Endothelial nitric oxide synthase-enhancing G-protein coupled receptor antagonist inhibits pulmonary artery hypertension by endothelin-1-dependent and endothelin-1-independent pathways in a monocrotaline model

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Abstract This study investigates whether endothelin-1 (ET-1) mediates monocrotaline (MCT)-induced pulmonary artery hypertension (PAH) and right ventricular hypertrophy (RVH), and if so, whether the G-protein coupled receptor antagonist KMUP-1 (7-{2-[4-(2-chlorobenzene)piperazinyl]ethyl}-1,3-dimethylxanthine) inhibits ET-1-mediated PA constriction and the aforementioned pathological changes. In a chronic rat model, intraperitoneal MCT (60 mg/kg) induced PAH and increased PA medial wall thickening and RV/left ventricle + septum weight ratio on Day 21 after MCT injection. Treatment with sublingual KMUP-1 (2.5 mg/kg/day) for 21 days prevented these changes and restored vascular endothelial nitric oxide synthase (eNOS) immunohistochemical staining of lung tissues. Western blotting analysis demonstrated
that KMUP-1 enhanced eNOS, soluble guanylate cyclase, and protein kinase G levels, and reduced ET-1 expression and inactivated Rho kinase II (ROCKII) in MCT-treated lung tissue over long-term administration. In MCT-treated rats, KMUP-1 decreased plasma ET-1 on Day 21. KMUP-1 (3.6 mg/kg) maximally appeared at 0.25 hours in the plasma and declined to basal levels within 24 hours after sublingual administration. In isolated PA of MCT-treated rats, compared with control and pretreatment with L-NG-nitroarginine methyl ester (100 μM), KMUP-1 (0.1–100 μM) inhibited ET-1 (0.01 μM)-induced vasoconstriction. Endothelium-denuded PA sustained higher contractility in the presence of KMUP-1. In a 24-hour culture of smooth muscle cells (i.e., PA smooth muscle cells or PASMCs), KMUP-1 (0.1–10 μM) inhibited Rhoa- and ET-1-induced Rhoa activation. KMUP-1 prevented MCT-induced PAH, PA wall thickening, and RVH by enhancing eNOS and suppressing ET-1/ROCKII expression. In vitro, KMUP-1 inhibited ET-1-induced PA constriction and ET-1-dependent/independent Rhoa activation of PASMCs. In summary, KMUP-1 attenuates ET-1-induced/ET-1-mediated PA constriction, and could thus aid in the treatment of PAH caused by MCT.

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Introduction

This study investigated the role of endothelin-1 (ET-1) in monocrotaline (MCT)-induced rat pulmonary artery (PA) constriction, chronic PA hypertension (PAH), and right ventricular hypertrophy (RVH), as well as the results of treatment with KMUP-1. ET-1 is involved in Rhoa activation and Rho kinase (ROCK) expression in hypoxic PAH, but its expression is less clear in MCT-induced PAH. In previous studies, KMUP-1 inhibited angiotensin II (Ang II)-induced Ca²⁺ influx and contraction of pulmonary vascular smooth muscle cells. KMUP-1 has been reported to inhibit PAH partly through the serotonin (5-hydroxytryptamine or 5-HT) pathway [1,2]. ET-1, Ang II, catecholamine, and 5-HT can all mediate pulmonary arterial Rhoa/ROCK activation by nonspecific G-protein coupled receptors (GPCRs) [1–4]. To date, bosentan is the representative PAH inhibitor for inhibiting ET-1-mediated response in the clinic. At present, no competitors of bosentan are more effective for inhibiting PAH. Antagonists of nonspecific GPCRs, including those involving the ET-1 receptor, with NO/endothelial nitric oxide synthase (eNOS)-enhancing activity may be alternatively used instead of, or in combination with, bosentan or sildenafil for inhibiting ET-1-mediated PAH caused by MCT.

PAH is a fatal disease characterized by increased pulmonary vascular pressure and progressive structural remodeling in the PA, leading to RV failure and premature death. In the development of PAH, altered production of various endothelial vasoactive mediators such as NO, prostacyclin, ET-1, 5-HT, chemokines, and thromboxane had been increasingly recognized in patients [5]. Among these mediators, ET-1 released from the endothelium, leading to PA vasoconstriction and cell proliferation, is mainly involved in the pathogenesis of endothelial dysfunction [6].

