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Novel Dual Endothelin Receptor Antagonist Macitentan Reverses Severe Pulmonary Arterial Hypertension in Rats

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Short Running title: Macitentan reverses severe PAH

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Abstract

The efficacy of ET receptor antagonist bosentan in patients with severe pulmonary arterial hypertension remains limited, partly because its higher doses for potential blockade of ET receptors have never been tested due to liver dysfunction. We hypothesized that rigorous blockade of ET receptors using the novel dual ET receptor antagonist macitentan would be effective in treating severe pulmonary arterial hypertension without major side effects in a preclinical model appropriately representing the human disorder. In normal rats, 30 mg/kg/day of macitentan completely abolished big ET-1-induced increases in RV systolic pressure. Adult male rats were injected with SU5416, a VEGF blocker, and exposed to hypoxia for 3 weeks and then to normoxia for an additional 5 weeks (total 8 weeks). In intrapulmonary arterial rings isolated from rats with severe pulmonary arterial hypertension, macitentan concentration-dependently inhibited ET-1-induced contraction. Long-term treatment with macitentan (30 mg/kg/day, from week 3 to 8) reversed the high RV systolic pressure with preserved cardiac output. Development of RV hypertrophy, luminal occlusive lesions and medial wall thickening were also significantly improved without increasing serum levels of liver enzymes by macitentan. In conclusion, efficacious blockade of ET receptors with macitentan would reverse severe pulmonary arterial hypertension without major adverse effects.

(199 words)

Key words: macitentan; pulmonary arterial hypertension; enthothelin; endothelin receptor antagonist; luminal occlusive lesions

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Introduction

Severe pulmonary arterial hypertension (PAH) is a fatal syndrome characterized by progressive narrowing of small pulmonary arteries and arterioles, which results in sustained increase in pulmonary arterial resistance and pressure.[1,2] Major contributors of pulmonary arterial narrowing are sustained vasoconstriction and fixed vascular remodeling.[3] Among many substances postulated in the pathogenesis of PAH, endothelin-1 (ET-1), one of the most potent vasoconstrictors, has been suggested to play a crucial role in the development of PAH.[4,5]

Endothelin receptor antagonists (ERAs) have been approved by the FDA for the treatment of PAH. Although it remains controversial whether dual or ET_A-selective receptor antagonism is better for the treatment of PAH, previous studies have suggested that a blockade of both receptors ET_A and ET_B is necessary to achieve optimal inhibition of pulmonary vasoconstriction and proliferation.[6,7] During the past decade, the conventional dual ERA bosentan has been described to delay clinical worsening of patients with moderate PAH.[8,9,10] However, the efficacy of bosentan alone, compared with epoprostenol, has been limited for the treatment of patients with severe PAH, partly because higher doses of bosentan that were reported to completely block ET receptors have never been tested due to their adverse effects including liver dysfunction.[8,9,11] In addition, any reversal effects of ERAs on severe PAH have not yet been tested in an appropriate preclinical model that closely simulates human PAH, because the major limitation of the frequently studied animal models of pulmonary hypertension (i.e., chronically hypoxic and monocrotaline-injected rats) is that they do not develop luminal

occlusive lesions.[11,12,13]

Macitentan, a novel dual ERA, presents sustained binding on ET receptors and a two-fold longer duration of action in comparison with bosentan.[14,15] Notably, macitentan induces little liver dysfunction, unlike bosentan.[16,17] In addition, it has been recently reported that acute administration with 30 mg/kg of macitentan further lowered pulmonary arterial pressure in monocrotaline-exposed rats with the maximal dose of bosentan (100 mg/kg).[18] We therefore hypothesized that efficacious blockade of ET receptors by macitentan could chronically reverse severe PAH in a preclinical model of occlusive PAH without major side effects including liver dysfunction.

We used the VEGF receptor blockade by SU5416 plus hypoxia/normoxia (SU/Hx/Nx) exposure in rats to establish a severe PAH model, which closely mimics human severe PAH histologically and hemodynamically.[19,20] To test our hypothesis, ET receptor blockade by macitentan was assessed by examining the inhibitory effects of macitentan on the big ET-1-induced increases in RV systolic pressure in catheterized normal rats and the ET-1-induced contraction in intrapulmonary arterial rings isolated from SU/Hx/Nx-exposed rats. Furthermore, we examined whether long-term treatment of PAH rats with macitentan would reverse high RV systolic pressure, RV hypertrophy and occlusive pulmonary arterial lesions without any major side effects including liver dysfunction.

Materials and Methods

Development of a Model of Pulmonary Artery Hypertension in Rats

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Kyushu University, Japan. Adult male Sprague-Dawley rats weighing 200-250 g were injected subcutaneously with SU5416 (20 mg/kg, Cayman Chemical, MI, USA) and exposed to hypoxia (10% O_2) for 3 weeks. They were then returned to normoxia (21% O_2) for an additional 5 weeks (total 8 weeks after SU5416 injection).[19]

Chemicals

SU5416 was suspended in CMC solution (0.5% [wt/vol] carboxymethylcellulose sodium, 0.9% [wt/vol] NaCl, 0.4% [vol/vol] polysorbate, 0.9% [vol/vol] benzyl alcohol in deionized water). Macitentan (provided by Actelion Pharmaceuticals Ltd. Allschwil, Switzerland) was dissolved in dimethyl sulfoxide for isometric tension study and in CMC solution for in vivo experiments. The stock solutions of ET-1 (0.1 mmol/L) and big ET-1 (0.1 mmol/L) were prepared in 0.1% acetic acid, and they were appropriately diluted in physiological saline solution (PSS) or water in the final use. The final concentration of acetic acid was lower than 0.0001%. ET-1 and big ET-1 were purchased from Peptide institute, inc. Osaka, Japan. All other chemicals/reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Tension Measurement with Isolated Intrapulmonary Arterial Rings

