Pulmonary arterial hypertension (PAH) is a fatal disease characterized by progressive obliteration of the vessel lumen and increased pulmonary artery pressure, leading to right ventricular (RV) failure and premature death. During the past few decades, increased understanding of PAH pathophysiology has led to the development of several effective therapies, including prostacyclin analogs and derivatives, endothelin receptor antagonists, PDE5 (phosphodiesterase

Identification of Celastramycin as a Novel Therapeutic Agent for Pulmonary Arterial Hypertension
High-Throughput Screening of 5562 Compounds


Rationale: Pulmonary arterial hypertension (PAH) is characterized by enhanced proliferation of pulmonary artery smooth muscle cells (PASMCs) accompanying increased production of inflammatory factors and adaptation of the mitochondrial metabolism to a hyperproliferative state. However, all the drugs in clinical use target pulmonary vascular dilatation, which may not be effective for patients with advanced PAH.

Objective: We aimed to discover a novel drug for PAH that inhibits PASMC proliferation.

Methods and Results: We screened 5562 compounds from original library using high-throughput screening system to discover compounds which inhibit proliferation of PASMCs from patients with PAH (PAH-PASMCs). We found that celastramycin, a benzoyl pyrrole-type compound originally found in a bacteria extract, inhibited the proliferation of PAH-PASMCs in a dose-dependent manner with relatively small effects on PASMCs from healthy donors. Then, we made 25 analogs of celastramycin and selected the lead compound, which significantly inhibited cell proliferation of PAH-PASMCs and reduced cytosolic reactive oxygen species levels. Mechanistic analysis demonstrated that celastramycin reduced the protein levels of HIF-1α (hypoxia-inducible factor 1α), which impairs aerobic metabolism, and NF-κB (nuclear factor-κB), which induces proinflammatory signals, in PAH-PASMCs, leading to reduced secretion of inflammatory cytokine. Importantly, celastramycin treatment reduced reactive oxygen species levels in PAH-PASMCs with increased protein levels of Nrf2 (nuclear factor erythroid 2-related factor 2), a master regulator of cellular response against oxidative stress. Furthermore, celastramycin treatment improved mitochondrial energy metabolism with recovered mitochondrial network formation in PAH-PASMCs. Moreover, these celastramycin-mediated effects were regulated by ZFC3H1 (zinc finger C3H1 domain-containing protein), a binding partner of celastramycin. Finally, celastramycin treatment ameliorated pulmonary hypertension in 3 experimental animal models, accompanied by reduced inflammatory changes in the lungs.

Conclusions: These results indicate that celastramycin ameliorates pulmonary hypertension, reducing excessive proliferation of PAH-PASMCs with less inflammation and reactive oxygen species levels, and recovered mitochondrial energy metabolism. Thus, celastramycin is a novel drug for PAH that targets antiproliferative effects on PAH-PASMCs.

Visual Overview: An online visual overview is available for this article. (Circ Res. 2019;125:309-327. DOI: 10.1161/CIRCRESAHA.119.315229.)

Key Words: cell proliferation ■ energy metabolism ■ hypertension ■ hypoxia-inducible factor 1 ■ reactive oxygen species

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pathophysiology has led to the development of several effective therapies, including prostacyclin analogs and derivatives, endothelin receptor antagonists, PDE5 (phosphodiesterase

Circulation Research is available at https://www.ahajournals.org/journal/res DOI: 10.1161/CIRCRESAHA.119.315229
What Is Known?

- Pulmonary arterial smooth muscle cells (PASMCs) from patients with pulmonary arterial hypertension (PAH) rigorously proliferate like cancer cells and finally occlude the distal pulmonary arteries, which is associated with increased production of inflammatory factors and adaptation of mitochondrial metabolism to a hyperproliferative state.
- Celastramycin is a benzoyl pyrrole-type compound originally found in a bacterial extract which suppresses NF-κB (nuclear factor κB–like transcriptional factors).

What New Information Does This Article Contribute?