Therefore, we measured the expression of colocalized enzyme and protein biomarkers such as eNOS, soluble guanylate cyclase (sGC), protein kinase G (PKG), ROCKII, and ET-1 in lung tissues from MCT-treated rats with and without treatment with KMUP-1. Regulation of upstream eNOS, sGC, and PKG expression and downstream Rhoa/ROCK activation in the cyclic guanosine monophosphate (cGMP)-signaling pathway affects vascular contractility and resistance [7]. Initial activation of eNOS in the endothelium to release NO provides smooth muscle relaxation through the cGMP/ROCK pathway, and colocalized eNOS, sGC, and PKG expression in the PA is important for inhibiting PAH [1,8–10]. However, the relationship between ET-1 and eNOS/sGC/PKG/Rhoa expression in PAH treated with KMUP-1 remains to be investigated. We hypothesized that pulmonary colocalized ET-1, Rhoa, and eNOS expression or activation of GPCRs are the major therapeutic targets in the treatment of PAH.

To date, statins, ROCK inhibitors, and the cGMP-enhancing phosphodiesterase 5A (PDE-5A) inhibitor sildenafil have been described as beneficial for treating PAH [11–17]. Unfortunately, sildenafil requires combination therapy for the treatment of children’s PAH [18]. More effective agents for treating PAH by inhibiting the GPCRs agonist ET-1/Ang II/5-HT and enhancing NO/eNOS are urgently needed for patients. Statins might exert pleiotropic effects by inhibiting small guanosine triphosphate (GTP)-binding proteins to enhance eNOS expression and decrease the transcription of prepro ET-1 messenger RNA (mRNA), resulting in the reduction of endothelial ET-1 [19]. The pleiotropic effects of statins encouraged us to search for a nonstatin molecule that could increase eNOS and reduce ET-1 expression of PA endothelium in lung tissue, thereby inhibiting ET-1 release-mediated PAH caused by MCT and associated hypoxia [20–22].

KMUP-1 is known to enhance eNOS/cGMP in various animal tissues [23–25]. In comparison, sildenafil enhances the activation of cGMP-dependent protein kinase (i.e., PKG) by inhibiting PDE-5A, which separately suppresses Rhoa activation and cell proliferation [26]. Notably, bosentan and sildenafil have been combined to achieve full inhibition of PAH, suppressing 5-HT and ET-1 [27,28]. However, it has been argued that eNOS expression can be potentiated without reduced expression of PDE-5A in PAH [1,17]. 5-HT plays an important role in PAH [5]. Whether KMUP-1 suppresses ET-1, in addition to inhibiting pulmonary 5-HT, in PAH remains unclear. In this study, the effects of KMUP-1 on MCT-induced
PAH pathogenesis, PA contractility, and expression of RhoA and eNOS in PA smooth muscle cells (PASMCs) were examined to expose the role of ET-1 in MCT-induced PAH \[29\].

**Methods**

**Compounds**

KMUP-1·HCl \(7\{-[4\{-2\{-chlorobenzene\}piperazinyl\}ethyl\}-1,3-dimethylxanthine·HCl\} was synthesized from KMUP-1 in our laboratory \[1\]. Sildenafil was supplied by Cadila Healthcare Ltd. (Maninagar, Gujarat, India). (3R,4R,5R,13aR,13bR)-4,5-dihydroxy-3,4,5-trimethyl-4,5,8,10,12,13,13a,13b-octahydro-2H-[1,6]dioxacycloundecino[2,3,4-gh]

**Animals**

All experimental procedures were approved by the Animal Center of Kaohsiung Medical University (KMU; Kaohsiung, Taiwan). The protocol for this study was approved by KMU for submission to this journal by the Institutional Review Board. In this study, adult male Wistar rats weighing 200–250 g received a single subcutaneous injection of MCT (60 mg/kg) or vehicle and were allowed 21 days to develop PAH. A sublingual preparation of KMUP-1 (2.5 mg/kg/day/25 μL propylene glycol) was applied by micropipette to the sublingual cavity. Micropipette application of KMUP-1 required less than 1 minute and analgesia was not needed. The rats were divided into four experimental groups, namely, Group 1 (normal controls), Group 2 (MCT treated), Group 3 (MCT-treated rats receiving sildenafil), and Group 4 (MCT-treated rats receiving sildenafil).

