*Full Paper***Tadalafil, a Long-Acting Inhibitor of PDE5, Improves Pulmonary Hemodynamics and Survival Rate of Monocrotaline-Induced Pulmonary Artery Hypertension in Rats**Fusae Sawamura^{1,*}, Masami Kato², Kazuhisa Fujita², Takahiro Nakazawa¹, and Anthony Beardsworth³¹Lilly Research Laboratories Japan, Eli Lilly Japan K.K., 7-1-5 Isogami-dori, Chuo-ku, Kobe, Hyogo 651-0086, Japan²Hashima Laboratory, Nihon Bioresearch Inc., 104, 6-chome, Majima, Fukuju-cho, Hashima, Gifu 501-6251, Japan³Cialis Global Development Team, Eli Lilly and Company Ltd., Erl Wood Manor, GU20 6PH, UK

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Abstract. The aim of this study was to assess the effect of tadalafil (0.5, 2.5, and 10 mg/kg per day) on the progression of pulmonary arterial hypertension (PAH) in early treatment and on the survival rate in late treatment on the monocrotaline (MCT)-induced PAH rat model. Tadalafil was administered once daily to rats for 3 weeks from the day of MCT-injection or 21 days after the injection. With early treatment, tadalafil at 10 mg/kg per day prevented the development of PAH by maintaining mean pulmonary artery pressure within the normal range and attenuated right ventricular hypertrophy. With late treatment, tadalafil tended to increase the partial pressure of oxygen in arterial blood and dose-dependently improved the survival rate by 55%, 60%, and 70% at 0.5, 2.5, and 10 mg/kg per day, respectively, versus 40% in the MCT-control group. Both early and late treatments with tadalafil were associated with elevated lung cyclic guanosine monophosphate (cGMP). These results suggest that tadalafil relaxes pulmonary arteries by elevating cGMP in lungs and extend survival time by improving pulmonary hemodynamics even when treatment occurs in the late phase of PAH. Thus, it is expected that tadalafil may be an effective, once-daily treatment option in humans with PAH.

Keywords: tadalafil, phosphodiesterase 5 (PDE5) inhibitor, pulmonary artery hypertension (PAH), monocrotaline (MCT), survival

Introduction

Pulmonary arterial hypertension (PAH) is a complex disease characterized by endothelial proliferation that results in progressive pulmonary vascular remodeling and elevation of the pulmonary arterial pressure, leading to right heart failure and ultimately death (1, 2). Conventional therapies, such as anticoagulants, diuretics, calcium channel blockers, or inotropic agents have been used to improve the symptoms of PAH, but none of them are formally approved for the treatment of PAH by regulatory agencies. In the last decade, specific targeted therapies, such as prostacyclins (3, 4), endothelin-receptor antagonists (5 – 8), and phosphodiesterase type 5

(PDE5) inhibitor (9), have achieved regulatory approval for the treatment of PAH. However, alternatives continue to be sought due to the insufficient effects (10) and unique tolerability and administration aspects (11 – 13) associated with these targeted therapies.

Tadalafil is a potent and selective inhibitor of PDE5 (14, 15) with a half life of about 17.5 h (16, 17) and is currently widely used for the treatment of erectile dysfunction. PDE5, which hydrolyzes cyclic guanosine monophosphate (cGMP), is a predominant PDE in the pulmonary vasculature (18, 19) and is associated with the pathobiology of PAH. Although the pathogenesis of PAH is not completely understood, it likely involves an imbalance in the normal relationships between vasodilators and vasoconstrictors. A decreased expression of nitric oxide (NO) synthase (20) has been reported in PAH patients that results in vasoconstriction and the excessive growth of the tunica media through the reduc-

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tion of NO-induced cGMP in the pulmonary vasculature. Thus, it is expected that tadalafil improves the vasoconstrictive-predominant condition in PAH patients by increasing cGMP levels in pulmonary vessels through the specific inhibition of PDE5. The effects of PDE5 inhibitors have already been demonstrated with sildenafil (21, 22) approved for PAH indication, but the effects of tadalafil on PAH may not be equivalent because they have different pharmacokinetics and selectivity to the PDEs (23). Different effects among the specific PDE5 inhibitors (sildenafil, vardenafil, and tadalafil) have been reported on the calcium handling in aorta (24) and pulmonary artery cytokine expression (25). The purpose of this study, therefore, was to investigate the potential effects of tadalafil on PAH progression and on survival benefit using the monocrotaline (MCT)-induced PAH rat model.