Rings of the fourth to fifth branch intrapulmonary artery were prepared from PAH rats at

3 weeks after SU5416 injection, as previously described.[21] Briefly, rats were heparinized (100 IU) and then euthanized by intravenous injection of an overdose of sodium pentobarbital (30 mg/kg, IP). The heart and lungs were immediately removed and were kept in normal PSS. Intrapulmonary arteries at sizes ranging from 200 to 300 μm in diameter were carefully isolated from the right lower lobe and left lobe under observation with a microscope. Each intact ring was mounted to a pair of steel wires attached to a force transducer to measure isometric force development (Minebea, Nagano, Japan). The preparations were equilibrated in normal PSS at 37°C for at least 60 minutes before starting the experiment. During the 60-minute equilibration period, the rings were stimulated with 60 mmol/L K^+ every 15 minutes, and the resting load was increased in a stepwise manner until a final adjustment of 50 mg, which was the minimal load that produced maximal tension in response to 60 mmol/L K^+.

Hemodynamic Measurements in Catheterized Normal Rats

All rats were placed on a controlled heating pad after they were anesthetized with pentobarbital sodium (30 mg/kg, IP). Hemodynamic measurements were performed under normoxic conditions. Briefly, a polyvinyl catheter (PV-1, internal diameter: 0.28 mm) was inserted into the right ventricle (RV) via the right jugular vein for measurement of RV systolic pressure (RVSP).[22] RVSP was generally measured to estimate systolic pulmonary arterial pressure, because catheterization of pulmonary artery was often difficult in these extremely hypertensive (RVSP > 80 mmHg) rats.[22] A microtip P-V catheter (FTH-1912B-8018, Transonic Inc.) was inserted into the right carotid artery and advanced into the left ventricle (LV) as described previously.[23] The signals were

continuously recorded by ML880/9 PowerLab 16/30 (AD Instruments) with Science ASVantage 5.0 control unit (FY097B, Transonic Inc.) and a personal computer. RVSP, heart rate (HR), mean systemic arterial pressure (MSAP), and cardiac output (CO) were measured. Cardiac index (CI) was expressed as CO divided by body weight. Total pulmonary vascular resistance index (TPRI) was estimated by dividing RVSP by CI.[23] Although HRs were monitored to be consistent (>300 bpm), the possibility that pentobarbital had minor cardiovascular suppressive effects cannot be excluded.[24]

Experimental Protocols

Effects of Macitentan on Big ET-1 Induced Pulmonary Contraction in vivo

Intravenous injection of ET-1 (3 nmol/kg) caused airway constriction, which hindered stable measurement of RVSP. The vasoconstrictor effect of big ET-1 was reported to be dependent on the conversion to ET-1.[25,26] Furthermore, the previous in vivo studies utilized big ET-1 instead of ET-1 for the purpose similar to that of the present study.[11,27] Therefore, the efficacy of macitentan in blocking the ET receptors was evaluated by examining the effect of mactitentan on the big ET-1-induced elevation of RVSP. Normal rats were exposed to increasing doses of big ET-1 in the following groups; (1) vehicle alone (CMC), (2) 3 mg/kg/day macitentan and (3) 30 mg/kg/day macitentan. All animals were treated with macitentan once daily by gavage for 3 days, and hemodynamic analyses were performed at 8 hr after final administration when the plasma concentration of macitentan is maximally increased as previously reported.[28] Ten minutes after the baseline measurements, big ET-1 (0.1, 1 and 3 nmol/kg, diluted in 0.5 ml distilled water) was intravenously and cumulatively injected,, and hemodynamic parameters including

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RVSP and MSAP were continuously monitored (the effect of big ET-1 reached a plateau within 10 minutes).[11] The response to big ET-1 was expressed by a percentage of an increase in RVSP / baseline RVSP.

Isometric tension study

A contraction-response curve to ET-1 (0.1 to 100 nM) was determined in intact intrapulmonary arterial rings isolated from 3-week SU/Hx/Nx-exposed PAH rats. To determine the inhibitory effects of macitentan on ET-1-induced contraction, concentration-response curves to macitentan (0.1 nM to 10 μM) were examined in arterial rings precontracted with ET-1 (10 nM). The data were expressed as 10 nM ET-1 / 60 mM K^+-induced contractions (mg) × 100 (%).[29]

Reversal Protocol of Macitentan on Established PAH

Rats were treated with macitentan (3 or 30 mg/kg/day) by gavage from week 3 to 8 after SU5416 injection. Hemodynamic measurements were performed at week 0, 3 and 8. Macitentan was stopped 24 hrs before hemodynamic measurements. At the end of each hemodynamic study, blood samples were taken directly from carotid arteries in 8-week PAH rats with and without macitentan. The levels of serum enzymes such as total bilirubin and transaminases indicating liver function were measured (SRL Co, Fukuoka, Japan). The rats were thereafter euthanized by an overdose of pentobarbital sodium, and the hearts were excised. The weight ratio of right ventricle / left ventricle + septum (RV / LV+S) was obtained for each animal as an indication of RV hypertrophy. The lungs were inflated with 1 % formalin plus 0.5 % agarose at 20 cm H_2O pressure, and fixed in 10 %

formalin overnight.[19,20] The left lobe was blocked and paraffin embedded.