**Western blotting analysis**

To measure protein expression levels, the total proteins of experimental rats were isolated and cut into small chips used in the Western blot analyses. Whole right lung tissues were extracted and Western blotting analyses were performed as described previously \[1\]. The eNOS, sGC1α, PKG, RhoA, ROCKII, and ET-1 expression in lung tissues after KMUP-1 and sildenafil administration were measured from the four experimental groups of rats. Mouse or rabbit monoclonal antibodies to eNOS (Millipore, Temecula, CA, USA), sGC1α (Sigma-Aldrich), PKG (Calbiochem, San Diego, CA, USA), RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ROCKII (Millipore), ET-1 (Abcam, London, UK), and the loading control protein β-actin (Sigma-Aldrich) were used in the Western blot analyses. Whole right lung tissues of experimental rats were isolated and cut into small chips to extract protein as described previously \[1\]. The protein extract was then boiled in a ratio of 5:1 with sample buffer (Tris 100 mM, pH 6.8, glycerol 20%, sodium dodecyl sulfate (SDS) 4%, and bromophenol blue 0.2%). Electrophoresis was performed using 10% SDS–polyacrylamide gel (2 hours, 100 V, 40 mA, 20-μg protein per lane). The separated proteins were transferred to polyvinylidene difluoride
membranes treated with 5% fat-free milk powder to block the nonspecific immunoglobulin G (IgG; 90 minutes, 100 V) and incubated for 1 hour with specific antibody. The blot was then incubated with antimouse or antigoat IgG linked to alkaline phosphatase (1:1000) for 1 hour. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibodies and subsequently enhanced chemiluminescence for the detection of the specific antigen. The intensity of the bands was measured by densitometry.

Preparation of PASMCs

MCT-treated or nontreated rats were killed with pentobarbital sodium (60 mg/kg). For culturing PA PASMCs, the PA rings were gently separated from the underlying connective tissue and then chopped and placed into a dish and incubated with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 0.24% NaHCO3 and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO2/95% air. The culture medium was changed every 3 days and cells were subcultured until confluence. Primary cultures of two to four passages were used in the experiments. Cells were examined by immunofluorescence staining for β-actin (Sigma-Aldrich) to confirm the purity of the PASMCs. Over 95% of the cell preparations were found to be composed of smooth muscle cells.

RhoA activation of PASMCs

RhoA activation was determined using an affinity precipitation assay, which binds only the active GTP-bound form of Rho. In brief, subconfluent PASMCs were grown to approximately 85–90% confluence and the cells were exposed to KMUP-1 (0.1 μM, 1 μM, and 10 μM) for 24 hours and to ET-1 (0.01 μM) for 30 minutes with 10% fetal bovine saline at 37°C prior to adding lysis buffer (25 mM hydroxyethyl piperazineethanesulfonic acid, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 10 mM MgCl2, 1 mM ethylenediaminetetraacetic acid and 10% glycerol, 1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM Na3VO4) for 15 minutes at 4°C. The plasma concentrations of KMUP-1 (lower range: 0.01 ng/mL, higher range: 0.1 ng/mL) for 24 hours and to ET-1 (0.01 ng/mL) for 30 minutes with 10% fetal bovine saline at 37°C prior to adding lysis buffer (25 mM hydroxyethyl piperazineethanesulfonic acid, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 10 mM MgCl2, 1 mM ethylenediaminetetraacetic acid and 10% glycerol, 1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM Na3VO4) for 15 minutes at 4°C. Cell lysates were centrifuged for 15 minutes at 4°C for 10 minutes. Equal volumes of lysates were incubated with agarose-conjugated rhotein Rho-binding domain for 45 minutes at 4°C, and then washed three times with lysis buffer. Agarose beads were boiled in SDS-polyacrylamide gel electrophoresis sample buffer to release active RhoA, and the sample was resolved on a 12% polyacrylamide gel followed by immunoblotting with anti-RhoA (Clone 55).

Hematoxylin–eosin staining

The right lobes of the rat lungs and the heart of six rats from each group were cut and soaked in formalin, dehydrated through graded alcohols, and embedded in paraffin wax. The lung-tissue specimens fixed with formalin were embedded in paraffin, cut into 4-μm-thick sections and subjected to hematoxylin–eosin (H&E) staining prior to performing light microscopic examination. The histopathological changes were evaluated in terms of the thickening of the medial wall of the small intrapulmonary arteries. In heart tissues, 4-μm paraffin sections were cut from paraffin-embedded tissue blocks. The tissue sections were deparaffinized by immersing them in xylene and rehydrated as described previously [1]. For H&E staining, the slices were dyed with H&E. After gentle rinsing with water, each slide was dehydrated through graded alcohols. Finally, they were soaked twice in xylene. Photomicrographs were obtained using Eclipse TE2000-S (Nikon, Tokyo, Japan) microscopes.