MCT is metabolized in the liver and changed to the active form (monocrotaline pyrrole) which, after a single subcutaneous injection, injures vascular endothelium of pulmonary vessels within several hours (26), activates platelets within 1 week (27, 28), and causes enhanced reactivity of pulmonary vessels to vasoconstrictive substances (29) and muscular hypertrophy of the media of pulmonary vessels (30). Mortality due to right cardiac failure begins 3 to 4 weeks after the injection and the survival decreases to about 30% by 6 weeks after the injection. The actual cause of death by MCT is not completely understood, but the monocrotaline pyrrole obviously causes injury of the lung (31) and induces right ventricular (RV) failure confirmed by the presence of ascites, pericardial, or pleural effusions (32). The effect of sildenafil on PAH has previously been demonstrated in this model (33, 34). The potential effects of tadalafil on PAH progression and survival benefit were investigated by treating rats for 3 weeks from the MCT injection when vascular remodeling is occurring (early treatment) or from 21 days after the MCT injection when PAH was established (late treatment), respectively. Sildenafil was used as a positive control.

Materials and Methods

Animals

Six-week-old male Sprague-Dawley (SD) rats were purchased from Charles River Laboratories Japan, Inc. (Hino Breeding Center, Shiga). The animals were housed individually in stainless steel 5-compartment cages (W: 755 × D: 210 × H: 170 mm) in an animal room set at 23°C and 55% relative humidity on a 12-h light and dark cycle (lighting: 7:00 a.m. – 7:00 p.m.) and had free access to food and water. Experiments were undertaken following basic guidelines for the use of

experimental animals in Institutions under the Jurisdiction of the Ministry of Health, Labour, and Welfare (Notification No. 0601005 of the Science Bureau, Japanese Ministry of Health, Labour, and Welfare, June 1, 2006) and guidelines for Management and Welfare of Experimental Animals (Hashima Laboratory, Nihon BioResearch Inc., April 2, 2007).

Hemodynamic measurement

MCT (Sigma-Aldrich, St. Louis, MO, USA) at 60 mg/kg (s.c.) was given to the male SD rats (Day 1); and tadalafil (0.5, 2.5, and 10 mg/kg per day, q.d.; Eli Lilly and Company, Indianapolis, IN, USA), sildenafil (25 mg/kg per day, b.i.d., Sequoia Research Products, Ltd., Pangbourne, UK), or vehicle (10% aqueous gum arabic) was given by oral administration up to Day 21. The groups treated once daily with tadalafil (q.d.) were given a vehicle at the time of the second daily dose of sildenafil or a vehicle so that the experimental condition would be the same. At the end of the study (Day 21), the animals were anesthetized with sodium pentobarbital (30 mg/kg, Nembutal® injection; Dainippon Sumitomo Pharma Co., Ltd., Osaka). The trachea cannula connected to a ventilator (55-3438; Harvard Apparatus, Holliston, MA, USA) was inserted and artificial respiration (frequency: 50 – 60 times/minute and tidal volume: 5 – 15 mL/kg) was performed. A polyethylene catheter (Intramedic PE-50; Beckton Dickinson and Company, Franklin Lakes, NJ, USA) was inserted into the right carotid artery and connected to a pressure transducer (TP-300T; Nihon Kohden Co., Tokyo) for measurement of arterial blood pressure (BP). A 22G hypodermic needle was inserted into RV and a catheter (catheter-type transducer) (FT111B; Scisence, Inc., Ontario, Canada) was passed through the lumen of RV into the pulmonary artery to measure pulmonary arterial pressure (PAP) (35, 36). Then RV pressure (RVP) was measured after the tip of the catheter was returned toward the RV. Changes in BP were amplified with an amplifier for blood pressure (Bridge Amp, ML110; AD Instruments Pty., Ltd., Castle Hill, Australia) or a control box for pressure (FP891B, Scisence Inc.) and recorded with a system for collection and analysis of data (PowerLab System, AD Instruments Pty., Ltd.) (sample rate: 1 kHz). To analyze each parameter for hemodynamics, mean values in 5 min with stable data were used for calculation of mean PAP (MPAP), systolic RVP (SRVP), mean BP (MBP), and heart rate (HR).

