PHARMACOLOGY OF MACITENTAN, AN ORALLY ACTIVE
TISSUE TARGETING DUAL ENDOTHELIN RECEPTOR
ANTAGONIST

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Abbreviations: ET, endothelin; IL, interleukin; TNF, tumor necrosis factor; INF, interferon; PH, pulmonary hypertension; CHO, Chinese hamster ovary; MCT, monocrotaline; MPAP: mean pulmonary arterial pressure; BW, body weight; RV, right ventricle; LV, left ventricle; S, septum; STZ, streptozotocin; TGF, transforming growth factor; VEGF, vascular endothelial growth factor

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ABSTRACT

Macitentan, also called Actelion-1 or ACT-064992, (N-[5-(4-Bromophenyl)-6-(2-(5-bromopyrimidin-2-yl)oxy)pyrimidin-4-yl]-N’-propylaminosulfonamide) is a new dual ET₄/ET₆ 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 ET₄ and ET₆ 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 (ET₄ receptors) and sarafotoxin S6c-induced contractions in isolated rat trachea (ET₆ receptors). In rats with pulmonary hypertension, macitentan prevented both the increase of pulmonary pressure and the right ventricle hypertrophy, and 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.
INTRODUCTION

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 (Hocher et al., 2000). Of the ET-1 produced by endothelial cells, 80% is secreted basolaterally towards 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 (Hocher et al., 1997; Hocher et al., 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 (ETB only) (Haug et al., 2001), vascular smooth muscle cells (Haug et al., 2001), fibroblasts (Shi-Wen et al., 2001), cardiomyocytes (Serneri et al., 2000), adrenal cells (Rossi et al., 1994) and stellate cells (ETA and ETB) (Yokomori et al., 2001).

In certain pathological situations, there is increased expression of both tissue ET-1 and ET receptors. This upregulation explains why the ET system plays a role in pathology
more than in physiology, and why ET receptor antagonists have so 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<sub>A</sub>/ET<sub>B</sub> ratio (Kakoki et al., 1999). In these pathological situations, both ET<sub>A</sub> and ET<sub>B</sub> receptors mediate the detrimental actions of ET-1, and dual blockade of both receptors may be necessary. Indeed, a cross-talk between ET<sub>A</sub> and ET<sub>B</sub> 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<sub>A</sub> and ET<sub>B</sub> receptors is likely to contribute to this cross-talk (Gregan et al., 2004). Accordingly, blocking of both ET<sub>A</sub> and ET<sub>B</sub> 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<sub>A</sub> and ET<sub>B</sub> receptor antagonist, but not by a selective ET<sub>A</sub> receptor antagonist (Mulder et al., 1997; Mulder et al., 2002; Yang et al., 2004). Survival of rats with monocrotaline-induced pulmonary hypertension is also prolonged by a dual ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist, but not by a selective ET<sub>A</sub> receptor antagonist (Jasmin et al., 2001).

Our goal was therefore 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<sub>A</sub> and ET<sub>B</sub> receptors and possessing a high partition coefficient as indicator of strong affinity for the tissues. Macitentan, also called Actelion-1 or ACT-064992 or (N-[5-(4-Bromophenyl)-6-(2-(5-bromopyrimidin-2-
xyloxy)ethoxy)pyrimidin-4-yl]-N’-propylaminosulfonamide) (Figure 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 which contributes to its pharmacological activity. We describe here the general pharmacology of macitentan, its pharmacokinetics, and its ability to protect against end-organ damage and improve survival in animal models.
METHODS

Physicochemical characterization and pharmacokinetics of macitentan

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

Distribution coefficient: logD. 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). Briefly, 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 minutes 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. LogD determination was similarly performed for bosentan and ambrisentan, (a dual ET_{A/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 i.v. 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 (LC-MS/MS) after plasma protein precipitation.
In vitro binding and functional assays