Immunohistochemistry

For light microscopic examination, samples of the right lung and the heart of six rats were obtained from each group. The samples were fixed in formalin and embedded in paraffin, and 4-μm sections were mounted onto Superfrost slides. The right lung and heart sections were stained with H&E to assess vascular and cardiac morphology. In each group, medial thickness (micrometer) and medial wall area (calculated as the area between the internal elastic lamina and the adventitia in micrometer) of the muscular layer of pulmonary arteries were determined using an Eclipse TE2000-S microscope (Nikon) coupled to a color video camera (Nikon). The relative weight ratio (i.e., right heart index) is calculated as follows:

\[
\text{Relative weight ratio} = \frac{\text{right ventricle (RV)}}{\text{left ventricle (LV)}} + \frac{\text{intraventricular septum (S)}}{\text{right ventricle (RV) + septum (RV/LV + S)}}
\]

The relative weight ratio was demonstrated by RV/left ventricle + septum (RV/LV + S) using H&E staining. Measurements were obtained using HistoLab software (GT Vision Ltd, Haverhill, UK). Six PA measurements in each animal were taken on average. The eNOS antibody (1:100) was selected to carry out immunostaining on the right lung sections.

Plasma concentrations of ET-1

The plasma concentration of ET-1 was determined using an enzyme immunoassay kit according to the manufacturer’s instructions (Biomedica Group, Wien, Austria). Cardiac needle puncture (19 G) was performed to obtain blood, followed by centrifugation for plasma sampling. Plasma samples (0.8–1.0 mL) were acidified with 0.6% trifluoroacetic acid and centrifuged (2000g, 4°C for 15 minutes).

Plasma concentrations of KMUP-1

The plasma concentrations of KMUP-1 (lower range: 0.1–20 ng/mL and higher range: 10–5000 ng/mL) in rats receiving 3.6 mg/kg KMUP-1·HCl dissolved in propylene glycol (25 μL) sublingually administered with a micropipette were measured by the liquid chromatography-tandem mass spectrometry method. In high-performance liquid chromatography conditions—mobile phase, 23% CH3CN + 1% HCOOH; column, Luna C18, 2.0 mm × 50 mm, 5 mm (Phenomenex, Torrance, CA, USA); and flow rate, 0.2 mL/minute—measurements were carried out using a mass spectrometer (Quattro Ultima, Micromass Ltd., Manchester, UK), concomitantly using an autosampler (Waters Alliance 2790 LC, Waters, MA, USA) and data processor (MassLynx...
version 3.5; Micromass Ltd.). Plasma concentrations were measured at 0 minutes, 2 minutes, 5 minutes, 10 minutes, and at 0.25 hours, 0.5 hours, 1 hour, 1.5 hours, 2 hours, 4 hours, 6 hours, 9 hours, and 24 hours.

**Statistical analyses**

The values are expressed as the mean ± standard error of the mean. The significance of difference was calculated by Dunnett’s test. A p value < 0.05 was taken to be significant.

**Results**

**MPAP, MABP, and body weight**

As shown in Table 1, the MPAP of normal control rats (Group 1) was 12.9 ± 0.9 mmHg (n = 6). This value sharply shifted to 30.9 ± 2.9 mmHg (n = 6) in MCT-treated rats (Group 2). After long-term treatment of MCT-treated rats with sublingual KMUP-1 (2.5 mg/kg/day) or sildenafil (2.5 mg/kg/day) for 21 days, PAH was markedly attenuated to 16.9 ± 1.1 mmHg (Group 3) and 19.8 ± 0.7 mmHg (Group 4; n = 6) on the last day, respectively. MABP and heart rate were not significantly changed after treatment with KMUP-1 or sildenafil. Body weight after treatment with KMUP-1 and sildenafil was increased from 310 ± 5.6 g (Group 2) to 334 ± 4.5 g (Group 3) and 328.6 ± 8.3 g (Group 4), respectively, compared with 373 ± 5.4 g in nontreated rats (Group 1).

MCT-induced MPAP was significantly reversed by treatment with sublingual KMUP-1 and sildenafil (p < 0.05, treatment vs. MCT). KMUP-1 was more potent than sildenafil for inhibiting MCT-induced MPAP.

**PA contractility**

The concentration-dependent effects of KMUP-1 (0.1–100 μM) on ET-1-induced vascular constriction were examined in endothelium-intact (Fig. 1A) and endothelium-denuded (Fig. 1B) PA preparations from control rats or PA preparations (Fig. 1C) from MCT-treated rats. KMUP-1 concentration-dependently inhibited ET-1-induced constriction in endothelium-intact PA preparations. In endothelium-denuded PA, KMUP-1 at concentrations from 0.1 μM to 10 μM failed to inhibit ET-1-induced constriction. At 100 μM, KMUP-1 sharply inhibited ET-1-induced constriction of endothelium-denuded PAs. In PAs from MCT-treated rats, KMUP-1-induced relaxation was reduced compared with non-MCT controls (Fig. 1C vs. Fig. 1A). L-NAME pretreatment did not alter the response of PAs from MCT rats to ET-1 and KMUP-1 (Fig. 1C and D), indicating endothelium dysfunction in PAs from MCT rats. The estimated inhibition of ET-1-induced PA contractility is shown in Fig. 1E.