Measurement of organ weight

At the end of the study, hearts were dissected, RV and left ventricle with ventricular septum (LV + S) were weighed, and the ratio of the RV to LV + S weight

[RV/(LV + S)] was calculated as an index of RV hypertrophy.

Survival analysis

Male SD rats were given MCT at 60 mg/kg by single subcutaneous injection. Three weeks after the injection (Day 21), each rat was allocated to each of the 5 groups including 3 doses of tadalafil (n = 20/group), sildenafil (n = 20), and MCT-control (n = 30) groups by random sampling to achieve equal body weights between groups. The animals received daily oral administration for 3 weeks up to 6 weeks (Day 41), and body weight was measured periodically. During the study period, the animals were observed daily for mortality, and median survival time in each group was calculated with Kaplan-Meier analysis.

Measurement of arterial blood gas

Rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) after the end of survival observation, and an 18G winged hypodermic needle for infusion was inserted into the abdominal aorta for blood sampling. Using the collected blood, pH, partial pressure of oxygen in arterial blood (PaO₂), partial pressure of carbon dioxide (PaCO₂), and arterial oxygen saturation (SaO₂) were measured using a blood analysis system (AVL OPTI CCA; Sysmex Co., Hyogo).

Measurement of cGMP level in lungs

Lungs in the rats at 3 weeks after MCT injection and at 6 weeks after MCT injection were removed and the wet weights were measured. Whole cGMP levels in the excised lungs were measured with radioimmunoassay using a cGMP assay kit (Yamasa Co., Chiba). Specifically, cGMP level in the standard solution and a sample solution were succinylated with succinylation reagents. The succinylated cGMP and ¹²⁵I-succinylated cGMP tyrosine methyl ester were competitively reacted with anti-cGMP monoclonal antibody (anti-serum). The ¹²⁵I-succinylated cGMP tyrosine methyl ester that had not been bound to the antibody during the competitive reaction was removed by absorption to dextran-coated activated carbon. The radioactivity of the supernatant was measured to calculate the cGMP level in the sample solution, and the amount (pmol) in pulmonary tissue (1 gram) was calculated.

Statistical analyses

Statistical evaluation was performed by statistical software (SAS, ver. 8.2; SAS Institute, Inc., Cary, NC, USA), and results are expressed as the mean ± S.E.M. To confirm the establishment of the MCT-induced PAH model and our assay sensitivity, data between two

groups (non-MCT control group vs. MCT-control group or MCT-control group vs. Positive control group) were analyzed using the F test followed by Student's *t*-test when the variances were homogeneous or by Aspin-Welch's *t*-test when the variances were heterogeneous. Multiple comparison tests, Bartlett's test for homogeneity of variance followed by Dunnett's test when the variances were homogeneous or by a Dunnett-type test using a rank order when the variances were heterogeneous, were performed among the MCT-control group and 3 tadalafil groups (0.5, 2.5, and 10 mg/kg per day). Survival curves were derived by the Kaplan-Meier method and compared by Log-Rank tests. A *P*-value less than 0.05 was considered statistically significant.

Results

Effects on hemodynamics

The effects of early treatment with tadalafil on MPAP are shown in Fig. 1. MPAP in the MCT-control group was 22.7 ± 1.3 mmHg, showing a significant increase in comparison to 15.1 ± 0.9 mmHg in the non-MCT control group (*P* < 0.01, Student's *t*-test). Sildenafil (25 mg/kg per day, b.i.d.), a positive control, significantly suppressed the MPAP (*P* < 0.01, Student's *t*-test) by 14.3 ± 1.8 mmHg. From these results, the test system (early treatment) was confirmed.

