able to block both receptors in vivo since they are above the inhibitory concentrations obtained on isolated aortic (ET_A) and tracheal (ET_B) preparations.

In vitro, macitentan and ACT-132577 both inhibit ET-1-mediated intracellular increase of calcium in smooth muscle cells and fibroblasts. Ex vivo experiments on isolated aortic and tracheal preparations indicated that both macitentan and ACT-132577 block ET_A and ET_B receptors. Thus, despite a lower potency in vitro compared with its parent compound, ACT-132577, following macitentan oral administration, accumulates and may participate to ET receptor antagonism of macitentan in vivo.

Although the respective contribution of an improved tissue penetration and dual ET receptor antagonism remains diffi-

cult to evaluate, acute and chronic studies in experimental models of systemic and pulmonary hypertension and diabetes mellitus confirmed that the combination of these two properties translates into high potency and beneficial long-term effects on end-organ damage and survival. In hypertensive rats, macitentan was 10 times more potent than bosentan in reducing arterial blood pressure and showed a trend toward higher efficacy in terms of maximal effect on blood pressure reduction compared with bosentan (-26 ± 4 versus 20 ± 5 mm Hg, N.S., respectively). As evidenced by the area between the curves of both compounds, the pharmacological activity of macitentan is superior to that of bosentan despite similar half-lives (2 and 3 h, respectively) for the two molecules. This difference might be attributed to tissue targeting of macitentan and to the longer half-life (8.4 h) of ACT-132577, the pharmacologically active metabolite of macitentan. In monocrotaline rats, macitentan fully prevented right ventricle hypertrophy and the development of pulmonary hypertension at a 10 times lower dose than bosentan (30 versus 300 mg/kg/day, respectively). Macitentan also decreased mortality in this animal model, in line with previous data confirming that dual blockade of ET receptors can improve survival (Mulder et al., 1997; Jasmin et al., 2001; Clozel et al., 2006). Macitentan was tested in a rat model of type I diabetes to assess its impact on end-organ damage. Preclinical and clinical data suggest that ET-1 is involved in the pathophysiology of diabetic nephropathy (Schrijvers et al., 2004) and retinopathy (Chakrabarti et al., 2000). Urinary ET-1 levels correlate with the severity of nephropathy in diabetic patients (Lee et al., 1994) and glomerular ET-1, not its receptors, is increased in STZ rats (Fukui et al., 1993). Accumulating evidence points to a role for ET-1 in renal cells proliferation and matrix turnover since ET-1 closely interacts with several growth factors such as transforming growth factor- β and VEGF (Schrijvers et al., 2004; Masuzawa et al., 2006). Hence, ET-1 transgenic mice with only slightly elevated tissue ET-1 concentrations develop glomerulosclerosis, interstitial fibrosis, renal cysts, and a progressive decline in glomerular filtration rate (Hochoer et al., 1997). Macitentan prevented end-organ damage in diabetic STZ-rats by markedly preventing renal vasoconstriction, renal vascular hypertrophy, and structural injury. This translated into a decrease of proteinuria and a higher glomerular filtration rate. These data are consistent with published data from our group indicating that bosentan also partially prevented the development of proteinuria and renal structural injury in a similar animal model of late overt diabetic nephropathy (Ding et al., 2003). Prevention of VEGF increase by macitentan in the retina of diabetic rats indicates potential antiedema activity since VEGF increases vascular permeability and is involved in early stage of retinal damage in this model (Cukiernik et al., 2004). Our results are also in line with a previous study in the same animal model, using another dual ET receptor antagonist, SB209670 (Masuzawa et al., 2006). Macitentan, by decreasing VEGF in diabetic rats, might therefore possess particular antiedema and antipermeability properties.

In conclusion, the efficacy of macitentan in different experimental models shows that targeting both ET receptors with a high tissue affinity is a promising therapeutic approach for the treatment of pathological disorders associated with tissue remodelling. Macitentan efficacy is partially due to the formation of an active, long-lasting, and dual ET receptor

antagonist ACT-132577. In vitro data have shown that macitentan and its metabolite are able to inhibit ET-1-mediated deleterious effects in various cell types. These properties translated into efficacy in experimental models of diabetes mellitus and pulmonary hypertension, in which macitentan prevented end-organ damage and improved survival. For these reasons, macitentan represents a new therapeutic approach in the management of pulmonary arterial hypertension and other diseases where the tissue ET system is activated.

Acknowledgments

We are grateful to Celia Mueller, Katalin Menyhart, Franck Haag, and Roland Ernst for expert technical assistance.