**Pulmonary vascular wall thickening and H&E staining**

The PA wall thickness (%) of MCT-treated rats was measured on Day 0 and Day 21 following right lung resection. As shown in Fig. 2A, sections stained with H&E indicated that muscularization of the distal PA was significantly lower in MCT-treated rats that were given KMUP-1 (Group 3) or sildenafil (Group 4), compared with vehicle only (Group 2). Results showed that small pulmonary arterial morphology was highly improved in KMUP-1-treated rats (Fig. 2B).

**Immunohistochemical staining of eNOS**

Morphometric immunostaining of Group 2 lung sections demonstrated a marked decrease of eNOS mainly in the PA endothelium, correlated with thickening. Treatments with KMUP-1 or sildenafil restored eNOS immunostaining. The negative control did not display brown endothelial staining (Fig. 2C).

**PAH-associated RVH**

MCT injection increased the relative weight and area of the rat RV/LV + S ratio, that is, the right heart weight index and area index, to 40.9% ± 4.5% and 35.2% ± 1.7%, respectively (Group 2) compared with normal rats (Group 1; Fig. 3A and B). KMUP-1 reduced the right heart weight index to 30.6 ± 1.8% (Group 3; Fig. 3A; p < 0.05, vs. MCT). Sildenafil decreased the right heart weight index to 31.1 ± 2.7% (Group 4; Fig. 3A; p < 0.05, vs. MCT).

**Expression of ET-1, ROCKII, eNOS, sGC1α, and PKG**

Changes in ET-1 expression are shown in Fig. 4A. We found that MCT induced significant ET-1 expression in lung parenchyma tissue compared with the control group. Both

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**Table 1 Effects on MCT-induced body weight, MABP, heart rate, and MPAP of rats treated with KMUP-1 and sildenafil.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>MABP (mmHg)</th>
<th>Heart rate (beats/min)</th>
<th>MPAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>373 ± 5.4</td>
<td>109 ± 4.4</td>
<td>375 ± 25</td>
<td>12.9 ± 0.9</td>
</tr>
<tr>
<td>MCT</td>
<td>310 ± 5.6*</td>
<td>102.7 ± 7.0</td>
<td>354 ± 37</td>
<td>30.9 ± 2.9*</td>
</tr>
<tr>
<td>MCT + KMUP-1</td>
<td>334 ± 4.5**</td>
<td>101 ± 3.8</td>
<td>370 ± 18</td>
<td>16.9 ± 0.1**</td>
</tr>
<tr>
<td>MCT + sildenafil</td>
<td>328.6 ± 8.3**</td>
<td>105.2 ± 4.0</td>
<td>381 ± 22</td>
<td>19.8 ± 0.9</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard error of the mean.

CTL = control; MABP = mean artery blood pressure; MCT = monocrotaline; MPAP = mean pulmonary arterial pressure.

*a p < 0.05, compared with the CTL group.

*b p < 0.05, compared with the MCT group (analysis of variance followed by Bonferroni test; n = 6).

**Effects on body weight, MABP, heart rate, and MPAP in rats treated with MCT, MCT + KMUP-1 or MCT + sildenafil at Day 21: The MCT injection sharply increased MPAP levels, and sublingual administration of KMUP-1 (2.5 mg/kg/day) markedly improved MPAP.**
KMUP-1 and sildenafil reduced the expression of ET-1 caused by MCT. Expression of ROCKII was increased to 354.3% in MCT-treated rats after 21 days. Treatment with KMUP-1 and sildenafil during this period reduced the increase of ROCKII to 150.2% and 97.1% respectively (Fig. 4B). KMUP-1 was not significantly different from sildenafil for decreasing ROCKII expression.

As shown in Fig. 4C and D, Western blotting analysis demonstrated that eNOS and sGC1α in lung tissues of MCT-treated rats were increased by KMUP-1 to 315.5% and 203.4% (Group 3) and by sildenafil to 180.3% and 109.2% (Group 4), respectively. KMUP-1 was more potent than sildenafil in increasing the expressions of eNOS and sGC. In addition, KMUP-1 increased PKG to 140.3% more potently than sildenafil 76.6% (Group 4), compared with the non-treated rats (Group 2; Fig. 4E).