Morphological and histological analyses

Luminal occlusive lesions: All small pulmonary arteries (more than 30 vessels per section in a whole left lobe cross section including the hilum, outer diameter < 100 μm) were randomly evaluated by at least 3 investigators who were unaware of the source of the sections. Vessels were assessed for occlusive lesions on Verhoeff–van Gieson stained slides and scored as: no evidence of neointimal formation (Grade 0), partial luminal occlusion (< 50%, Grade 1) and severe-luminal occlusion ((=>50%, Grade 2). Pulmonary artery (PA) occlusion rate was expressed as percentage of each grade. [20]

Medial wall thickening: In each tissue section all circular muscular arteries (outer diameter between 50 and 100 μm were analyzed at x 400 magnification (vessels vessels < 50 μm were excluded because most normal PA of this size are non-muscular and two elastic laminae could not be identified). Medial wall thickening (MWT) was evaluated as medial thickness/outer diameter x 100 (%) as described previously.[20]

Plasma Concentration of Macitentan

As mentioned above, the concentration of macitentan has been reported to reach a peak at 8 hrs after oral administration.[30] We therefore collected each blood sample 8 hr after the last administration in normal rats that had received macitentan (30 mg/kg, once daily by gavage) for 3 days, when the plasma concentration of macitentan was considered to be stable. The blood samples were collected directly from the LV of rats euthanized by

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intravenous injection of an overdose of sodium pentobarbital. Plasma concentrations of macitentan and its active metabolite ACT-132577 were measured by a high performance liquid chromatography(HPLC) method.[28]

Statistical Analysis

Values are means ± SEM. Comparisons between groups were made with Student's *t* test or ANOVA with Scheffe's post hoc test for multiple comparisons. Differences were considered significant at $P<0.05$.

Results

Effects of Macitentan on Big ET-1-induced Increases in RVSP in Vivo

In normal rats, 30 mg/kg/day macitentan, compared with 3 mg/kg/day, completely inhibited the increase in RVSP induced by 3 nmol/kg big ET-1 (Figure 1), suggesting that this dose of macitentan can completely antagonize ET receptors to abolish pulmonary contraction induced by big ET-1 in vivo. HR was not significantly changed in each group (data not shown).

Inhibitory Effects of Macitentan on ET-1 Contraction of Intrapulmonary Arterial Rings Isolated from SU/Hx/Nx-Exposed PAH Rats

ET-1 concentration-dependently contracted intact intrapulmonary arteries isolated from PAH rats at 3 weeks after SU5416 injection (Figure 2A). In addition, the contraction-response curve to ET-1 was significantly shifted rightward by 1 and 10 μmol/L macitentan. Ten nmol/L ET-1 induced a submaximal contraction, which was 73 % of maximal contraction obtained with 100 nM ET-1. Ten nM ET-1 was therefore used to evaluate the inhibitory effect of macitentan on ET-1-induced contraction. Macitentan concentration-dependently inhibited ET-1-induced contraction (Figure 2B). The significant inhibitory effect of macitentan was obtained with 0.1 nmol/L and higher concentrations. These effects of macitentan appear to be selective to ET receptors blockade, as 10 μM macitentan had no significant effect on the contraction induced by 60 mM K^+ (data not shown).

Reversal Effects of Macitentan on Pulmonary Hypertension in SU/Hx/Nx-Exposed Rats

Compared to the normal rats, SU/Hx/Nx-exposed rats developed PAH as reflected in RVSP at the 3- and 8-week time points (Figure 3A). Five-week treatment with macitentan (30 mg/kg/day, from week 3 to 8) reversed the high RVSP to the normal level. CI was significantly decreased in 8-week SU/Hx/Nx-exposed rats, compared with normal and 3-week SU/Hx/Nx-exposed rats (Figure 3B). Macitentan completely reversed the decreased CI to the normal level. TPRI was substantially increased in 3-week and 8-week SU/Hx/Nx-exposed rats as compared with normal rats (Figure 3C). This increase in TPRI was greatly inhibited by macitentan treatment. Macitentan had no effect on MSAP and HRs (Figures 3D and 3E). Macitentan also reversed the RV hypertrophy (Figure 3F). In contrast, treatment with a lower dosage of macitentan at 3 mg/kg/day caused no significant effects on both RVSP and RV hypertrophy (120 ± 4 vs. 103 ± 11 mmHg in RVSP, 0.68 ± 0.05 vs. 0.61 ± 0.08 in RV/LV+S, n=6 vs. 3, $P > 0.05$, 8-week SU/Hx/Nx vs 3 mg/kg/day of macitentan).

Improvement of Occlusive Lesions and Medial Wall Thickening by Macitentan in SU/Hx/Nx-Exposed Rats

At 3- and 8-weeks after SU5416 injection, the fraction of Grade 2 occlusive lesions in small pulmonary arteries (OD =< 50μm) progressively increased from 0% (normal) to 16% (week 3) and 44% (week 8) (Figures 4A and 4C). Treatment with 30 mg/kg/day of macitentan significantly inhibited the progression of the occlusive lesions, and the fraction of Grade 2 occlusive lesions observed with macitentan at week 8 (18%) was

similar to that seen without macitentan at week 3. In larger pulmonary arteries (OD: 50 – 100 μm), no Grade 2 occlusive lesions were observed at week 3 and 8, and Grade 1 occlusive lesions were only modestly observed (Figures 4B and 4D). However, there were no significant difference in the fraction of Grade 1 occlusive lesions between week 3 and week 8, and the treatment with macitentan had no significant effect on the occlusive lesions in larger pulmonary arteries. In addition, 3 mg/kg/day of macitentan showed a moderate but insignificant effect on the rate of occlusive lesions in both small and large pulmonary arteries (Grade 0: 32 and 88 %, Grade 1: 42 and 12 %, Grade 2: 26 and 0 %). Medial wall thickening progressively increased from 11% (normal) to 16% (week 3) and 22% (week 8) in larger pulmonary arteries (Figures 5A and 5B). Macitentan treatment also inhibited these progressions. The fraction of the lesion obtained with macitentan at week 8 was similar to that obtained without macitentan at week 3.