References

- Allen RI, Box KJ, Comer JE, Peake C, and Tam KY (1998) Multiwavelength spectrophotometric determination of acid dissociation constants of ionizable drugs. *J Pharm Biomed Anal* 17:699–712.
- Amiri F, Virdis A, Neves MF, Iglarz M, Seidah NG, Touyz RM, Reudelhuber TL, and Schiffrin EL (2004) Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation* 110:2233–2240.
- Böhm F, Ahlborg G, Johansson BL, Hansson LO, and Pernow J (2002) Combined endothelin receptor blockade evokes enhanced vasodilatation in patients with atherosclerosis. *Arterioscler Thromb Vasc Biol* 22:674–679.
- Breu V, Ertel SI, Roux S, and Clozel M (1998) The pharmacology of bosentan. *Expert Opin Investig Drugs* 7:1173–1192.
- Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, Bruns C, Prieschl E, Baumruker T, Hiestand P, et al. (2002) The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* 277:21453–21457.
- Cardillo C, Campia U, Bryant MB, and Panza JA (2002) Increased activity of endogenous endothelin in patients with type II diabetes mellitus. *Circulation* 106:1783–1787.
- Cardillo C, Kilcoyne CM, Cannon RO 3rd, and Panza JA (2000) Interactions between nitric oxide and endothelin in the regulation of vascular tone of human resistance vessels in vivo. *Hypertension* 35:1237–1241.
- Cardillo C, Kilcoyne CM, Wacławski M, Cannon RO 3rd, and Panza JA (1999) Role of endothelin in the increased vascular tone of patients with essential hypertension. *Hypertension* 33:753–758.
- Chakrabarti S, Cukiernik M, Hileto D, Evans T, and Chen S (2000) Role of vasoactive factors in the pathogenesis of early changes in diabetic retinopathy. *Diabetes Metab Res Rev* 16:393–407.
- Clozel M and Gray GA (1995) Are there different ETB receptors mediating constriction and relaxation? *J Cardiovasc Pharmacol* 26 (Suppl 3):S262–S264.
- Clozel M, Hess P, Rey M, Iglarz M, Binkert C, and Qiu C (2006) Bosentan, sildenafil, and their combination in the monocrotaline model of pulmonary hypertension in rats. *Exp Biol Med (Maywood)* 231:967–973.
- Clozel M, Ramuz H, Clozel JP, Breu V, Hess P, Löffler BM, Coassolo P, and Roux S (1999) Pharmacology of tezosentan, new endothelin receptor antagonist designed for parenteral use. *J Pharmacol Exp Ther* 290:840–846.
- Cukiernik M, Hileto D, Evans T, Mukherjee S, Downey D, and Chakrabarti S (2004) Vascular endothelial growth factor in diabetes induced early retinal abnormalities. *Diabetes Res Clin Pract* 65:197–208.
- Ding SS, Qiu C, Hess P, Xi JF, Zheng N, and Clozel M (2003) Chronic endothelin receptor blockade prevents both early hyperfiltration and late overt diabetic nephropathy in the rat. *J Cardiovasc Pharmacol* 42:48–54.
- Fukui M, Nakamura T, Ebihara I, Osada S, Tomino Y, Masaki T, Goto K, Furuichi Y, and Koide H (1993) Gene expression for endothelins and their receptors in glomeruli of diabetic rats. *J Lab Clin Med* 122:149–156.
- Gregan B, Jürgensen J, Papsdorf G, Furkert J, Schaefer M, Beyer mann M, Rosenthal W, and Oksche A (2004) Ligand-dependent differences in the internalization of endothelin A and endothelin B receptor heterodimers. *J Biol Chem* 279:27679–27687.
- Haug C, Schmid-Kotsas A, Zorn U, Schuett S, Gross HJ, Gruenert A, and Bachem MG (2001) Endothelin-1 synthesis and endothelin B receptor expression in human coronary artery smooth muscle cells and monocyte-derived macrophages is up-regulated by low density lipoproteins. *J Mol Cell Cardiol* 33:1701–1712.
- Hochoer B, Schwarz A, Fagan KA, Thöne-Reineke C, El-Hag K, Kusserow H, Elitok S, Bauer C, Neumayer HH, Rodman DM, et al. (2000) Pulmonary fibrosis and chronic lung inflammation in ET-1 transgenic mice. *Am J Respir Cell Mol Biol* 23:19–26.
- Hochoer B, Thöne-Reineke C, Rohmeiss P, Schmager F, Slowinski T, Burst V, Siegmund F, Quertemous T, Bauer C, Neumayer HH, et al. (1997) Endothelin-1 transgenic mice develop glomerulosclerosis, interstitial fibrosis, and renal cysts but not hypertension. *J Clin Invest* 99:1380–1389.
- Jasmin JF, Lucas M, Cernacek P, and Dupuis J (2001) Effectiveness of a nonselective ET(A/B) and a selective ET(A) antagonist in rats with monocrotaline-induced pulmonary hypertension. *Circulation* 103:314–318.
- Kakoki M, Hirata Y, Hayakawa H, Tojo A, Nagata D, Suzuki E, Kimura K, Goto A, Kikuchi K, Nagano T, et al. (1999) Effects of hypertension, diabetes mellitus, and hypercholesterolemia on endothelin type B receptor-mediated nitric oxide release from rat kidney. *Circulation* 99:1242–1248.
- Lee YJ, Shin SJ, and Tsai JH (1994) Increased urinary endothelin-1-like immuno-