RhoA inactivation

Fig. 5A shows that KMUP-1 inhibited RhoA activation in PASMCs in a concentration-dependent manner. RhoA activation was directly inhibited by KMUP-1 in PASMCs treated for 24 hours.

ET-1-induced RhoA activation

Fig. 5B shows that ET-1 (0.01 μM) induced RhoA activation in cultured PASMCs. RhoA activation was raised in cells stimulated with ET-1 for 30 minutes compared with control cells (p < 0.05). Pretreatment of PASMCs with KMUP-1 (0.1 μM, 1.0 μM, and 10 μM) for 24 hours also concentration-dependently suppressed ET-1-induced RhoA activation, which was fully achieved at 10 μM.

Plasma ET-1

Changes in ET-1 plasma concentration due to MCT injection and treatment with KMUP-1 are shown in Fig. 6. The MCT + KMUP-1 group (Group 3) displayed a lower level of ET-1 (0.05 ± 0.02 ng/mL), compared with the MCT-treated group (Group 2; 0.16 ± 0.06 ng/mL), but higher than the control group (Group 1; 0.007 ± 0.002 ng/mL).

Plasma KMUP-1

Changes in KMUP-1 plasma concentration are shown in Fig. 7A and B. Sample concentrations below 0.1 ng/mL
were reported as below the lower limit of quantification. The concentration was detectable within 6 hours, maximally at 0.25 hours, and trace KMUP-1 was found at 24 hours.

**Discussion**

KMUP-1 inhibits PAH by increasing eNOS, sGC, and PKG and decreasing ROCKII and ET-1 expression in MCT-treated rat.

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**Figure 2.** Effects of KMUP-1 and sildenafil on (A) pulmonary vascular morphology, (B) wall thickness, (C) eNOS immunoreactivity. After intraperitoneal administration of MCT (60 mg/kg), KMUP-1 and sildenafil (2.5 mg/kg) were administered once daily for 21 days. PAs were stained with hematoxylin–eosin (A) for eNOS immunoreactivity (brown) in PA endothelium (C). Scale bar = (A) 100 μm and (C) 20 μm. Bar chart represents the mean ± standard error of the mean of six experiments. *p < 0.05, compared with CTL, and **p < 0.05, compared with MCT (analysis of variance followed by Bonferroni test). CTL = control; eNOS = endothelial nitric oxide synthase; MCT = monocrotaline-treated group; MCT + KMUP-1 = rats treated with MCT and KMUP-1; MCT + sildenafil = rats treated with MCT and sildenafil; negative CTL = control rats without immunostaining; PA = pulmonary artery.

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**Figure 3.** Effects of KMUP-1 and sildenafil on MCT-induced RVH. (A) MCT + sildenafil. (B) KMUP-1 and sildenafil improved MCT-induced RVH represented by weight ratio. KMUP-1 or sildenafil was sublingually administered once daily for 3 weeks (2.5 mg/kg/day). Bar chart represents the mean ± standard error of the mean of six experiments. *p < 0.01, compared with CTL; **p < 0.01, compared with MCT (analysis of variance followed by Bonferroni test). CTL = control group; LV = left ventricle; MCT = rats treated with monocrotaline; MCT + KMUP-1 = MCT rats treated with KMUP-1; MCT + sildenafil = MCT rats treated with sildenafil at 21 days; RV = right ventricle; RVH = right ventricular hypertrophy; S = septum.
Three treatment strategies for PAH are addressed in this study, namely: (1) activating eNOS/sGC to accumulate cGMP or increase PKG, targeting vascular relaxation [1]; (2) inhibiting ROCKII to desensitize Ca\(^{2+}\), targeting the reduction of vascular resistance [30]; and (3) inhibiting the activity of ET-1 to attenuate PA constriction, targeting the reduction of vascular constriction [5]. We measured the effects of KMUP-1 and sildenafil on MCT-induced PAH and related expression of eNOS, sGC, PKG, ROCKII, and ET-1, which are linked to PA function. Inhibition combined with ET-1, 5-HT, and \(\alpha_{1A/1D}\) receptors suggests that KMUP-1 is a GPCRs antagonist inhibiting PAH and related RhoA/ROCK activation [1,2,31,32].

