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Oral Administration of a Novel Long-Acting Prostacyclin Agonist With Thromboxane Synthase Inhibitory Activity for Pulmonary Arterial Hypertension

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Background: Continuous administration of prostacyclin has improved the survival of patients with pulmonary arterial hypertension (PAH). However, this treatment has some problems, including its short duration of activity and difficult delivery. Therefore, we developed ONO-1301, an orally active, long-acting prostacyclin agonist with thromboxane synthase inhibitory activity.

Methods and Results: We investigated whether oral administration of ONO-1301 can both prevent and reverse monocrotaline (MCT)-induced PAH in rats. Rats were randomly assigned to receive repeated oral administration of ONO-1301 twice daily beginning either 1 or 8 days after subcutaneous injection of MCT. A control group received oral saline, and a sham group received a subcutaneous injection of saline instead of MCT. MCT-treated controls developed significant pulmonary hypertension. Treatment with ONO-1301 from day 1 or 8 significantly attenuated the increases in right ventricular systolic pressure and the increase in medial wall thickness of pulmonary arterioles. Kaplan-Meier survival curves demonstrated that the effect of ONO-1301 was equivalent to that of an endothelin receptor antagonist and a phosphodiesterase-5 inhibitor. A single oral dose of ONO-1301 increased plasma cAMP levels for up to 6 h. Treatment with ONO-1301 significantly decreased urinary 11-dehydro-thromboxane B_2 and increased the plasma hepatocyte growth factor concentration.

Conclusions: Oral administration of ONO-1301 ameliorated PAH in rats, an effect that may occur through cAMP and hepatocyte growth factor. (*Circ J* 2013; **77:** 2127–2133)

Key Words: cAMP; Hepatocyte growth factor; Monocrotaline; Pulmonary arterial hypertension; Thromboxane synthase

Pulmonary arterial hypertension (PAH) is a rare but life-threatening disease characterized by progressive pulmonary hypertension that leads to right ventricular failure and death.[1,2] Continuous intravenous infusion of prostacyclin has become recognized as a therapeutic breakthrough[3–5] and the dramatic success of long-term intravenous prostacyclin has led to the development of prostacyclin analogs.[6–9] Nevertheless, treatment with prostacyclin or its analogs has some problems in the clinical setting, viz., short-term activity and difficulty of drug delivery.

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ONO-1301 has long-lasting prostacyclin activity in association with thromboxane synthase inhibitory effects.[10,11] We have reported that repeated subcutaneous injections of this compound ameliorated PAH induced by monocrotaline (MCT) in rats.[10,11] However, whether oral administration has the same beneficial effects on PAH remains unknown, so in the present study we investigated the effects of oral ONO-1301 on pulmonary hemodynamics and survival in MCT rats.

Thus, the aims of this study were as follows: (1) to investi-

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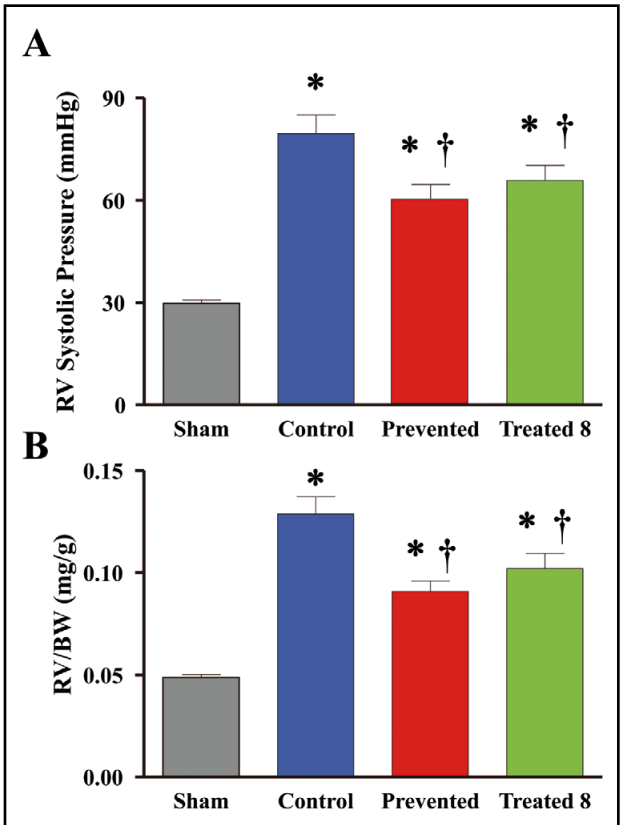