**Binding assay.** The cDNAs for human ET\textsubscript{A} and ET\textsubscript{B} receptors were cloned, sequenced and stably overexpressed in Chinese hamster ovary (CHO) cells. Membranes were prepared from these cells as previously described (Clozel et al., 1999). The competition binding assay was performed in 200 μl 50 mM Tris/HCl buffer, pH 7.4, including 25 mM MnCl\textsubscript{2}, 1 mM EDTA, 0.5% (w/v) BSA (fraction V), and 50 μg/ml AEBSF (Pefablock, Sigma A-8456) in polypropylene microtiter plates. Membranes containing 0.5 μg protein (ET\textsubscript{A}) or 0.2 μg protein (ET\textsubscript{B}) were incubated for 2 h at room temperature with 16 pM \textsuperscript{125}I-ET-1 (8000 cpm) and increasing concentrations of unlabelled test compounds. Maximum and minimum binding was determined in samples without and with 100 nM unlabeled ET-1 (Bachem H-6995), respectively. After 2 h of incubation, the membranes were filtered onto filterplates containing GF/C filters (Unifilterplates from Canberra Packard S.A. Zürich, Switzerland). To each well, 50 μl of scintillation cocktail was added (MicroScint 20, Canberra Packard S.A. Zürich, Switzerland) and the filter plates counted in a microplate counter (TopCount, Canberra Packard S.A. Zürich, Switzerland). Test compounds were dissolved, diluted and added to the assay in DMSO. The final concentration of DMSO in the assay was 2.5%, which was found not to interfere with the binding. IC\textsubscript{50} was calculated as the concentration of antagonist inhibiting 50% of the specific binding of ET-1.

**Inhibition of intracellular calcium mobilization.** hPASMC (primary human pulmonary arterial smooth muscle cells, Cambrex) were grown in the supplied medium for at least 7 days before trypsinization and plating into gelatin-coated 384-well FLIPR (Fluorometric Imaging Plate Reader, Molecular Devices, USA) plates at a density of 10,000 cells/well.
The rat aortic smooth muscle cell line (A10) was grown in DMEM/20% FBS/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 DMEM/10% FBS/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 over night, the medium was discarded and cells were incubated with 25 μl of dye buffer (3 μM fluo4-AM, 0.0375% NaHCO₃, 20 mM HEPES, 5 mM probenecid in HBSS, pH 7.4). After 60 min incubation at 37°C, cells were washed 3 times with 50 μl of wash buffer (0.0375% NaHCO₃, 20 mM HEPES, 2.5 mM probenecid, 0.1% BSA in HBSS, pH 7.4), supplemented with 50 μl 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 μM and the IC₅₀ 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 μM). Endothelium denudation was assessed by the absence of relaxation to acetylcholine (10 μM). 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 to 1 μM) or ACT-132577 (0.3 to 3 μM). 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 and 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 to 30 μM) or ACT-132577 (10 to 70 μM), 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 represent the geometric mean (Brinkmann et al., 2002) of at least 3 independent experiments. For isolated organs tests, 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 (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 pKB value were used as indices of functional inhibitory potency.
In vivo pharmacology

Animals. Normotensive male Wistar rats were delivered from RCC Ltd (Biotechnology and Animal Breeding Division 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 hours 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 hours 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, 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 DOCA (deoxycorticosterone acetate, 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 Ketamin-HCl (Ketavet, Parke-Davis, Berlin FRG) and 10 mg/kg xylazin (Rompun, Bayer, Leverkusen, Germany) administered intraperitoneally. The pressure transmitter was implanted into the peritoneal cavity under aseptic conditions and a sensing catheter
(model TA11PA-C40) 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). A receiver platform (RPC-1, Data Sciences) connected the radio signal to digitized input that was sent to a dedicated personal computer (Dell, Optiplex, GX270). 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).