- Celastramycin inhibits cell proliferation of PASMCs from patients with PAH dose-dependently with small effects on control PASMCs. Celastramycin also inhibits inflammation and reactive oxygen species and recovers mitochondrial energy metabolism.
- Treatment with celastramycin ameliorates pulmonary hypertension in rodent models.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>BRD4</td>
<td>bromodomain-containing protein 4</td>
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<tr>
<td>CO</td>
<td>cardiac output</td>
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<tr>
<td>DDI</td>
<td>Drug Discovery Initiative</td>
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<td>ECAR</td>
<td>extracellular acidification rate</td>
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<tr>
<td>eNOS</td>
<td>endothelial NO synthase</td>
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<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
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<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor 1α</td>
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<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
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<td>Nrf2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
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<tr>
<td>OCR</td>
<td>oxygen consumption rate</td>
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<tr>
<td>PAECs</td>
<td>pulmonary artery endothelial cells</td>
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<td>PAH</td>
<td>pulmonary arterial hypertension</td>
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<tr>
<td>PAH-PASMCs</td>
<td>PASMCs from patients with PAH</td>
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<tr>
<td>PARP-1</td>
<td>poly (ADP-ribose) polymerase-1</td>
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<tr>
<td>PASMCs</td>
<td>pulmonary artery smooth muscle cells</td>
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<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
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<tr>
<td>PDK1</td>
<td>pyruvate dehydrogenase lipoamide kinase isozyme 1</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator-activated receptor γ co-activator 1α</td>
</tr>
<tr>
<td>PH</td>
<td>pulmonary hypertension</td>
</tr>
<tr>
<td>PPARA</td>
<td>peroxisome proliferator-activated receptor-α</td>
</tr>
<tr>
<td>PPARD</td>
<td>peroxisome proliferator-activated receptor-δ</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RVSP</td>
<td>right ventricular systolic pressure</td>
</tr>
<tr>
<td>SDHA</td>
<td>succinate dehydrogenase complex subunit A</td>
</tr>
<tr>
<td>TFAM</td>
<td>mitochondrial transcription factor A</td>
</tr>
<tr>
<td>ZFC3H1</td>
<td>zinc finger C3H1 domain-containing protein</td>
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type 5) inhibitors, and an sGC (soluble guanylate cyclase) stimulator. However, all the drugs in clinical use for PAH are essentially pulmonary vasodilators, which have limited efficacy in patients with advanced PAH. Indeed, despite the improvements in treatment options, overall survival still remains unsatisfactory. Thus, it is important to develop novel drugs that possess different mechanisms of action.

It is known that the characteristics of pulmonary artery smooth muscle cells (PASMCs) from patients with PAH (PAH-PASMCs) are different from those from healthy controls (control PASMCs). Indeed, PAH-PASMCs rigorously and continuously proliferate like cancer cells and finally occlude the distal pulmonary resistant vessels. Thus, effective treatment that achieves reverse remodeling needs to be developed for patients with advanced PAH. When we consider the abnormal phenotype of excessive proliferation and apoptosis-resistance in PAH-PASMCs, the abnormal phenotype itself can be a target for the development of novel drugs. Recently, the Drug Discovery Initiative (DDI) has been founded in Japan as a hub of the national collaborative research network for drug discovery, which provides consultation, technical assistance, and public chemical samples to researchers who will begin chemical screening (http://www.ddi.u-tokyo.ac.jp/en/). Tohoku University, a screening and library point of the DDI, has a unique library containing 5562 original compounds and automated machines to perform high-throughput screening (http://www.pford.med.tohoku.ac.jp/index.html). Celastramycin is a benzoyl pyrrole-type compound originally found in a bacteria extract, which arose from functional screenings using an ex vivo culture system in Drosophila. Celastramycin attenuates TNF (tumor necrosis factor)-α-mediated induction of IL (interleukin)-6 and IL-8 in human endothelial cells and lung cancer cells. It is also known that inflammation promotes cell proliferation by up-regulation of cytokines/chemokines and growth factors, some of which directly affect cell proliferation, migration, and differentiation of PASMCs. In animal models of pulmonary hypertension (PH), inflammation precedes vascular remodeling, suggesting that altered immunity is one of the primary events in the development of PAH. Cytokines and growth factors increase reactive oxygen species (ROS), which augment inflammation again.
Mounting evidence has implicated oxidative stress as an important pathogenic mechanism in PAH. Additionally, most of the cytokines directly affect mitochondrial function in PASMCs.15,16

In the present study, we screened the original library of Tohoku University using a high-throughput screening system and discovered that celastramycin inhibits PAH-PASMC proliferation with anti-inflammatory and antioxidant effects, leading to the recovery of mitochondrial function and amelioration in 3 rodent models of PH. Our data suggest that celastramycin is a novel and promising drug for the treatment of PAH.