Liver Enzymes

Five-week treatment with 30 mg/kg/day of macitentan caused no significant ($P > 0.05$) changes in serum levels of aspartate aminotransferases (AST), alanine aminotransferase (ALT) and total bilirubin (115 ± 42 vs. 127 ± 79 IU/L in AST, 34 ± 2 vs. 40 ± 17 IU/L in ALT, 0.03 ± 0.01 vs. 0.03 ± 0.00 mg/d in total bilirubin; n=4, normal vs. 30 mg/kg/day of macitentan).

Plasma Concentrations of Macitentan

The plasma concentration of macitentan and its active metabolite were 1,328 ± 89 ng/ml (2.3 ± 0.2 μM, n=4) and 10,100 ± 482 ng/ml (18.5 ± 0.9 μM, n=4) respectively at 8 hours

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after the last oral administration of macitentan (30 mg/kg/day).

Discussion

The major findings of the present study are the followings; 1) chronic treatment with 30 mg/kg/day but not 3 mg/kg/day of macitentan reversed the severely elevated RVSP to normal levels with preserved CI, and improved the occlusive pulmonary arterial lesions without adverse effects on liver function in SU/Hx/Nx-exposed rats, and 2) the maximal effect of macitentan was associated with the dose fully blocking ET-1/big ET-1-induced pulmonary arterial contractions in vitro and in vivo. These results provide the novel evidence that efficacious blockade of ET receptors with macitentan can hemodynamically and histologically reverse severe PAH without major adverse effects including liver dysfunction.

ET-1 endogenously binds to two types of its receptors, ET_A and ET_B, in smooth muscle cells, and potentially contributes to vasoconstriction and proliferation during the development of PAH.[4,6] The expression of both ET receptors has been reported to be up-regulated in concentric occlusive and plexiform lesions in patients with severe PAH.[5] These data suggest that the ET-1 signaling pathway contributes to the pathogenesis of severe PAH. However, the efficacy of the antagonist bosentan alone has been limited in the severe PAH patients with WHO functional class IV, although it delays clinical worsening of moderate class II or III PAH patients.[8,9,10] One possible explanation for these disappointing clinical data in severe PAH may partly be attributable to the fact that higher doses of bosentan, which are supposed to completely eliminate ET-1 effects, have not been tested due to its side effects including liver dysfunction (the occurrence of liver dysfunction by the clinical dose of bosentan (250 mg per day) was approximately 14 % in

PAH patients).[9] This speculation is supported by previous preclinical studies indicating that only high doses of bosentan (100 to 250 mg/kg/day) could effectively block pulmonary arterial contractions induced by big ET-1 and prevent the development of pulmonary hypertension in experimental models including Su/Hx-exposed model.[11,31,32,33] Thus, more efficacious blockade of ET receptors is presumably needed to better treat severe PAH.

Macitentan, a novel dual ERA, was developed by improving its tissue distribution and receptor binding affinity compared with bosentan.[14,15] although pA2 values from the previous papers were similar among macitentan, ACT-132577 and bosentan (table).[14,15,27] Regarding the receptor selectivity of macitentan and bosentan, ET_A/ET_B selectivity ratio were 50/1 for macitentan, 16/1 for its metabolite ACT-132577, and 25/1 for bosentan,[15,27]suggesting that macitentan has a dual ET_A/ET_B activity with higher affinity for ET_A than that of ACT-132577 or bosentan.

As shown in figure 2B, macitentan exhibited an apparent biphasic inhibitory effect on the ET-1-induced contraction. The inhibition plateaued at the concentrations ranging 10 nmol/L to 1 μmol/L. The higher concentrations appeared to cause additional inhibition. The biphasic inhibition could be due to either non-specific inhibitory effect on the contraction or differential inhibitory effect on ET_A and ET_B. However, macitentan, even at 10 μmol/L, exhibited a little inhibitory effect on the contraction induced by 60 mmol/L K^+ (data not shown). The contribution of non-specific inhibitory effect to the biphasic effect is less likely. As shown in Table, macitentan shows differential pA_2 values for inhibition of ET_A (7.6) and ET_B (5.9). The inhibition seen with higher concentrations could be due to inhibitory effect on ET_B. However, the contribution of ET_B to the ET-1-

induced contraction in the pulmonary artery ring preparations isolated from SU/Hx/Nx-exposed rats remains to be evaluated.

The slow dissociation rate at the ET receptors make also macitentan behave as an insurmountable antagonist and thus very effective at high ET-1 concentrations.[14] In addition, macitentan has a prolonged half-life and an improved liver safety profile compared to bosentan. The recent Phase 3 study SERAPHIN demonstrated that macitentan (10 mg per day) significantly reduced the events related to PAH or death (46 % vs. 31 %, placebo vs. macitentan group) without liver dysfunction in PAH patients with WHO functional class II or III.[16] Little, however, has yet been investigated regarding the ability of macitentan to reverse severe PAH in either patients or preclinical models.

The present study used the SU/Hx/Nx-exposed rat as a severe PAH model. This model is considered to be a more appropriate preclinical model than the conventional chronically-hypoxic and monocrotaline-injected models, because this is the only rat model that replicates the severity of pulmonary hypertension, the formation of occlusive intimal lesions in small pulmonary arteries and the progressive hemodynamic deterioration as those seen in the patients with severe PAH.[19,20,34] We demonstrated that 30 mg/kg/day, but not 3 mg/kg/day of macitentan, reversed high RVSP to normal levels, and that it also markedly improved RV hypertrophy and histological findings including reduced occlusive lesions and medial wall thickening without increasing liver enzymes. Our acute in vivo data showing that the higher dose of macitentan prevented big ET-1-induced increases in RVSP in catheterized rats strongly support the speculation that complete blockade of ET receptors is the main reason why the higher dose of macitentan was so effective in reversing PAH.