- reactivity excretion in NIDDM patients with albuminuria. *Diabetes Care* **17**:263–266.
- Löffler BM, Breu V, and Clozel M (1993) Effect of different endothelin receptor antagonists and of the novel non-peptide antagonist Ro 46-2005 on endothelin levels in rat plasma. *FEBS Lett* **333**:108–110.
- Lund W (1994) The Pharmaceutical Codex: Principles and Practice of Pharmaceutics, 12th ed., pp 70–71, The Pharmaceutical Press, London, UK.
- Masuzawa K, Jesmin S, Maeda S, Zaedi S, Shimojo N, Miyauchi T, and Goto K (2006) Effect of endothelin dual receptor antagonist on VEGF levels in streptozotocin-induced diabetic rat retina. *Exp Biol Med (Maywood)* **231**:1090–1094.
- Mulder P, Boujedaini H, Richard V, Henry JP, Renet S, Münster K, and Thuillez C (2002) Long-term survival and hemodynamics after endothelin-a receptor antagonism and angiotensin-converting enzyme inhibition in rats with chronic heart failure: monotherapy versus combination therapy. *Circulation* **106**:1159–1164.
- Mulder P, Richard V, Derumeaux G, Hogue M, Henry JP, Lallemand F, Compagnon P, Mace B, Comoy E, Letac B, et al. (1997) Role of endogenous endothelin in chronic heart failure: effect of long-term treatment with an endothelin antagonist on survival, hemodynamics, and cardiac remodeling. *Circulation* **96**:1976–1982.
- Muller DN, Mervaala EM, Schmidt F, Park JK, Dechend R, Genersch E, Breu V, Löffler BM, Ganten D, Schneider W, et al. (2000) Effect of bosentan on NF-kappaB, inflammation, and tissue factor in angiotensin II-induced end-organ damage. *Hypertension* **36**:282–290.
- Porter KE, Olojugba DH, Masood I, Pemberton M, Bell PR, and London NJ (1998) Endothelin-B receptors mediate intimal hyperplasia in an organ culture of human saphenous vein. *J Vasc Surg* **28**:695–701.
- Rossi G, Albertin G, Belloni A, Zanin L, Biasolo MA, Prayer-Galetti T, Bader M, Nussdorfer GG, Palù G, and Pessina AC (1994) Gene expression, localization, and characterization of endothelin A and B receptors in the human adrenal cortex. *J Clin Invest* **94**:1226–1234.
- Sauvageau S, Thorin E, Caron A, and Dupuis J (2007) Endothelin-1-induced pulmonary vasoreactivity is regulated by ET(A) and ET(B) receptor interactions. *J Vasc Res* **44**:375–381.
- Schrijvers BF, De Vriese AS, and Flyvbjerg A (2004) From hyperglycemia to diabetic kidney disease: the role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines. *Endocr Rev* **25**:971–1010.
- Serneri GG, Cecioni I, Vanni S, Paniccia R, Bandinelli B, Vetere A, Janming X, Bertolozzi I, Boddi M, Lisi GF, et al. (2000) Selective upregulation of cardiac endothelin system in patients with ischemic but not idiopathic dilated cardiomyopathy: endothelin-1 system in the human failing heart. *Circ Res* **86**:377–385.
- Shi-Wen X, Denton CP, Dashwood MR, Holmes AM, Bou-Gharios G, Pearson JD, Black CM, and Abraham DJ (2001) Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1. *J Invest Dermatol* **116**:417–425.
- Teerlink JR, Carteaux JP, Sprecher U, Löffler BM, Clozel M, and Clozel JP (1995) Role of endogenous endothelin in normal hemodynamic status of anesthetized dogs. *Am J Physiol* **268**:H432–H440.
- Verhaar MC, Strachan FE, Newby DE, Cruden NL, Koomans HA, Rabelink TJ, and Webb DJ (1998) Endothelin-A receptor antagonist-mediated vasodilatation is attenuated by inhibition of nitric oxide synthesis and by endothelin-B receptor blockade. *Circulation* **97**:752–756.
- Wagner OF, Christ G, Wojta J, Vierhapper H, Parzer S, Nowotny PJ, Schneider B, Waldhäusl W, and Binder BR (1992) Polar secretion of endothelin-1 by cultured endothelial cells. *J Biol Chem* **267**:16066–16068.
- Yang LL, Gros R, Kabir MG, Sadi A, Gotlieb AI, Husain M, and Stewart DJ (2004) Conditional cardiac overexpression of endothelin-1 induces inflammation and dilated cardiomyopathy in mice. *Circulation* **109**:255–261.
- Yang Z, Krasnic N, and Lüscher TF (1999) Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ETA and ETB receptor blockade. *Circulation* **100**:5–8.
- Yokomori H, Oda M, Yasogawa Y, Nishi Y, Ogi M, Takahashi M, and Ishii H (2001) Enhanced expression of endothelin B receptor at protein and gene levels in human cirrhotic liver. *Am J Pathol* **159**:1353–1362.

Address correspondence to: Dr. Martine Clozel, Drug Discovery Department, Actelion Pharmaceuticals Ltd., Gewerbestrasse 16, CH-4123 Allschwil, Switzerland. E-mail: martine.clozel@actelion.com