Fig. 1. Effects of tadalafil on mean pulmonary arterial pressure (MPAP) in monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) in rats (early treatment). Data of MPAP after 3 weeks treatment of tadalafil, sildenafil, or vehicle are reported as the mean ± S.E.M. (n = 5/group), and the shaded zone (15.1 ± 0.9 mmHg) represents the range of MPAP in the non-MCT control group. The hatched bar chart represents the result of the positive control. ***P* < 0.01: Paired comparison between MCT-control group and non-MCT control group, Student's *t*-test. ††*P* < 0.01: Multiple comparison among tadalafil groups and MCT-control group, Dunnett's test. ##*P* < 0.01: Paired comparison between sildenafil group and MCT-control group, Student's *t*-test.

Table 1. Effects on hemodynamics and lung cGMP level at 3 weeks after MCT injection (early treatment)

Parameters	Groups	Non-MCT control	MCT-control	Tadalafil (mg/kg per day)			Sildenafil (mg/kg per day)
				0.5	2.5	10	25
Hemodynamics							
(n = 5/group)							
MPAP (mmHg)		15.1 ± 0.9	22.7 ± 1.3 ^{##}	17.4 ± 1.6	19.1 ± 1.4	15.4 ± 1.5 ^{††}	14.3 ± 1.8 ^{**}
SRVP (mmHg)		24.7 ± 1.5	43.3 ± 3.3 ^{##}	35.3 ± 3.5	36.7 ± 2.3	30.3 ± 2.7 [†]	28.9 ± 2.3 ^{**}
MBP (mmHg)		108.4 ± 9.3	85.3 ± 9.2	78.5 ± 12.8	73.2 ± 8.2	102.0 ± 9.2	74.4 ± 11.6
HR (bpm)		450 ± 15	406 ± 30	431 ± 28	430 ± 23	472 ± 15	402 ± 47
Lung cGMP level							
cGMP (pmol/g)		42 ± 5	59 ± 9	81 ± 23	118 ± 18	135 ± 24 [†]	150 ± 15 ^{**}
(vs. MCT-control)				(37%) [↑]	(100%) [↑]	(129%) [↑]	(154%) [↑]
		(n = 7)	(n = 8)	(n = 5)	(n = 5)	(n = 5)	(n = 5)

Data show the mean ± S.E.M. MCT, monocrotaline; MPAP, mean pulmonary arterial pressure; SRVP, systolic right ventricular pressure; MBP, mean systemic arterial pressure; HR, heart rate. ^{##}*P*<0.01: Paired comparison between MCT-control group vs. non-MCT control group, Student's *t*-test. [†]*P*<0.05, ^{††}*P*<0.01: Multiple comparison among Tadalafil groups and MCT-control group, Dunnett's test. ^{**}*P*<0.01: Paired comparison between Sildenafil group and MCT-control group, Student's *t*-test.

Tadalafil (0.5, 2.5, and 10 mg/kg per day, q.d.) suppressed the increased MPAP observed in the MCT-control group at all dose levels, with statistical significance observed with tadalafil at 10 mg/kg per day (*P*<0.01, Dunnett's test). The MPAP value (15.4 ± 1.5 mmHg) at 10 mg/kg per day of tadalafil remained in the normal range (15.1 ± 0.9 mmHg) as shown in the non-MCT control group. Additional hemodynamic effects are shown in Table 1. SRVP for tadalafil at all dose levels were lower than that in the MCT-control group and a statistically significant reduction was observed only with 10 mg/kg per day of tadalafil as well as MPAP. No significant differences were observed in the other hemodynamic parameters measured. Overall effects on hemodynamics with tadalafil at 10 mg/kg per day (q.d.) were comparable to those seen with sildenafil at 25 mg/kg per day (b.i.d.).