Methods

Additional detailed methods are included in the online-only Data Supplement. The data that support the findings of this study are available from the corresponding author on reasonable request.

Human Lung Samples

Lung tissues were obtained from patients at the time of lung transplantation or surgery for lung cancer at a site far from the tumor margins as previously described.17-19 All patients provided written informed consent for the use of their lung tissues for the present study.

Study Approval

All protocols using human specimens were approved by the Institutional Review Board of Tohoku University, Sendai, Japan (No. 2013-1-160). All animal experiments were performed in accordance with the protocols approved by the Tohoku University Animal Care and Use Committee (No. 2015-Kodo-007) based on the Animal Research: Reporting of In Vivo Experiments guideline.

High-Throughput Screening

We used the original libraries of Tohoku University containing 5562 unique compounds in the DDI in Japan. PAH-PASMCs were used for the first (proliferation assay) and second (repeatability assays, counter assays, and concentration-dependent assays) screening and control PASMCs were used for counter assay (proliferation assay). We optimized screening conditions (cell number, time-course of plating cells, and adding stimulus) beforehand. PAH-PASMCs were grown in DMEM with 10% FBS up to 80% confluence, which were plated at 1000 cells/45 μL mediums in each well of a 384-well plate (Greiner Bio-One, Austria) using the Multidrop Combi (Thermo Fisher Scientific, Waltham). They were then placed in the automated incubator at 37°C for 24 hours. Diluted compounds (final concentration, 5 μmol/L) were added to columns of every plate by the Biomek NXP (Molecular Devices). The intraplate and interplate variability showed the existing information, which includes stability, toxicology, and complexity in each compound (Online Table II). Thus, we selected the existing information, which includes stability, toxicity, and complexity in each compound (Online Table II). The intraplate and interplate variability showed a coefficient of variance of 5.9% and 4.0%, respectively.

Animal Experiments

All animal experiments were performed in accordance with the protocols approved by the Tohoku University Animal Care and Use Committee (No. 2015-Kodo-004) based on the Animal Research: Reporting of In Vivo Experiments guidelines and the recent recommendations with thorough randomization on the optimal preclinical studies in PAH.20,21 All the operations and analyses were performed in a blinded manner. We selected male rats because male rats were used in most previous studies as monocrotaline-induced PH or SUGEN/hypoxia-induced PH in rats, which are evidenced by accumulated papers with PH model animals.22-24 We were unable to conduct formal sample size and power calculations because the primary goal of this study was to explore the effect of an intervention (celastramycin treatment) in vivo for the first time.

Statistical Analyses

All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student t test for normally distributed cases or the bootstrap method25 for not normally distributed cases with unequal variances. Comparisons of means among ≥3 groups were performed by 1-way or 2-way ANOVA for normally distributed cases followed by the Tukey honestly significant difference method or the Dunnett method for multiple comparison, as appropriate. The normality of the underlying distributions was confirmed by the Shapiro-Wilk normality test. The multiplicity of the testing for not normally distributed cases was adjusted by the Holm method for pairwise 2-sample comparison.26 Linear associations between 2 continuous variables were analyzed using linear regression model. The ratio of fully muscularized vessels was analyzed by the Poisson regression with the offset equals to the sum of total vessels with multcomp 1.4-6 package of R. Statistical significance was evaluated with GraphPad Prism 7.02 (GraphPad Software, Inc, La Jolla, CA), JMP 12 (SAS Institute Inc, Cary), or R version 3.3.2 (http://www.R-project.org/). All reported P values are 2-tailed, with a P value of <0.05 indicating statistical significance.17,27