KMUP-1 in MCT-treated rats is suggested to activate upstream eNOS and sGC by NO and to reduce downstream ROCK expression or RhoA activation in the cGMP pathway. PAH is partly attributed to impairment of cGMP/PKG signaling, involving increased ROCK expression to retard PKG-induced vasodilatation in hypoxia [33]. Downstream of the cGMP pathway, phosphorylation of RhoA interferes with ROCK and phosphorylation of inositol 1, 4, 5-trisphosphate (IP3) receptor-associated cGMP kinase substrate (IRAG) inhibits IP3/IRAG-mediated Ca\(^{2+}\) release from endoplasmic reticulum [1,34–37]. Increased expression of ROCK is associated with vascular resistance, which is attributed to Ca\(^{2+}\) sensitization [16]. KMUP-1 may attenuate MCT-induced PAH through the activation of eNOS and the inhibition of ROCK-associated Ca\(^{2+}\) desensitization in PASMCs. We demonstrated that the pharmacological activity of KMUP-1 is more potent than sildenafil in enhancing the expressions of eNOS, sGC, and PKG, and therefore KMUP-1 could be of greater benefit for inhibiting MCT-induced pulmonary vascular thickness and RVH. However, further investigations are needed in this regard.

l-NAME blocked the relaxation of the PA by KMUP-1 after ET-1-induced constriction, exposing the important role of eNOS expression and ET-1 in the PA. Western blotting analyses of ET-1 and eNOS in lung tissue, morphologic immunostaining of eNOS, and measurement of ET-1

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**Figure 4.** Effects of KMUP-1 and sildenafil on MCT-induced expression of (A) ET-1, (B) ROCKII, (C) eNOS, (D) sGC, and (E) PKG in lung tissues. Each value represents the mean \pm standard error of the mean of six experiments. *p < 0.05, compared with CTL; **p < 0.01 and ***p < 0.001, compared with MCT; "p < 0.05, compared with MCT + sildenafil (analysis of variance followed by Dunnett’s test). CTL = normal control group; ET-1 = endothelin-1; eNOS = endothelial nitric oxide synthase; MCT = rats treated with monocrotaline; MCT + KMUP-1 = rats treated with MCT and KMUP-1; MCT + sildenafil = rats treated with MCT and sildenafil; PKG = protein kinase G; ROCKII = Rho kinase II; sGC = soluble guanylate cyclase.
concentration in plasma confirm their interactive roles in PA endothelium. By preventing elevated ET-1 and suppressing eNOS in the dysfunctional endothelium, KMUP-1 is beneficial for inhibiting vascular constriction and PAH. Activation of eNOS/NO enhances vascular relaxation and can inhibit ET-1-induced PA constriction [5]. Sildenafil is unable to inhibit the concentration of ET-1 in plasma [32]. Reports [17,27,32] on mRNA determination, morphologic examination, aorta-banded operation, heart failure, hypoxia and reoxygenation, and administration of MCT have suggested that sildenafil does not decrease ET-1 concentration or expression, unlike KMUP-1. This could be another advantage of using KMUP-1 to treat PAH-induced vascular resistance.

Endothelium dysfunction can signal eNOS reduction in MCT-treated rats, such as the rarefaction of eNOS immunostaining restored by KMUP-1. Therefore, eNOS expression by KMUP-1 is suggested to increase the production and bioavailability of endothelium-derived NO. ROCK is an important therapeutic target in cardiovascular medicine [15,30]. In the PA, ROCK and eNOS expression display a counterregulation relationship in endothelium [38-41]. Upregulated eNOS and downregulated ROCKII/ET-1 in the PA might be an important mechanism of action (Fig. 8) underlying the cardiovascular protective effect of KMUP-1 in PAH and associated RVH [42,43].

The use of the PDE-5 inhibitor sildenafil to treat PAH by enhancing cGMP in the PA encouraged us to use KMUP-1 as a cGMP-dependent ROCK inhibitor [1,2]. Sildenafil has been described as potentiating compensatory upregulation of NO/cGMP signaling in hypoxic PAH, instead of PDE-5 inhibition. PDE-5 was either unchanged in long-term hypoxia, or even significantly decreased along with unchanged activity [14,44]. In this study, we demonstrated that KMUP-1 enhances eNOS, but not PDE-5A, contributing to an increase in PKG levels.

Both KMUP-1 and sildenafil preserve eNOS/sGC/PKG and reduce ROCKII/ET-1 expression caused by MCT. KMUP-1 displays more potent eNOS/sGC/PKG expression than sildenafil in MCT-treated rats. Administration of KMUP-1 or sildenafil can inhibit ET-1/ROCKII expression and enhance the NO/cGMP pathway, displaying antiproliferation and antihyperplasia activity in the PA. Similar to statins and ROCK inhibitors, inhibition of ROCK by KMUP-1 in the
endothelium might increase or activate eNOS expression and sustain PA relaxation activity to arrest or reverse the MCT-induced pathogenesis of PAH. KMUP-1 appears more soluble than sildenafil and is more favorable for sublingual administration to obtain an effective blood concentration. Sublingual KMUP-1 inhibited ET-1 expression and ET-1 concentration in MCT-induced PAH. This fact encouraged us to further investigate whether KMUP-1 directly inhibits ET-1-induced GTP-RhoA in PASMCs. We found that KMUP-1 clearly inhibits the activation of RhoA in a concentration-dependent fashion and also inhibits ET-1-induced activation of RhoA in PASMCs. ROCK expression is also related to an