Figure 1. Effects of ONO-1301 on right ventricular (RV) systolic pressure (**A**) and RV weight to body weight (RV/BW) (**B**) in sham rats given vehicle (Sham), monocrotaline (MCT) rats given vehicle (Control), MCT rats administered 10 mg/kg ONO-1301 from day 1 after MCT injection (Prevented) and MCT rats administered 10 mg/kg ONO-1301 from day 8 after MCT injection (Treated 8). Data are mean±SEM. *P<0.05 vs. Sham; †P<0.05 vs. Control.

gate whether oral administration of ONO-1301 attenuates PAH induced by MCT in rats, (2) to compare the effect of this compound with that of conventional treatments, and (3) to assess the underlying mechanisms of its therapeutic effect.

Methods

Animal Model

In this study to evaluate the effects of oral administration of ONO-1301 on MCT-induced PAH, we used 60 5-week-old male Wistar rats weighing 100–120 g; 45 were chosen randomly to receive a subcutaneous injection of 60 mg/kg MCT on day 1 of the trial, while the remaining 15 received a subcutaneous injection of 0.9% saline. The MCT-injected rats were then assigned to one of 3 treatment protocols: oral treatment with ONO-1301 from day 1 (prevented group, n=15), oral treatment with ONO-1301 from day 8 (treated 8 group, n=15), and oral 0.9% saline (control group, n=15). Saline-injected rats received 0.9% saline orally from day 1 (sham group, n=15). In addition, 65 rats were studied to evaluate plasma ONO-1301 (n=20), and 11-dehydro-thromboxane B_2 (TXB_2) level (n=45) concentration s. Furthermore, the effect of ONO-1301 on survival of MCT rats was also evaluated.

In Vivo Experimental Protocol

Following anesthesia by intraperitoneal injection of 30 mg/kg pentobarbital, rats were given a subcutaneous injection of either 60 mg/kg MCT or 0.9% saline. Subsequently, 10 mg/kg ONO-1301 was administered twice daily by oral gavage from either the 1st or 8th day after MCT injection. It has been previously shown that 10 mg/kg is the maximum dose that does not cause significant hypotension. Hemodynamic measurements and histologic analyses were performed on day 25; this time point was based on survival curve analyses. Hemodynamic measurements were performed with the rats anesthetized by continuous inhalation of 1% isoflurane. A polyethylene catheter (model PE-50; BD Biosciences, San Jose, CA, USA) was inserted into the right carotid artery to measure heart rate and mean arterial pressure. A second catheter was inserted through the right jugular vein into the right ventricle (RV) for the measurement of pressure. Heart rate, mean arterial pressure, and systolic RV pressure were calculated from 20 consecutive heart beats in each rat. Finally, cardiac arrest was induced by blood collection through the catheter. Blood was immediately transferred into a chilled glass tube containing disodium ethylenediaminetetraacetic acid (EDTA; 1 mg/ml) and aprotinin (500 U/ml) and centrifuged. The ventricles and lungs were excised and weighed. The ratio of RV weight to body weight (RV/BW) was calculated as an index of ventricular hypertrophy, as previously reported.[12] All protocols were performed in accordance with the guidelines of the Animal Care and Ethics Committee of the National Cardiovascular Center Research Institute (Osaka, Japan).

Morphometric Analysis of Pulmonary Arterioles

Paraffin sections of 4 μm thickness were obtained from the lower region of the right lung and stained with hematoxylin-eosin. Analysis of the medial wall thickness of the pulmonary arterioles was performed as described previously.[13] In brief, the external diameter and medial wall thickness were measured in 20 muscular arteries (25–100 μm external diameter) per lung section. For each artery, the medial wall thickness was expressed as follows: %wall thickness = ([medial thickness × 2]/external diameter) × 100.