Safety is obviously a concern for translation from preclinical to clinical usage of drugs. It has been demonstrated in a Phase I study with healthy subjects that the higher doses of macitentan (up to 30 mg per day in the multiple ascending dose study) are well tolerated without major side effects.[30,35] In this latter clinical report, the peak concentrations of macitentan (10 and 30 mg per day for 10 days) in healthy subjects were approximately 0.6 and 1.3 μM respectively, which comes close to the range of the plasma concentrations determined (approximately 2.3 μM) in our rats treated with 30 mg/kg/day of macitentan. However, the plasma concentrations of the active metabolite of macitentan, ACT-132577, were 5 to 10 fold higher in rats than in healthy subjects treated with 10 and 30 mg doses of macitentan respectively.[35] As ACT-132577 has been reported to be 5-time less potent on ET_A receptor and 2-time less potent on ET_B receptors in vitro when compared to macitentan, it is likely to contribute to overall effects of macitentan.[15] Its higher concentration in rats together with its long half-life (8h) could contribute to an overestimation of the effect of macitentan observed in rats compared to what could be expected in human. Keeping this in mind, we suggest that higher dose of macitentan could be more efficacious in patients with severe PAH, although further long-term clinical trials are clearly needed. Concerning the other adverse effects of ERA, such as RV contractility and hypotension, [36] 30 mg/kg/day of macitentan had no effects on cardiac output and systemic arterial pressure in 8-week SU/Hx/Nx-exposed PAH rats. In fact, the Phase I study has demonstrated that acute high doses of macitentan (up to 600 mg per day) induce no significant changes of vital signs including systemic arterial pressure in human subjects.[31]

In conclusion, maximal dose of ET receptors blockade with macitentan

completely reverse severe occlusive PAH in a preclinical model appropriately representing the human disorder without major adverse effects including liver dysfunction. These results raise the possibility that efficacious blockade of ET receptors can improve the outcome of patients with severe PAH.

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Table

	pA2		ET_A/ET_B selectivity ratio
	Aorta (ET_A)	Trachea (ET_B)	
Macitentan [a]	7.6	5.9	50/1
ACT-132577 [a]	6.7	5.5	16/1
Bosentan [b]	7.3	5.9	25/1

[a], values from reference 15; [b], values from reference 27

Figures and Figure Legends

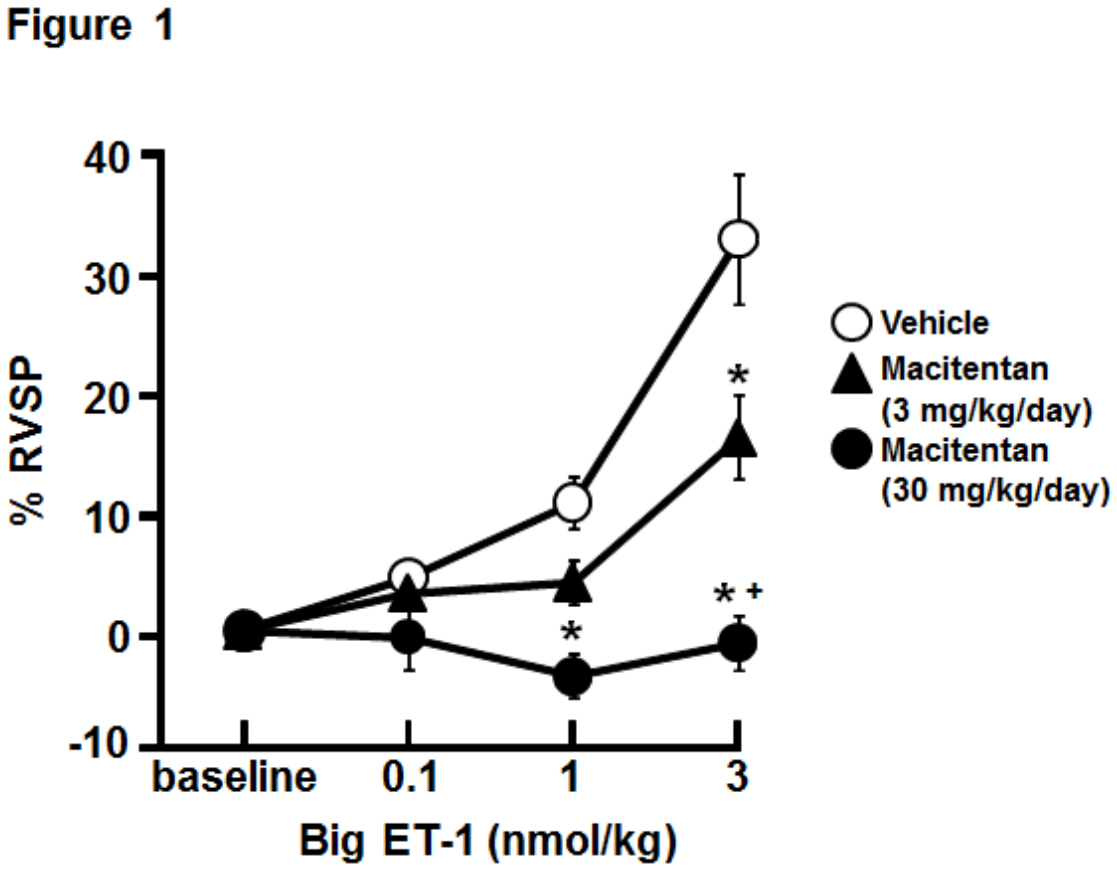

Figure 1. Effects of macitentan on increases in right ventricular systolic pressure induced by big ET-1.