Effects on survival benefit

The body weights in the MCT-control group at 3 weeks after MCT injection were significantly lower than those in the non-MCT control group (*P*<0.01, Student's *t*-test). In the MCT-control group, body weight showed no increase and actually decreased further, from around 1 month after MCT injection, while the non-MCT control group showed a constant gain during the study period (Fig. 2A). Neither tadalafil nor sildenafil affected the changes observed in the MCT-control group. Approximately 1 month after MCT injection, deaths began to be observed and the survival rate in the control group was 40% at the end of the study (Fig. 2B). Sildenafil improved the survival rate by 75% with median survival time of 30 days (*P*<0.05, Log-

Rank test). From these results, the test system (late treatment) was also confirmed as well.

Tadalafil at 0.5, 2.5, and 10 mg/kg per day improved the survival rates, in a dose-dependent manner, by 55%, 60%, and 70%, respectively. In the tadalafil groups, since the survival rates did not drop below 50%, log-logistic distribution was applied to estimate the median value of 50% survival time. In the MCT-control group, the median survival time was 16 days (36 days after MCT administration), while the values in the groups treated with tadalafil at 0.5, 2.5, and 10 mg/kg per day were 22 days (42 days after MCT administration), 24 days (44 days after MCT administration), and 27 days (47 days after MCT administration, *P*<0.05, Log-Rank test), respectively, showing the dose-dependent extension of the survival time. The survival benefit of tadalafil at 10 mg/kg per day was similar to that observed with sildenafil at 25 mg/kg per day.

Effect on blood gas

Summary of pH and partial pressure of blood gas is shown in Table 2. The PaO₂ and SaO₂ in the MCT-control group was significantly reduced compared to those in the non-MCT control group by 66 ± 4 mmHg (*P*<0.01, Student's *t*-test) and 86 ± 3% (*P*<0.01, Aspin-Welch's *t*-test), respectively. Tadalafil at 10 mg/kg per day prevented the reduction and improved them (PaO₂ and SaO₂) by 87 ± 5 mmHg and 92 ± 2%, respectively, but they were not significantly different from those of the MCT-control. Sildenafil at 25 mg/kg per day had also no significant effect on the values (PaO₂, 73 ± 5 mmHg; SaO₂, 88 ± 2%) as well as the other parameters measured.

Fig. 2. Effect of tadalafil on changes of body weights and survival rates (late treatment). A) Body weight was measured twice a week and the figure shows the mean $\pm$ S.E.M. for $n = 10 - 15$ /group. $^{##}P < 0.01$: Paired comparison between MCT-control group and non-MCT control group, Student's t -test. B) Mortality was observed daily and the figure shows the survival rate at each time point, setting the survival rate at the start of treatment on Day 21 as 100%. $^{##}P < 0.01$: Paired comparison between the MCT control group and non-MCT control group, Log-Rank test. $^{\dagger}P < 0.05$: Multiple comparison among tadalafil groups and MCT-control group, Log-Rank test adjusted for multiplicity with Bonferroni analysis. $^{*}P < 0.05$: Paired comparison between sildenafil group and MCT-control group, Log-Rank test.

Table 2. Effects of tadalafil on arterial blood gases and pH (late treatment)

Group	Dose (mg/kg per day)	pH	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	SaO ₂ (%)
Non-MCT control	0	7.45 $\pm$ 0.01 (10)	39 $\pm$ 2 (10)	97 $\pm$ 4 (10)	95 $\pm$ 1 (10)
MCT-control	0	7.43 $\pm$ 0.03 (11)	38 $\pm$ 4 (11)	66 $\pm$ 4 $^{##}$ (11)	86 $\pm$ 3 ** (11)
Tadalafil	0.5	7.44 $\pm$ 0.03 (10)	37 $\pm$ 4 (10)	79 $\pm$ 6 (10)	88 $\pm$ 4 (10)
	2.5	7.39 $\pm$ 0.05 (11)	37 $\pm$ 6 (11)	79 $\pm$ 9 (11)	88 $\pm$ 3 (11)
	10	7.44 $\pm$ 0.03 (14)	32 $\pm$ 2 (14)	87 $\pm$ 5 (14)	92 $\pm$ 2 (14)
Sildenafil	25	7.46 $\pm$ 0.01 (15)	33 $\pm$ 2 (15)	73 $\pm$ 5 (15)	88 $\pm$ 2 (14)

Arterial blood gases and pH in rats surviving up to 6 weeks after MCT injection were measured. Data show the mean $\pm$ S.E.M. The numbers in parentheses indicate the number of animals in each group. $^{##}P < 0.01$: Paired comparison between MCT-control group and non-MCT control group, Student's t -test. $^{**}P < 0.01$: Paired comparison between MCT-control group and non-MCT control group, Aspin-Welch's t -test.