Comparison With Conventional Drugs

To evaluate the effect of oral administration of ONO-1301 on survival in MCT rats, the following survival analyses were performed: whether an oral dose of ONO-1301 improved the survival rate in MCT rats as compared with vehicle (Control), an endothelin receptor antagonist (bosentan) or a phosphodiesterase-5 inhibitor (sildenafil).

Survival was evaluated from the date of MCT injection to the death of the rat or 6 weeks after injection.

Assay of Plasma ONO-1301 and cAMP Levels

To investigate whether prostacyclin activity induced by a single, oral dose of ONO-1301 is comparatively equivalent with that with a subcutaneous injection, we measured the plasma level of ONO-1301. Rats were assigned to receive a single, oral or subcutaneous dose of 10 mg/kg ONO-1301 (n=10 in each group), and blood was drawn at 0, 1, 2, 4, 8, 12, and 24 h. Blood was immediately transferred to a chilled glass tube containing 1 mg/ml disodium EDTA and centrifuged. The plasma ONO-1301 level was measured by liquid chromatography tandem mass spectrometry assay.

We investigated whether a single, oral dose of ONO-1301 induced long-lasting prostacyclin activity in MCT rats. In this study, 20 rats were assigned to orally receive the dose of ONO-

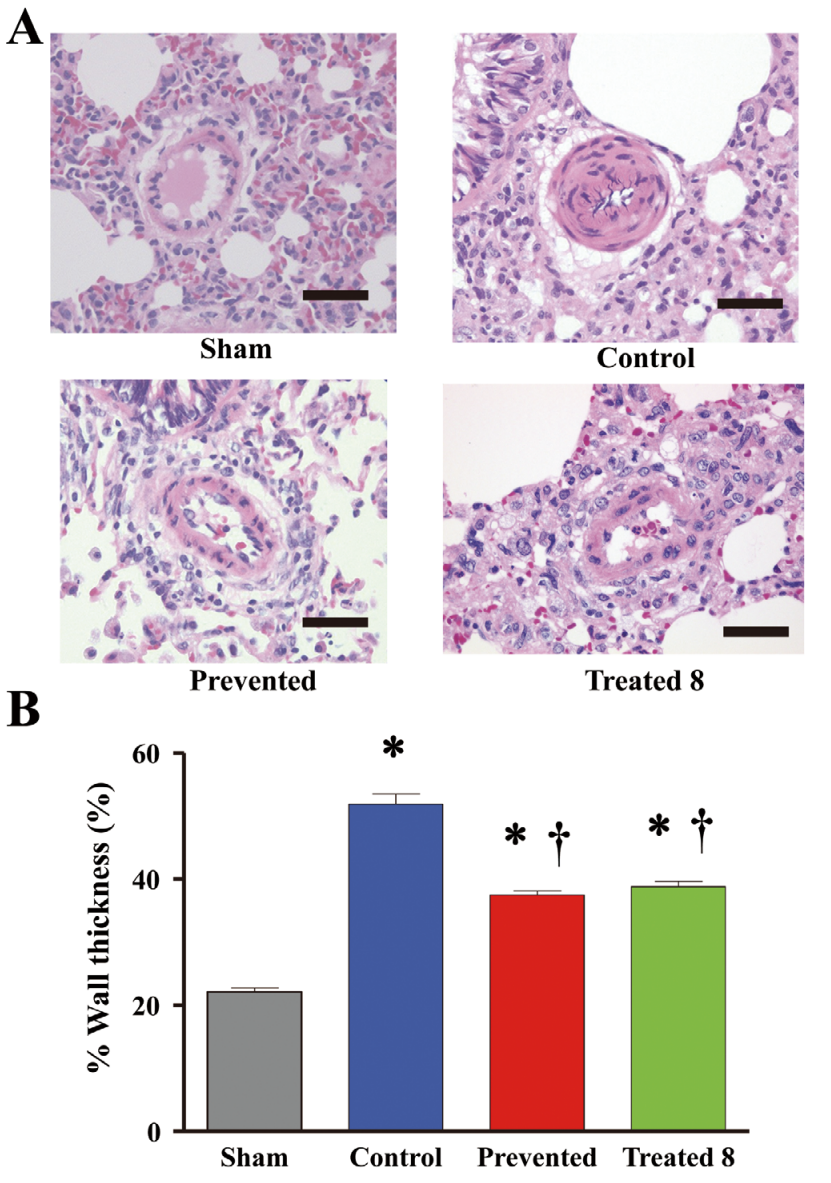

Figure 2. (**A**) Representative photomicrographs of pulmonary arterioles on day 25 after monocrotaline (MCT) injection. Scale bars = 20 μm. (**B**) Quantitative analysis of percent wall thickness in pulmonary arterioles. Data are mean ± SEM. *P<0.05 vs. Sham; †P<0.05 vs. Control.

1301 (10 mg/kg) or vehicle (n=10 each). Blood was drawn from the right carotid artery at baseline and at 1, 2, 6, 8, 12 and 24 h after ONO-1301 administration. Plasma cAMP levels were measured with a radioimmunoassay kit (cAMP assay kit; Yamasa Shoyu, Chiba, Japan), as reported previously.[14]

Assay of Urine 11-Dehydro Thromboxane B_2 Level