\[ \text{Figure 7.} \quad \text{Changes of KMUP-1 concentration in blood after sublingual administration within (A) 60 minutes and (B) within 24 hours. Each value represents the mean \pm standard error of the mean of six experiments. Conc. = concentration.} \]

\[ \text{Figure 8.} \quad \text{Proposed mechanism of action of KMUP-1 on the PA in lung tissues. MCT induced endothelial dysfunction, leading to upregulation of ET-1/ROCKII and downregulation of eNOS in the PA. Treatment with KMUP-1 activates NO/sGC/cGMP, inhibits ROCKII expression, and reduces expression and plasma concentrations of ET-1, protecting tissue from MCT-induced endothelial dysfunction, vascular hyperplasia, and right ventricular hypertrophy. Both 5-HT and KMUP-1 activate endothelial 5-hydroxytryptamine receptor 2B-coupled eNOS (2), which is also increased by sildenafil. Both KMUP-1 and bosentan inhibit ET-1-mediated activation of GPCRs and reduce the downstream activation of Rhoa/ROCK enhanced by MCT and associated hypoxia through active oxygen species in PA endothelium and smooth muscles. 5-HT = 5-hydroxytryptamine; cGMP = cyclic guanosine monophosphate; ET-1 = endothelin-1; eNOS = endothelial nitric oxide synthase; GPCRs = G-protein coupled receptors; MCT = monocrotaline; PA = pulmonary artery; PDE 5A = phosphodiesterase 5A; PKG = protein kinase G; ROCKII = Rho kinase II; sGC = soluble guanylate cyclase.} \]
agonist activity of ET-1 on GPCRs of the PA, associated with PA remodeling and vascular hyperplasia caused by MCT [5, 31, 34, 35]. Similar to statins, KMUP-1 inhibits the expressions of both ROCKII activation and ET-1, demonstrating GTP binding of RhoA and Rhoa activation in PASMCs and preventing the hyperplasia of the PA in PAH caused by MCT [1, 19, 31, 32].

Several studies suggest that RhoA/ROCK activation and expression of ET-1 contribute to vasoconstruction as well as proliferation of smooth muscle cells in chronic PAH. In parallel, expression of eNOS contributes not only to the local regulation of vascular smooth muscle tone but also to the inhibition of ROCKII, leading to the reduction of cell proliferation in smooth muscles [7, 41, 42]. PAH can lead to vascular resistance in the PA. In our preventive study using MCT-treated rats, MPAP, RV divide, LV divide, septum, and small pulmonary arterial morphologic changes were highly improved in the treated rats. KMUP-1 causes specific PA vasodilatation and has an antihyperplasia effect, leading to a significant decrease of MPAP. Therefore, KMUP-1 inhibits PAH by a combination of cGMP-enhancing and ET-1/ROCKII-reducing activity in the PA to inhibit long-term MCT-induced PAH and associated RVH.

KMUP-1 has been described as an inhibitor of nonspecific GPCRs [1–3]. ET-1 receptor belongs to the GPCRs family; therefore, nonselective inhibition of GPCRs by KMUP-1 is theoretically a reasonable way to inhibit ET-1-mediated activation of GPCRs resulting in smooth muscle constriction and RhoA activation in the PA. Because MABP and heart rate were not significantly changed after KMUP-1 administration, PAH inhibition by a GPCRs antagonist would be useful for the treatment of PAH. Sublingual KMUP-1 was well absorbed and showed pharmacologic benefits to reduce the unfavorable release of ET-1 after MCT administration. KMUP-1 was able to prevent MCT-induced PAH and ET-1 release. Sublingual administration of KMUP-1 is suggested to provide better bioavailability than oral administration for inhibiting ET-1 release, expression, and blood concentration in the cardiac and pulmonary circulation systems.

In conclusion, sublingual administration of KMUP-1 inhibits PAH and associated ventricular hypertrophy by suppressing ET-1, inactivating RhoA/ROCK, and enhancing eNOS/sGC/PKG expression. KMUP-1 is suggested to be a GPCR antagonist, instead of an ET-1 receptor antagonist, for inhibiting MCT-induced and ET-1-mediated PAH.

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