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 gelatine 7.5% in 6 to 9 rats. Vehicle (gelatine solution 7.5%) was also tested. Data were collected every 5 minutes. Hourly means of blood pressure were calculated. Each rat served as its own control, with the blood pressure data of the last 24 hours before treatment taken as reference. Efficacy was measured by both the maximal reduction in blood pressure and the measurement of the area between curves (ABC). ABC was calculated using the hourly means of blood pressure between data collected 24 hours prior to administration of the compound and the data collected during the treatment period. Mean arterial blood pressure was expressed in millimeters of mercury (mmHg).

**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 per dose) or bosentan (10-300 mg/kg/day in food...
admix, n=14-15 per dose) were initiated immediately after subcutaneous monocrotaline (MCT) injection (60 mg/kg, Sigma Chemicals, St. Louis, MO, USA) 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 treatment phase, the rats were anesthetized by intraperitoneal injection of 100 mg/kg thiobutabarbitral-Na (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 inserted in the pulmonary artery via the right jugular vein for measurement of MPAP (Clozel et al., 2006). Measurements were recorded for 15 minutes 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 (BW) 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 3 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 served as control.
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 one-week adaptation period, all male Wistar rats were anesthetized with a mixture of Ketamine-Rompun (50 mg/kg -5mg/kg, i.p.) and the right kidney was removed in order to hasten the development of diabetic nephropathy. Two weeks later, the rats were made diabetic by intravenous injection of streptozotocin (STZ, Sigma Chemical Co., St. Louis, MO, USA) (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 per group) was initiated immediately after STZ injection, for a duration of 24 weeks. The rats were placed in individual metabolic cages for 24-hour urine collection every 4 weeks. Total 24-hour urinary protein was measured using the Bradford assay (Bio-Red, Hercules, CA, USA), and bovine serum albumin was used to generate the standard curve.

After 24 weeks of treatment, a renal clearance experiment was conducted as previously described (Ding et al., 2003). Briefly, 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, AD Instruments, Hastings, UK) for recording of arterial blood pressure (BP) and periodic sampling of blood. During surgery, the rat received an intravenous infusion of 1 ml synthetic plasma and a 0.5 ml bolus of 0.9 % NaCl containing 40 mg/ml inulin (Sigma Chemicals, St. Louis, MO, USA) and p-
aminohippurate (0.5%, Merck Sharp & Dohme, West Point, PA, USA). Subsequently, a continuous intravenous infusion of 0.9% NaCl containing the same concentrations of inulin and p-aminohippurate at a rate of 40 μl/min was initiated. After a 45-minute equilibration period, renal clearance experiments were begun in which 2 consecutive 20-minute 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 μm thickness were stained with hematoxillin 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 previously described (Ding et al., 2003). Briefly, to assess glomerulosclerosis, 50 glomeruli in each kidney were observed at x400 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%; 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 to 3): grade 0, normal; 1, lesions involving < 25%; 2, lesions involving 25-50%; 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 mm x 2 mm area of retina above the optic nerve was cut, homogenized in phosphate buffer and centrifuged at 12000 rpm. The concentration of VEGF in the supernatant was measured by ELISA kit (Shanghai Senxiong BioTech Ltd).

**Expression of results**

Results are expressed as mean ± S.E.M. ANOVA for repeated measures and Dunnett's test was 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 (ANOVA) using Statistica (StatSoft, Berikon, Switzerland) and Student-Newman-Keuls procedure for multiple comparisons. Survival analysis was performed with a log-rank test.

**Compounds**

$^{125}$I-labeled ET-1 tracer was obtained from ANAWA Trading SA, Wangen, Switzerland. ET-1 and sarafotoxin S6c were obtained from Bachem (Bubendorf, Switzerland). Test compounds (macitentan, ACT-132577, bosentan and ambrisentan) were synthesized by Actelion Pharmaceuticals Ltd.
RESULTS

Physicochemical characterization and pharmacokinetics

A distribution coefficient (logD) favoring partition to lipids and a high pKa value reflecting a high non-ionized fraction were used as markers of high affinity for lipidic milieu and tissue targeting. Macitentan exhibits a pKa of 6.2 and consequently a relatively high percentage of non-ionized form in an aqueous environment at physiological pH 7.4 when compared to 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 has also 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 presents a 40 and 2000-fold increased affinity for the lipid phase vs. bosentan and ambrisentan, respectively.