To investigate the effect of ONO-1301 on thromboxane synthesis in rats, we measured the urinary level of 11-dehydro thromboxane B_2 (11-DTXB_2), a metabolite of thromboxane A_2 (TXA_2), after oral administration of ONO-1301 (10 mg/kg) or vehicle (n=15 in each group). Urine samples were collected for 24 h on day 14 from rats in metabolic cages, and the urine 11-DTXB_2 level was measured with an enzyme immunoassay kit (11-DTXB_2 assay kit; Cayman Chemical Co, Ann Arbor, MI, USA). The urinary 11-DTXB_2 level was expressed as a ratio to the urinary creatinine level, as reported previously.[15]

Assay for Plasma Hepatocyte Growth Factor (HGF) Level and Survival Rate After Injection of Anti-HGF Antibody

To investigate whether ONO-1301 acts by increasing the plasma HGF level, that factor was measured with an enzyme immunoassay kit (Rat HGF EIA, Institute of Immunology, Tokyo, Japan).

To evaluate the effect of oral administration of ONO-1301 on survival of MCT rats, the following survival analyses were performed: whether an oral dose of ONO-1301 (Prevented) improved survival rate in MCT rats compared with (1) vehicle (Control), (2) vehicle with anti-HGF antibody (Control + anti-HGF antibody) or (3) ONO-1301 with anti-HGF antibody (Prevented + anti-HGF antibody).

Survival was estimated from the date of MCT injection to the death of the rat or 6 weeks after injection. Anti-HGF antibody (8 mg/kg) was injected intraperitoneally once every 4 days for 42 days after MCT injection.

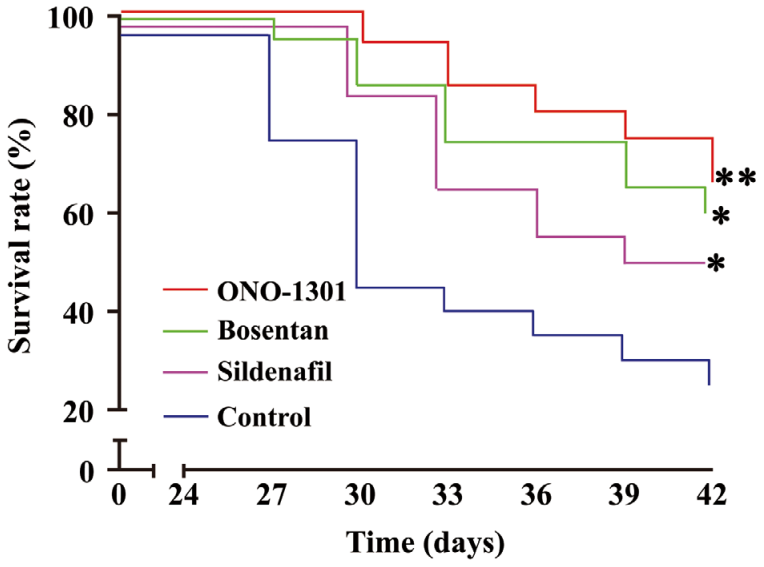

Figure 3. Kaplan-Meier survival curves showing survival rate in MCT + vehicle (Control, blue line), MCT + ONO-1301 (ONO-1301, red line), MCT + bosentan (Bosentan, green line) and MCT + sildenafil (Sildenafil, pink line). Data are mean values. Statistical significance was determined by log-rank test. *P<0.05 vs. Control. **P<0.01 vs. Control. MCT, monocrotaline.