Percentage changes of right ventricular systolic pressure (RVSP) are shown at baseline and after incremental bolus dosages of big ET-1 in normal rats pretreated with 30 mg/kg/day (closed circle) and 3 mg/kg/day of macitentan (closed triangle) and vehicle (open circle). Values are means ± SE of n=3-4. * $P < 0.05$ vs. vehicle. + $P < 0.05$ vs. a lower dose of macitentan.

Figure 2

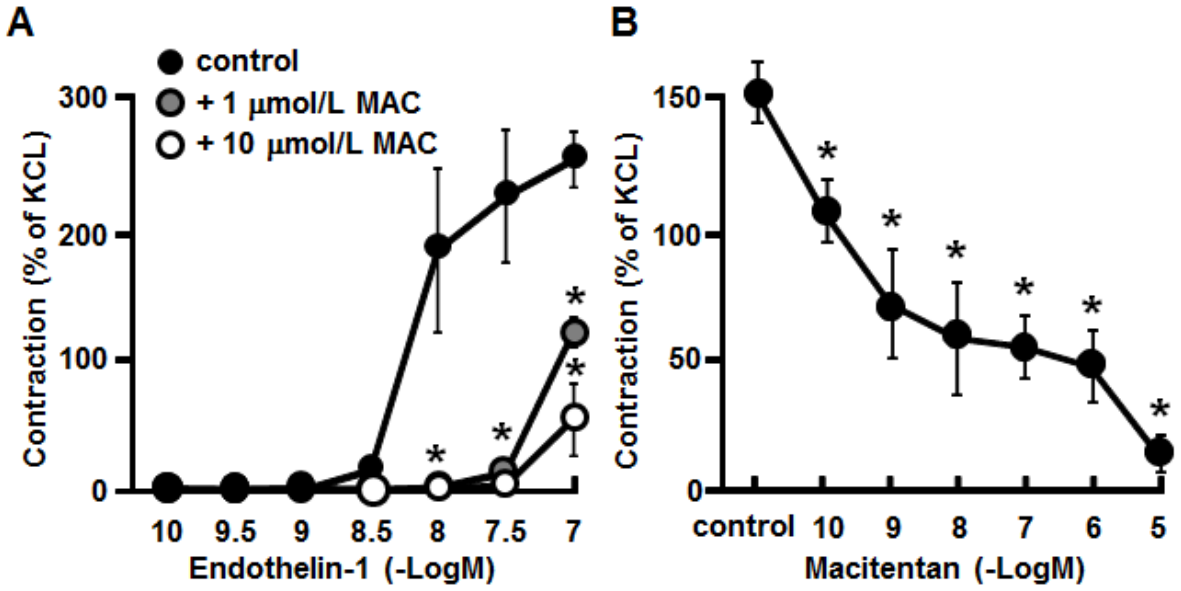

Figure 2. Inhibitory effects of macitentan on the contraction induced by endothelin-1(ET-1) in intrapulmonary arterial rings isolated from SU5416/hypoxia/normoxia (SU/Hx/Nx)-exposed PAH rats at the 3-week time point.

Concentration-response curves for contractile responses to endothelin-1 (ET-1) obtained in the absence and presence of 1 μmol/L and 10 μmol/L macitentan (MAC) (A), and concentration-dependent inhibitory effects of macitentan on the contraction induced by 10 nmol/L ET-1 (B). In panel B, the level of contraction obtained without macitentan was shown as control. Values are means ±SE of n=6-7, each. * $P < 0.05$ vs. control.