After intravenous administration, macitentan has a terminal half-life of 2 hours 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 (Figure 1 and 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 (AUC\textsubscript{0-24}) 4- to 5-fold higher than those of the parent compound, reaching plasma concentrations (C\textsubscript{max}) for the two molecules above the IC\textsubscript{50} 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 (CHO) 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 non-recombinant 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 around IC\(_{50}\) = 1 nM and the metabolite IC\(_{50}\) was around 10 fold higher when 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, vs. 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 ten-fold lower dose than with bosentan, indicating a more potent pharmacological activity in vivo (Figure 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 $-26 \pm 4$ mmHg at a dose of 10 mg/kg (ED$_{50}$, 1 mg/kg). For bosentan, the maximal reduction in mean arterial pressure was $-20 \pm 5$ mmHg and was achieved at a ten-times higher dose of 100 mg/kg (ED$_{50}$, 10 mg/kg) (Figure 3). Area between curves comparison indicated a better maximal efficacy of macitentan in terms of coverage of the blood pressure reduction (Figure 3). At the maximal effective dose, the duration of the blood pressure response to macitentan was approximately 40 hours, whereas with bosentan the response duration was 20 hours (data not shown). Thus, in the DOCA-salt rat model of hypertension, macitentan is 10-fold more potent than bosentan, presents a higher efficacy as shown by the trends towards 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/d for macitentan (Figure 4). At this dose, no effect on systemic mean arterial blood pressure was observed (105.6±3.1 mmHg vs. 103.9±4.6 in treated and untreated monocrotaline rats, respectively). The maximal efficacious dose of bosentan was 300 mg/kg/d. Chronic oral administration of macitentan
at 30 mg/kg/d significantly improved the 42-day survival in monocrotaline rats (83 % vs. 50% survival in macitentan vs. vehicle-treated rats, 66% reduction of mortality at 42 days, p<0.002, Figure 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 (Figure 6A), an increase of renal vascular resistance leading to decreased renal blood flow (Table 5), and increase of filtration fraction (0.35±0.01 vs 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 vs 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 (Figure 6A). Macitentan slightly decreased systemic blood pressure (Table 5) and had no effect on body weight (274±8 vs. 271±8 g in untreated diabetic rats). Three vehicle-treated rats died during the study versus one in the macitentan-treated group. At the retinal level, macitentan markedly prevented the increase in VEGF content observed in diabetic rats (Figure 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 pKa, resulting in an increased percentage of the non-ionized form of the molecule in aqueous milieu at physiological pH. Only the non-ionized form is able to cross cell membranes and an increased concentration gradient of the non-ionized molecule drives the net flux across lipophilic cell membranes. Furthermore, chemical modifications resulting in increased logD values also lead to increased affinity for the tissue as logD is considered to be an indicator of distribution between plasma and tissue.

Macitentan was also optimized for its potency and dual 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 non-selective vasodilation by stimulation of endothelial ET receptors, since their effect can be blocked by inhibition of nitric oxide synthase by L-
NAME (Cardillo et al., 2000), but that in pathology the upregulation of contractile SMC 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 upregulated 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 (Bohm 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 (Loffler 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 as compared to bosentan.