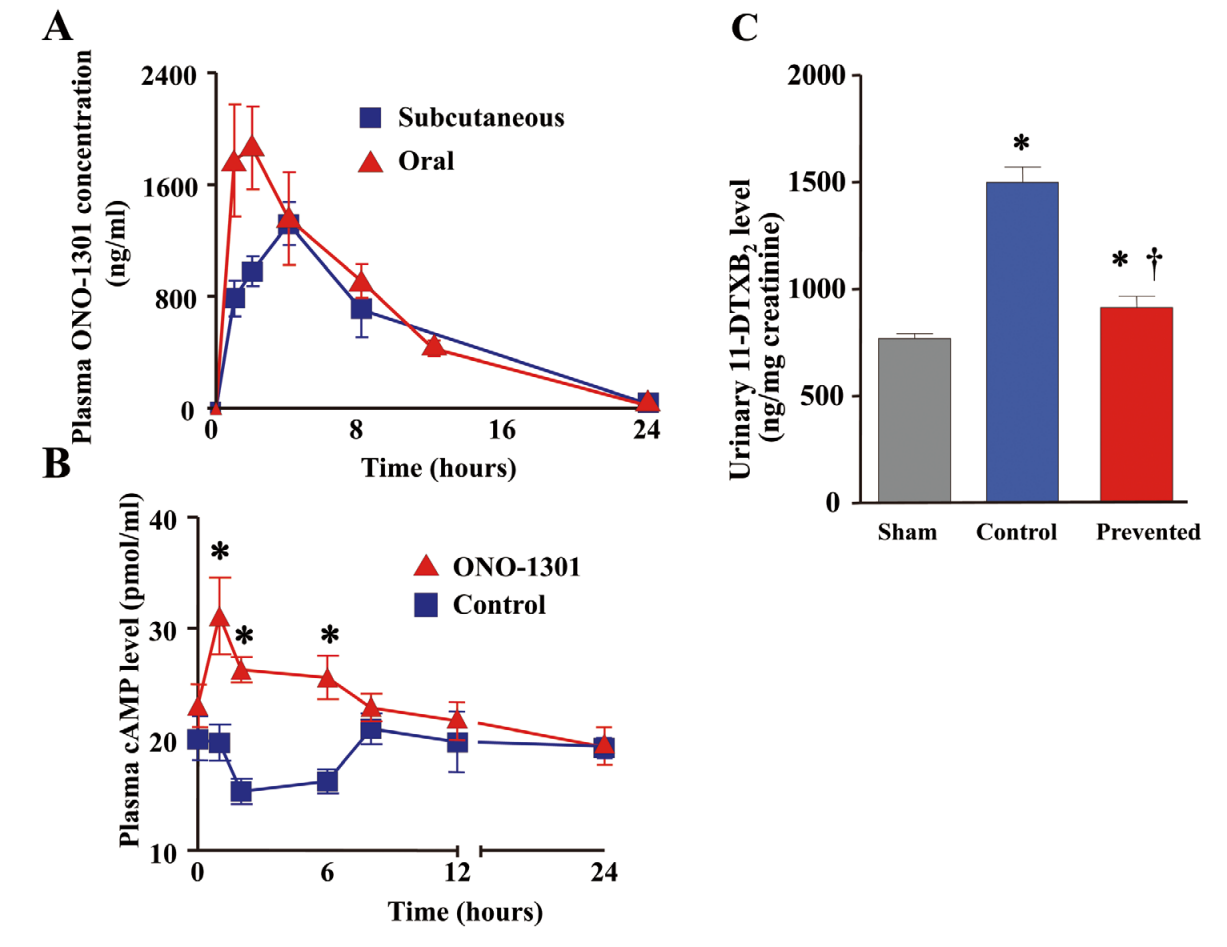

Figure 4. (**A**) Plasma ONO-1301 concentration after subcutaneous or oral administration. (**B**) Changes in plasma cAMP level after oral administration of ONO-1301 (ONO-1301) or vehicle (Control). Data are mean±SEM. *P<0.05 vs. Control. (**C**) Effect of ONO-1301 on plasma level of 11-dehydro-thromboxane B_2 (TXB_2), a metabolite of thromboxane A_2. Data are mean±SEM. *P<0.05 vs. Sham; †P<0.05 vs. Control.

Statistical Analysis

All data are expressed as mean±SEM. Comparisons of parameters among the 3 groups were made by 1-way analysis of variance (ANOVA), followed by Newman-Keuls' test. Comparisons of the time course of parameters between 2 groups were made by 2-way ANOVA for repeated measures, followed by Newman-Keuls' test. A value of $P<0.05$ was considered statistically significant. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test. $P<0.05$ was considered statistically significant.

Results

Effects of Orally Administered ONO-1301 on Pulmonary Hemodynamics and Vascular Remodeling

RV systolic pressure was significantly increased in all MCT-treated groups on day 25 after MCT injection (Figure 1A). However, this increase was significantly attenuated by twice-daily oral administration of ONO-1301 from day 1 or day 8 after MCT injection. Similarly, the increase in RV/BW in MCT rats was significantly attenuated by treatment with ONO-1301 (Figure 1B). There was no significant difference in heart rate or mean arterial pressure among the 4 groups. Hypertrophy of the pulmonary vessel wall after MCT injection was less in MCT rats treated with ONO-1301 than in control rats (Figure 2A). Quantitative analysis demonstrated a significant increase in the percent wall thickness after MCT injection, but this change was ameliorated by