Effects on RV weight ratio

RV weight ratio, RV/(LV + S), is shown in Fig. 3. In the early treatment study, at 3 weeks after MCT-injection, the RV/(LV + S) in the MCT-control group was 0.27 ± 0.02 , which was significantly higher than that (0.19 ± 0.01) in the non-MCT control group ($P < 0.05$, Aspin-Welch's t -test). No significant difference was observed in the RV/(LV + S) with any tadalafil dose (Fig. 3A). In the late treatment study, 6 weeks after MCT-injection, the RV/(LV + S) in the MCT-control group was 0.50 ± 0.02 , more than 3 times higher than the value of the non-MCT control group (0.15 ± 0.01), showing significant RV hypertrophy ($P < 0.01$, Aspin-Welch's t -test). Similar increases were observed for all tadalafil doses, although this was less marked with tadalafil at 10 mg/kg per day (Fig. 3B). The RV ratios

with sildenafil at 25 mg/kg per day showed marked reduction in both treatments.

Effects on lung cGMP

In the early treatment study, 3 weeks after MCT-injection, there was no significant change in pulmonary cGMP levels in the MCT-control group in comparison to that in the non-MCT control group (Table 1). Tadalafil dose-dependently increased pulmonary cGMP levels, but statistical significance was only observed at the highest dose of tadalafil ($P < 0.05$, Dunnett's test). In the late treatment study, 6 weeks after MCT-injection, tadalafil (0.5, 2.5, and 10 mg/kg per day) significantly increased the pulmonary cGMP at all dose levels by 119 ± 11 ($P < 0.05$), 187 ± 26 ($P < 0.01$), and 187 ± 23 pmol/g ($P < 0.01$, Dunnett-type mean rank test), respec-

Fig. 3. Effects of tadalafil on right ventricular (RV) weights in early and late treatment. After tadalafil, sildenafil, or vehicle was administered orally for 3 weeks, cardiac weights, RV, and left ventricle with ventricular septum (LV + S) were measured at 3 weeks (early treatment) (A) and 6 weeks (late treatment) (B) after MCT injection, respectively. Data are shown as the mean $\pm$ S.E.M., and the numbers in parentheses indicate the number of animals in each group. The shaded zones in the figures represent the normal range of the RV/(LV + S) in the non-MCT control group. The hatched bar chart represents the result of the positive control. $^{\#}P < 0.05$, $^{##}P < 0.01$: Paired comparison between MCT control group and non-MCT control group, Aspin-Welch's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$: Paired comparison between sildenafil and MCT-control group, Student's *t*-test.

Fig. 4. Effects of tadalafil on pulmonary cGMP levels (late treatment). Pulmonary cGMP in rats surviving up to 6 weeks after MCT injection was measured as described in Materials and Methods. The figure shows values of the mean $\pm$ S.E.M., and the numbers in parentheses indicate the number of animals in each group. The hatched bar chart represents the result of the positive control. $^{\#}P < 0.05$: Paired comparison between MCT control group and non-MCT control group, Aspin-Welch's *t*-test. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$: Multiple comparison among tadalafil groups and MCT-control group, Dunnett-type mean rank test. $^{**}P < 0.01$: Paired comparison between sildenafil and MCT-control group, Student's *t*-test.

tively (Fig. 4). Sildenafil had also increased the cGMP by 150 ± 15 ($P < 0.01$, Student's *t*-test) and 243 ± 34 pmol/g ($P < 0.01$, Student's *t*-test) in early and late treatment, respectively, as expected for a PDE5 inhibitor.