Pharmacokinetic experiments in rat show that macitentan has a long half-life (2 hours) 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 \(\mu\)M) greater than that of macitentan (2.6 \(\mu\)M) 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\textsubscript{A}) and tracheal (ET\textsubscript{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\textsubscript{A} and ET\textsubscript{B} receptors. Thus, despite a lower potency in vitro compared to 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 difficult 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 that bosentan in reducing arterial blood pressure and showed a trend towards higher efficacy in terms on maximal effect on blood pressure reduction when compared to bosentan (-26±4 vs 20±5 mmHg, NS, 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 3h 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 vs. 300 mg/kg/d 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 point to a role for ET-1 in renal cells proliferation and matrix turnover since ET-1 closely interacts with several growth factors such as TGFβ 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 (Hocher 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 anti-edema 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 anti-edema and anti-permeability 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.
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FOOTNOTES

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LEGENDS FOR FIGURES

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

Figure 2: ET-1 plasma concentrations in conscious Wistar rats 6 hours after oral administration of macitentan and bosentan. Veh=Vehicle (n=4 per group). Data are presented as means±S.E.M. ***p<0.001 compared to vehicle-treated rats.

Figure 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 to 9 per group). Veh=Vehicle, MAP=Mean Arterial Blood Pressure. ABC=Area Between Curves. Data are presented as means±S.E.M. ***p<0.001 compared to bosentan-treated rats.

Figure 4: Dose-response relationship for the effect of 4-weeks 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). MPAP=Mean Pulmonary Arterial Pressure, RV=Right Ventricular weight, LV+S=Left Ventricular+Septal weight, Veh=Vehicle, MCT=Monocrotaline. Data are presented as means±S.E.M. +++p<0.001 compared to control rats. *p<0.05, **p<0.01, ***p<0.001 compared to monocrotaline + vehicle.
Figure 5: Effect of oral administration of macitentan on survival of Wistar rats with monocrotaline-induced pulmonary hypertension (n=30 per group, except control group (n=10)). MCT=Monocrotaline.

Figure 6: Effect of chronic administration of macitentan on (A) proteinuria and (B) retinal VEGF content in streptozotocin-induced diabetic rats (n=14 per group). STZ=Streptozotocin. Data are presented as means±S.E.M. *p<0.05, **p<0.01, ***p<0.001 compared to control rats. *p<0.05, **p<0.01, ***p<0.001 compared to streptozotocin rats + vehicle.
**Table 1**: Physicochemical parameters of macitentan, bosentan and ambrisentan

<table>
<thead>
<tr>
<th></th>
<th>Distribution coefficient D (n-Octanol/Aqueous buffer)</th>
<th>LogD</th>
<th>pKa</th>
<th>non-ionized form at pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macitentan</td>
<td>800 / 1</td>
<td>2.9</td>
<td>6.2</td>
<td>6%</td>
</tr>
<tr>
<td>Bosentan</td>
<td>20 / 1</td>
<td>1.3</td>
<td>5.1</td>
<td>1%</td>
</tr>
<tr>
<td>Ambrisentan</td>
<td>1 / 2.5</td>
<td>-0.4</td>
<td>3.5</td>
<td>0.01%</td>
</tr>
</tbody>
</table>
Table 2: Pharmacokinetic parameters of macitentan and ACT-132577 in male rats after single and multiple administration of macitentan.

<table>
<thead>
<tr>
<th></th>
<th>Single dose</th>
<th></th>
<th>Multiple dose (26 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose i.v.</td>
<td>Half-life</td>
<td>Vd</td>
</tr>
<tr>
<td></td>
<td>(mg/kg)</td>
<td>(h)</td>
<td>(l/kg)</td>
</tr>
<tr>
<td>Macitentan</td>
<td>0.5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>ACT-132577</td>
<td>8.4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Vd: Volume of distribution, C<sub>max</sub>: Maximal plasma concentration, AUC<sub>0-24</sub>: Area Under the Curve (0 to 24h).
Table 3: Inhibition of ET-1-induced intracellular Ca^{2+} increase in 3 different non-recombinant cell types.