ONO-1301 treatment (Figure 2B). However, there were no significant differences in the physiological and morphological parameters between the group given ONO-1301 treatment beginning on day 1 (Prevented group) and that given it on day 8 (Treated 8 group) after MCT injection.

Effect of Orally Administered ONO-1301 on Survival

Kaplan-Meier survival curves demonstrated that oral administration of ONO-1301 significantly improved survival rate in MCT rats compared with vehicle (Figure 3). Interestingly, Kaplan-Meier survival curves also demonstrated that the effect of ONO-1301 may be equal to that of an endothelin receptor antagonist (bosentan) and a phosphodiesterase type 5 inhibitor (sildenafil). These results suggested that repeated oral administration of ONO-1301 has a beneficial effect on the survival of MCT rats.

Long-Lasting Activity After Oral Administration of ONO-1301

We measured plasma ONO-1301 concentrations after oral or subcutaneous administration of ONO-1301 (10 mg/kg). The increase in the plasma ONO-1301 concentration by a single, oral dose peaked at 2 h. The increase in plasma ONO-1301 concentration showed hardly any difference between oral and subcutaneous administration (Figure 4A). In addition, a single oral dose of ONO-1301 significantly increased the plasma cAMP levels in the rats (Figure 4B). The increase in plasma cAMP level peaked at 1 h and lasted for at least 6 h after ONO-1301 administration. These results suggested that oral administration of ONO-1301 had long-lasting activity in the rats.

Inhibitory Effect of ONO-1301 on Thromboxane Synthesis

The urinary 11-dehydro-TXB_2 levels were markedly elevated on day 14 after MCT injection (Figure 4C). However, oral administration of ONO-1301 significantly attenuated the increase in urinary 11-dehydro-TXB_2.