Discussion

The effects of tadalafil on PAH progression and survival benefit were investigated in MCT-induced PAH rats, employing the sildenafil group as a positive control. In our two studies, early and late treatment studies, the efficacy of sildenafil at 25 mg/kg per day (b.i.d.) was expectedly detected with statistical significance on MPAP and survival rate as already reported (34), which indicates that the MCT-induced PAH model had enough sensitivity to evaluate the efficacy of tadalafil. The doses of sildenafil (25 mg/kg per day, b.i.d.) and tadalafil (0.5, 2.5, and 10 mg/kg per day, q.d.) were expected to provide exposures in rats similar to those at the human clinical doses. The maximum plasma concentration after administration of sildenafil at 100 mg to humans ($C_{\max}$: 440 ng/mL, FDA sildenafil label 7/8/2005) was comparable to the $C_{\max}$ after administration of sildenafil at 45 mg/kg to rats (477 ng/mL, Application No. 020895), and thereby, sildenafil at 25 mg/kg in rats is considered to correspond to the approved clinical dose (60 mg/day) in humans. Tadalafil at 40 mg/day is the estimated highest clinical dose for PAH patients, with an expected exposure level in humans of 12,193 ng $\cdot$ h/mL. In rats treated with tadalafil at 10 mg/kg, the plasma exposure was 12,600 ng $\cdot$ h/mL. The lowest dose in this model was set at 0.5 mg/kg based on the lowest dose of 2.5 mg/day (596 ng $\cdot$ h/mL) tested in human clinical trials.

Fig. 5. Proposed mechanisms for the effects of tadalafil on each stage of PAH progression. Normal: The numbers presented on the image of the circulatory system show the normal values of mean blood pressure or systolic/diastolic pressure (mmHg) at each site, that is, the top and bottom grids show the normal pressure of pulmonary circulation and precapillary circulation, respectively, and the middle ones show the values in the normal heart (RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle). PAH: Left side figure shows the process of the PAH progression. Increase of mean pulmonary arterial pressure (MPAP) leads to the increase of mean right ventricular pressure (MRVP), and its overload to RV concomitantly causes compensatory RV hypertrophy. The enlarged RV shows significant phosphodiesterase 5 (PDE5) expression, which would reduce cGMP responses by atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) and contribute to the deterioration of RV failure. Tadalafil: A selective and sustained inhibitor of PDE5 that has a potential to maintain high levels of cGMP needed to relax pulmonary arteries, improve pulmonary hemodynamics through relaxation, and delay the progression to RV failure. In addition, even in the condition of RV failure, tadalafil may be able to maintain necessary pulmonary circulation through the improvement of RV contractility by cGMP-dependent inhibition of PDE3 in the enlarged RV and provide a good prognosis with improved partial pressure of oxygen in arterial blood (PaO_2), arterial oxygen saturation (SaO_2), and responses of ANP and BNP.

In the early phase treatment study, 3 weeks after MCT injection, only tadalafil at 10 mg/kg per day significantly decreased MPAP and SRVP relative to the MCT-injected control group and markedly increased pulmonary cGMP levels by 130% relative to the MCT-control group. In the late phase treatment study, 6 weeks after MCT injection, tadalafil significantly increased pulmonary cGMP at all dose levels with an approximately 200% maximum increase relative to the MCT-control group. These data suggest that the effect of tadalafil on pulmonary cGMP elevation is more potent in the late phase than the early phase of PAH. These results may support the effectiveness of tadalafil in patients with advanced PAH, such as WHO class IV.