<table>
<thead>
<tr>
<th>IC_{50} (nM)</th>
<th>hPASMC</th>
<th>rASMC</th>
<th>m3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macitentan</td>
<td>0.90</td>
<td>0.80</td>
<td>1.84</td>
</tr>
<tr>
<td>ACT-132577</td>
<td>14.0</td>
<td>5.80</td>
<td>10.9</td>
</tr>
</tbody>
</table>

hPASMC: primary human Pulmonary Arterial Smooth Muscle Cells, rASMC: rat Aortic Smooth Muscle Cell Line, m3T3: mouse fibroblast cell line.
Table 4: Inhibition (pA₂) 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.

<table>
<thead>
<tr>
<th>pA₂</th>
<th>Aorta (ETₐ)</th>
<th>Trachea (ETₐ)</th>
<th>ETₐ/ETₐ selectivity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macitentan</td>
<td>7.6</td>
<td>5.9</td>
<td>50/1</td>
</tr>
<tr>
<td>ACT-132577</td>
<td>6.7</td>
<td>5.5*</td>
<td>16/1</td>
</tr>
</tbody>
</table>

* pKₐ
Table 5: Effect of chronic administration of macitentan on hemodynamics and renal morphometry in diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>MAP</th>
<th>RBF</th>
<th>GFR</th>
<th>RVR</th>
<th>KW</th>
<th>GSA</th>
<th>GS</th>
<th>TIL</th>
<th>VL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmHg</td>
<td>ml/min/100g</td>
<td>ml/min/100g</td>
<td>mmHg/ml/min</td>
<td>g/100g</td>
<td>μm²x10³ (index)</td>
<td>(score)</td>
<td>(score)</td>
<td></td>
</tr>
<tr>
<td>CTL</td>
<td>116±2</td>
<td>2.89±0.10</td>
<td>0.58±0.03</td>
<td>4.3±0.2</td>
<td>0.24±0.02</td>
<td>9.8±0.1</td>
<td>0.01±0.01</td>
<td>-</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>STZ + vehicle</td>
<td>107±2 **</td>
<td>1.86±0.09 ***</td>
<td>0.64±0.03</td>
<td>11.0±0.7 ***</td>
<td>0.83±0.01 ***</td>
<td>17.3±0.1 ***</td>
<td>0.30±0.03 ***</td>
<td>0.67±0.07 ***</td>
<td>0.52±0.06 ***</td>
</tr>
<tr>
<td>STZ + macitentan</td>
<td>99±2 †</td>
<td>2.40±0.11 †††</td>
<td>0.75±0.03 †</td>
<td>7.9±0.3 †††</td>
<td>0.79±0.01</td>
<td>14.7±0.1 †††</td>
<td>0.14±0.02 †† †</td>
<td>0.41±0.04 †† †</td>
<td>0.25±0.03 †† †</td>
</tr>
</tbody>
</table>

CTL: Control, STZ: Streptozotocin, 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 to control rats. † p<0.05, †† † p<0.001 compared to streptozotocin rats + vehicle.
Figure 1

Macitentan

ACT-132577
Figure 2

ET-1 concentration (pg/ml)

Macitentan (mg/kg)

Bosentan (mg/kg)

Veh 0.1 0.3 1 3 10 30

*** *** ***

Veh 1 3 10 30 100 300

*** *** ***
Figure 3
Figure 4
Figure 5

![Graph showing survival (%) over time (days) for different groups: Control, MCT + vehicle, MCT + macitentan. The graph indicates a statistically significant difference (p = 0.002) between the MCT + macitentan group and the other two groups.](https://jpet.aspetjournals.org/content/jpet/113/4/142976.full.pdf)
Figure 6

A

- O Control
- ■ STZ + vehicle
- H STZ + macitentan

Proteinuria (mg/24hr)

Time (weeks)

0 6 12 18 24

0 50 100 150 200

B

VEGF concentration (pg/ml)

Control Vehicle Macitentan

0 100 200 300 400 500

+++ +++

+++ +++

+++ +++

+++ +++

+++ +++

+++ +++

+++ +++

+++ +++