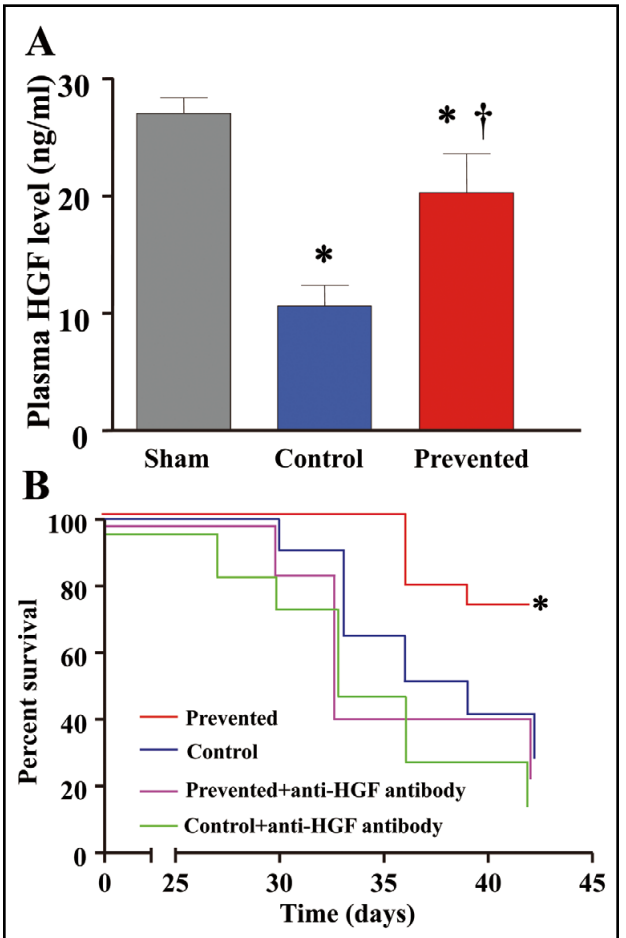

Figure 5. (**A**) Effects of ONO-1301 on plasma level of hepatocyte growth factor (HGF). Data are mean±SEM. *$P<0.05$ vs. Sham; †$P<0.05$ vs. Control. (**B**) Kaplan-Meier survival curves showing survival rate in MCT + vehicle (Control, blue line), MCT + ONO-1301 (Prevented, red line), MCT + vehicle + anti-HGF antibody (Control + anti-HGF antibody, green line) and MCT + ONO-1301 + anti-HGF antibody (Prevented + anti-HGF antibody, pink line). Data are mean values. Statistical significance was determined by log-rank test. *$P<0.05$ vs. Control, and †vs. Prevented + anti-HGF antibody. MCT, monocrotaline.

Effect of ONO-1301 on Endogenous HGF

The plasma endogenous HGF levels were significantly decreased 3 weeks after MCT injection (Figure 5A). However, oral administration of ONO-1301 significantly increased it. Although oral administration of ONO-1301 significantly improved the survival rate in MCT rats compared with vehicle, the effect was significantly attenuated by injection of anti-HGF antibody (Figure 5B).

Discussion

In the present study, we first investigated that orally administered prostacyclin agonist (ONO-1301) has beneficial effects on PAH using rats treated with MCT. We demonstrated that oral administration of ONO-1301 ameliorated the development of MCT-induced pulmonary hypertension and also improved the survival rate in MCT rats, that this compound decreased a urinary thromboxane metabolite, and attenuated the decrease in plasma HGF level in MCT rats.

We have developed a novel prostacyclin agonist with a long half-life of 5.6 h.[10] We previously reported that subcutaneous injection of ONO-1301 improved PAH in MCT rats.[10,11] However, the effect of oral administration of this compound remained unknown, so in the present study, we examined whether orally administered ONO-1301 has therapeutic potential for PAH. We found that repeated oral administration of ONO-1301 beginning on either day 1 or day 8 after MCT injection markedly attenuated the development of MCT-induced PAH, as indicated by significantly lower RV systolic pressure and RV weight in the ONO-1301-treated rats relative to control MCT rats. Furthermore, ONO-1301 attenuated the increase in medial wall thickness of the pulmonary arterioles in the MCT rats. Beginning oral ONO-1301 administration on either day 1 or day 8 after MCT treatment did not change its efficacy.