Although the maximum cGMP level was similar for tadalafil at both 2.5 and 10 mg/kg per day (Fig. 4), survival time extended in dose-dependent fashion with significance at the highest dose. These results suggest that survival benefit would depend on the duration of functional cGMP levels in the lungs that can reduce

MPAP rather than a maximum level, because the retention time in lungs will correlate with the exposure of tadalafil that is proportionally elevated with the doses, and the longer sustained relaxation of the pulmonary vasculature will reduce RV stress and delay the time to RV failure. In addition, there have been reports that inhibition of PDE5 potentiated the response to natriuretic peptides (NPs) in failing hearts (37, 38). NPs such as atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) act protectively on the cardiovascular system through diuresis, vasodilation, and inhibition of the renin-angiotensin system. Although NPs increase in patients with heart failure, insufficient cGMP may be the reason this increase does not translate into benefit, as shown in a decreased ratio of cGMP/NPs, an index of NP resistance (37, 39, 40). Therefore, tadalafil may extend the survival time in advanced PAH by potentiating the NPs response. While the long term benefit of tadalafil is associated with the increase of cGMP in lungs, other factors might also be playing a

role.

Compared with the MCT-injected control group, the 10 mg/kg per day dose of tadalafil induced a significantly higher probability of survival with a tendency to increase the PaO_2 and SaO_2 to approximately normal levels. The oxygen delivery that tissues require for survival will depend on adequate ventilation, gas exchange, and circulatory distribution. In the MCT-control group, PaO_2 and SaO_2 in arterial blood displayed significant reduction but PaCO_2 and pH values were comparable to those in the non-MCT control group. If pulmonary blood flow decreases, the PaO_2 but not PaCO_2 will decrease because the diffusion velocity of O_2 is about 40 times slower than that of CO_2 . Therefore, it is suggested that the significant reduction of PaO_2 in the MCT-control group would be attributed to the decreased pulmonary blood flow, and the elevation of PaO_2 by tadalafil is a consequence of an improvement of pulmonary blood circulation.

It is interesting, however, that the improved survival, the elevated cGMP levels, and the apparently improved pulmonary blood flow afforded by tadalafil did not translate into a reduction in RV hypertrophy, especially at the low and middle doses of tadalafil. Nagendran et al. has shown no expression of PDE5 in the normal RV and significant upregulation of PDE5 in hypertrophied RV (RVH) in rats and humans (41). In addition, they have demonstrated PDE5 inhibitors significantly increased RV contractility accompanied by increased cGMP and cAMP in RVH but not in normal RV (41). Thus, it has been hypothesized that tadalafil inactivates cGMP-sensitive PDE3 through the cGMP increased by the specific inhibition of PDE5, which elevates cardiac cAMP to improve RV contractility and so maintain the pulmonary blood flow. Thus, it is possible that the low and middle doses of tadalafil were insufficient to significantly decrease pulmonary blood pressure and so RV hypertrophy developed, but since the PDE3 is inactivated by cGMP, tadalafil could improve the attenuated RV contractility by blocking the rapid degradation of cGMP caused by the upregulated PDE5 and so improve O_2 gas exchanges in lungs. On the other hand, the highest dose of tadalafil was able to relieve the RV overload by significantly reducing pulmonary blood pressure and thereby maintaining pulmonary circulation and attenuating RV hypertrophy much better than the other doses.

There apparently seems to be a slight difference in the maximum effects between tadalafil at 10 mg/kg per day and sildenafil at 25 mg/kg per day on each measurement item, which might be coming from the difference of pharmacokinetics in lungs and the heart and so on, and other unknown mechanisms; however, overall efficacies

are quite similar in for both of the PDE5 inhibitors at the selected doses and frequency of the treatment.

Taken together, tadalafil at 10 mg/kg per day attenuated PAH progression and reduced mortality in the MCT-induced PAH rat model. The effects on pulmonary hemodynamics were also associated with elevated cGMP in lungs. These results suggest that tadalafil, by selective and sustained inhibition of PDE5, can maintain the high levels of cGMP needed to relax pulmonary arteries, improve pulmonary hemodynamics and blood gas exchange, and delay the progression to RV failure (Fig. 5). The significant effects of tadalafil at 10 mg/kg per day (q.d.), whose dose is comparable to a human dose of 40 mg/day, were demonstrated at both early and late disease stages of PAH, and these effects were similar to those observed with sildenafil at 25 mg/kg per day (b.i.d). Thus, it is expected that tadalafil would be an effective, once daily treatment option in humans with PAH.

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