Figure 3

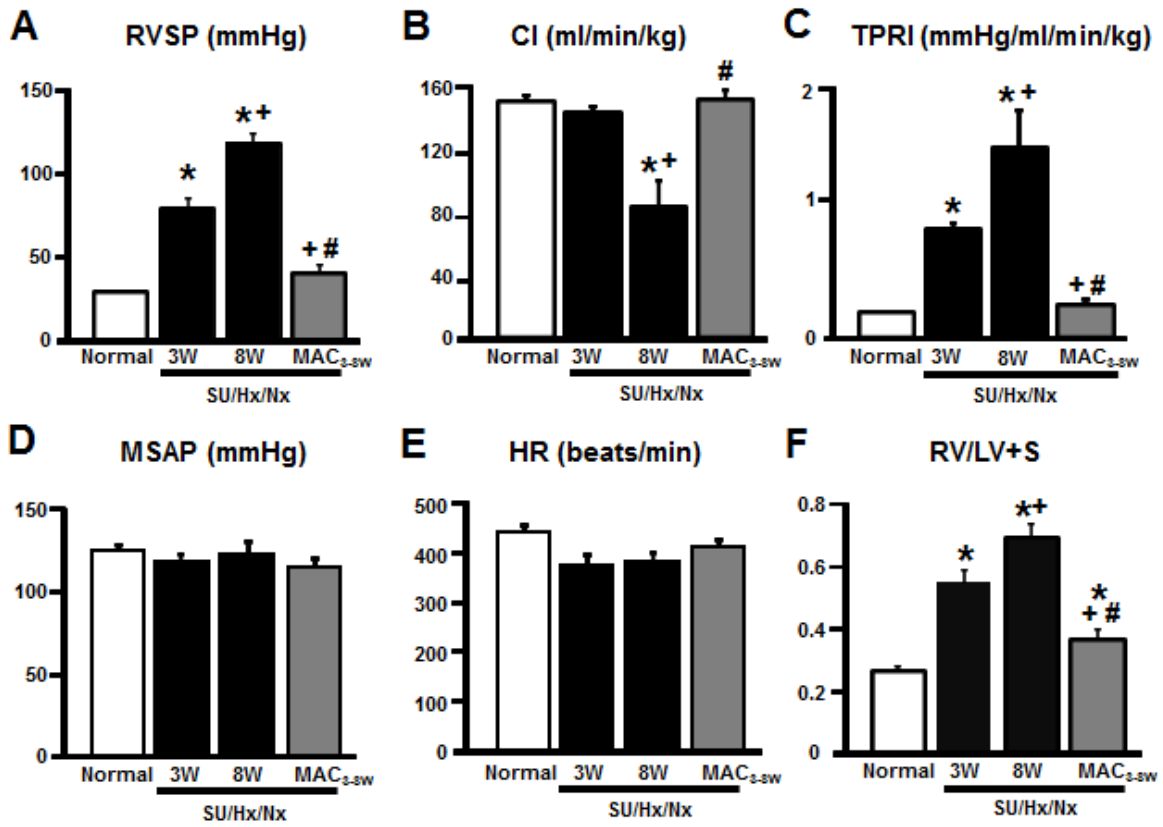

Figure 3. Macitentan improves hemodynamic parameters and RV hypertrophy in SU5416/hypoxia/normoxia (SU/Hx/Nx)-exposed PAH rats.

A: Right ventricular systolic pressure. B: Cardiac index (CI). C: Total pulmonary resistance index (TPRI). D: Mean systemic arterial pressure (MSAP). E: Heart rate (HR). F: Right ventricle/left ventricle + septum (RV/LV+S) weight ratio. Normal, rats received only vehicle of macitentan; 3W and 8W, rats at week 3 and 8 after SU5416 injection; $MAC_{3\text{-}8W}$: rats of SU/Hx/Nx, treated with macitentan (30 mg/kg/day) from week 3 to 8, orally. Values are means ± SE of n=4-9. *P<0.05 vs. Nor. +P<0.05 vs. SU/Hx/Nx 3W. #P<0.05 vs. SU/Hx/Nx 8W.

Figure 4

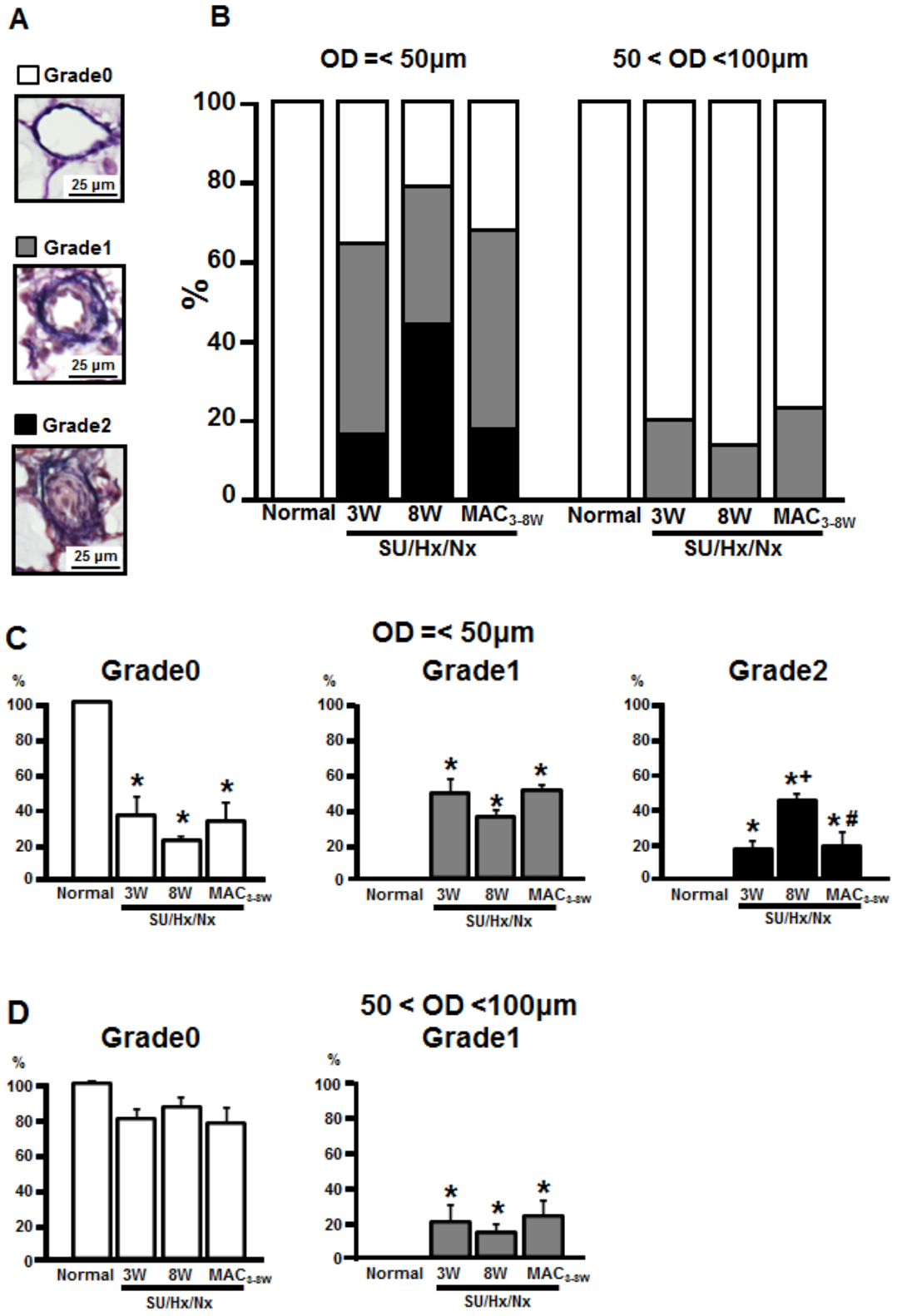
A
Grade0
25 μm
Grade1
25 μm
Grade2
25 μm
B
OD =< 50μm
50 < OD <100μm
%
100
80
60
40
20
0
Normal
3W
8W
MAC3-8W
SU/Hx/Nx
C
OD =< 50μm
Grade0
Grade1
Grade2
D
50 < OD <100μm
Grade0
Grade1