Subcutaneous injection of MCT causes a marked inflammatory response soon after injection, and promotes the subsequent proliferation of vascular smooth muscle cells (VSMCs).[12] Many compounds have been reported to prevent MCT-induced PAH, but we believe it was also important to evaluate the potential of ONO-1301 to not only prevent, but also reverse, MCT-induced changes. By beginning ONO-1301 administration on day 8 after MCT injection, we examined its ability to reverse the inflammation and VSMC proliferation associated with MCT treatment. Our results suggest that administration of ONO-1301 from day 8 after MCT injection may suppress not only the inflammation, but also the proliferation of VSMCs that commonly occurs in MCT rats. We found that beginning ONO-1301 administration on day 1 or day 8 following MCT treatment did not significantly alter the improvement in RV systolic pressure, RV/BW and pulmonary arterial wall thickness seen with both treatment protocols. In the present study, the reversing effect of ONO-1301 was determined to the same extent as the prevention protocol, suggest that ONO-1301 has the potential to reverse PAH.

Activation of prostacyclin receptors has been shown to suppress the growth of VSMCs through a cAMP-dependent pathway. Indeed, we showed that the plasma cAMP level increased and its activity was sustained long after oral administration of ONO-1301. Thus, ONO-1301 may attenuate the development of pulmonary vascular remodeling, at least in part via a cAMP-dependent pathway. As another mechanism, ONO-1301 has thromboxane synthase inhibitory activity. Indeed, markedly elevated levels of urinary 11-dehydro-TXB_2 (a metabolite of TXA_2) were significantly diminished by treatment with ONO-1301. Earlier studies have shown impaired prostacyclin synthesis and increased thromboxane production in patients with PAH, suggesting that imbalance of the release of thromboxane and prostacyclin plays an important role in the development of pulmonary hypertension.[15–17] ONO-1301 has a 3-pyridine radical, which is known to inhibit thromboxane synthase through interaction with carboxylic acid via a hydrogen bond. Rich et al have shown that inhibition of thromboxane synthase modestly improves pulmonary hemodynamics in patients with PAH.[18] It is possible that ONO-1301 attenuates MCT-induced pulmonary hypertension partly via improvement of the prostacyclin/thromboxane imbalance.

In the present study, we demonstrated that repeated oral administration of ONO-1301 improved the survival of MCT rats. In the clinical setting, it has been reported that bosentan and sildenafil are effective in treating PAH.[19–21] The effect of ONO-1301 was equivalent to that of both those compounds. Considering that ONO-1301 has different actions, it shows promise for the treatment of PAH. Interestingly, we also demonstrated that the effect of ONO-1301 was attenuated by injection of anti-HGF antibody, suggesting that the therapeutic potential ONO-1301 is mediated by HGF. We have already reported that ONO-1301 showed a beneficial effect by inhibiting the MEK/ERK pathway,[11] and we also reported that repeated administration of ONO-1301 attenuated the development of bleomycin-induced pulmonary fibrosis and improved survival of the affected mice, at least in part by inhibiting TXA_2 synthesis and activating the cAMP/PKA pathway.[22] Binding of ONO-1301 to the IP receptor stimulates adenylate cyclase activity, which increases the level of cAMP, which then induces endogenous HGF production and increases PKA activity.[23] PKA has been shown to induce endogenous HGF production[23] and inhibit the MEK/ERK pathway.[24] A recent study demonstrated that continuous intravenous delivery of human HGF led to prolonged survival of animals with MCT-induced PAH.[25] In the present study, anti-HGF antibody reversed the favorable effect of ONO-1301 on survival. Also, repeated oral administration of ONO-1301 increased the plasma HGF level. These findings suggest that ONO-1301 attenuates MCT-induced pulmonary hypertension through the cAMP/PKA/HGF pathway.

In conclusion, oral administration of ONO-1301 ameliorated PAH in rats, in association with an increase in cAMP, a decrease in thromboxane A_2 and an increase in endogenous HGF levels.

Acknowledgment

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Author Contributions

Drs Nakamura, Nagaya, Obata, Y Sakai, and Kimura were responsible for study concept, design and data interpretation. Drs Nakamura, Nagaya, Yoshikawa, Hamada, and Kimura were responsible for drafting and revising the manuscript. Drs Nakamura, Nagaya, Hamada, Obata, Y Sakai, K Sakai, Matsumoto and Kimura were responsible for data acquisition. Drs Nakamura, Nagaya, Y Sakai, and Kimura were responsible for data analysis.

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