Figure 4. Macitentan improves occlusive lesions in SU5416/hypoxia/normoxia (SU/Hx/Nx)-exposed PAH rats.

A: Representative photomicrographs of Verhoeff-van Gieson-stained pulmonary arterial cross sections. Pulmonary arterial occlusions were graded as 0 (no luminal occlusion; white), 1 (=<50% occlusion; gray) and 2 (>50% occlusion; black). B: Percentage (mean) of grade 0, 1 or 2 lesion in pulmonary arteries of outer diameter (OD) (left; < 50 μm, right; 50 < OD < 100 μm). C: Percentages of grade 0 (left), 1 (middle) and 2 (right) in pulmonary arteries of outer diameter (OD) of <50 μm. D: Percentages of grade 0 (left), 1 (middle) and 2 (right) in pulmonary arteries in 50 < OD < 100 μm. Normal, rats received only vehicle of macitentan; 3W and 8W, rats at week 3 and 8 after SU5416 injection; $MAC_{3\text{-}8W}$: rats of SU/Hx/Nx, treated with macitentan (30 mg/kg/day) from week 3 to 8, orally. N=4, each. *P<0.05 vs. Normal. +P<0.05 vs. SU/Hx/Nx 3W. #P<0.05 vs. SU/Hx/Nx 8W.

Figure 5

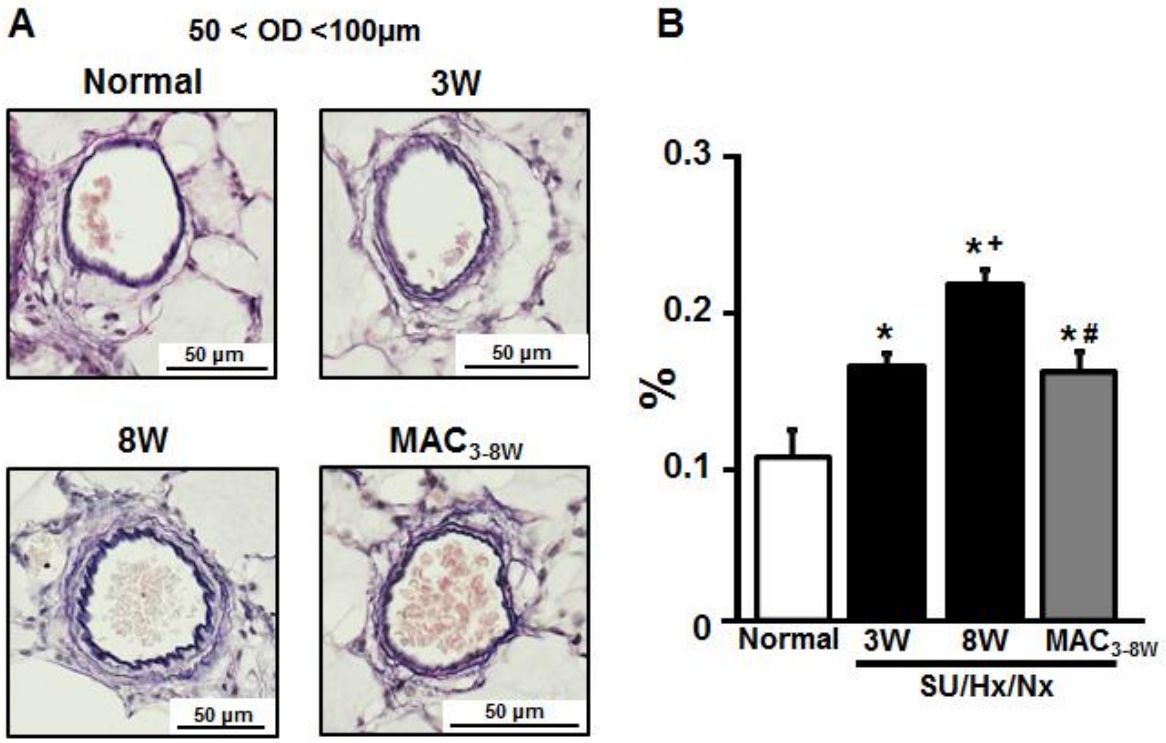

Figure 5. Macitentan suppresses medial wall thickening (MWT) in SU5416/hypoxia/normoxia (SU/Hx/Nx)-exposed PAH rats.

A: Representative photomicrographs showing medial wall thickening by Verhoeff-van Gieson staining. B: MWT was expressed as medial thickness/outer diameter (MWT/OD) x 100 (%) in pulmonary arteries of OD between 50 and 100 μm. Nor: Normal rats. $MAC_{3\text{-}8W}$: Treatment with macitentan (30 mg/kg/day) from week 3 to 8, orally. Values are means ± SE. N=4, each. *P<0.05 vs. Nor. +P<0.05 vs. SU/Hx/Nx 3W. #P<0.05 vs. SU/Hx/Nx 8W.