Results

Identification of Celastramycin by High-Throughput Screening

To discover a novel drug for patients with severe PAH, we used the screening system of the DDI with 5562 original compounds and derivatives in the original chemical library of Tohoku University. For the screening procedure, we established cell libraries of primary cultured PAH-PASMCs from patients undergoing lung transplantation and evaluated their inhibitory effects on cell proliferation after treatment with each compound (Figure 1A, Online Table I). We performed high-throughput screening to identify compounds that reduced proliferation of PAH-PASMCs in a dose-dependent manner (Figure 1B). In the first screening, PAH-PASMCs were incubated with each compound in 384-well plates for 24 hours. Among the compounds, we initially selected 80 that effectively inhibited PAH-PASMC proliferation (Figure 1C). In the second screening, we performed repeatability assays and counter assays for the 80 compounds and selected 9 compounds that inhibited PAH-PASMC proliferation with relatively small effects on control PASMCs from healthy volunteers (Figure 1D, Online Figure I). Next, in the second screening, we performed a concentration-dependent assay for the 9 compounds in PAH-PASMCs and control PASMCs (Figure 1E). In the process of final selection, we also considered the existing information, which includes stability, toxicity, and complexity in each compound (Online Table II). Thus, we finally selected celastramycin, as it has a promising structure and showed minimal effects on control PASMCs. We further confirmed the antiproliferative effects of celastramycin on PAH-PASMCs in 6 different lines. Interestingly, inhibition rate of PAH-PASMC proliferation positively correlated with the pulmonary vascular resistance in each patient (Online Figure II).

Because cardiac toxicity is an important issue when considering RV failure in patients with PAH, we confirmed that celastramycin inhibits PAH-PASMC proliferation without harmful effects on human adult cardiomyocytes (Online Figure IIIA). Interestingly, celastramycin treatment downregulated the expression of brain natriuretic peptide (NPPB) in human adult cardiomyocytes compared with vehicle control (Online Figure IIIB). In contrast, celastramycin treatment significantly upregulated the expressions of antioxidant genes, superoxide dismutase 2 (SOD2) and glutamate-cysteine ligase catalytic subunit (GCLC), in human adult
cardiomyocytes compared with vehicle control (Online Figure IIIIB). Moreover, we further performed experiments with pulmonary artery endothelial cells (PAECs) from patients with PAH and control PAECs (Online Figure IV). Celastramycin treatment suppressed proliferation and apoptosis-resistance in PAECs from patients with PAH with relatively small

Figure 1. Screening of a novel compound that inhibits pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension (PAH-PASMC) proliferation. A, The schema of the primary culture of PAH-PASMCs and screening of the Tohoku University Compound Library (5562 compounds). B, Schematic outline of high-throughput screening to identify celastramycin (CEL) that inhibit PAH-PASMC proliferation with minimal harmful effects. C, Results of the first screening of 5562 compounds. The ratio of assay units by 3-(4,5-di-methylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay after treatment with 5562 compounds (5 μmol/L) or vehicle for 24 h compared with day 0. Blue represents the MTT levels of PAH-PASMCs after treatment with 5562 compounds and we selected 80 compounds in the yellow square. D, Results of the second screening of 80 compounds. The ratio of assay units by MTT assay of PAH-PASMCs or PASMCs from healthy donors (control PASMCs) after treatment with 80 compounds (5 μmol/L) or vehicle for 48 h compared with day 0. Blue represents the MTT levels of PAH-PASMCs and green represents control PASMCs after treatment with 80 compounds and we selected CEL in the yellow square. E, Results of concentration-dependent assays with 9 compounds in PAH-PASMCs and control PASMCs. The ratio of assay units by MTT assay after treatment with different concentrations (0, 0.1, 1, and 5 μmol/L) of 9 compounds for 48 h as compared to day 0 (n=8 each). The 9 compounds are CEL, Maleimide, Gitoxigenin, Puromycin, Emetine, C6H3IN2O4, 1,4-Naphthoquinone, C22H33Cl2N7O6, and C11H14N2O3. F, RealTime-Glo assay, in which cell viability was measured intermittently after treatment with different concentrations (0, 0.1, 1, 5, and 10 μmol/L) of CEL (n=8 each). G, Results of concentration-dependent apoptotic assays stained for Annexin V and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) in PAH-PASMCs after treatment with vehicle or CEL for 48 h (20 images/group). Scale bars, 50 μm. Data represent the mean±SEM. *P<0.05. Comparisons of parameters were performed with 1-way ANOVA followed by Dunnett test for multiple comparisons.
effects on control PAECs from healthy donors (Online Figure IVA and IVB). Indeed, it has been reported that PAECs from patients with PAH have highly proliferative and apoptosis-resistant features that induce occlusion of pulmonary arteries.26 Moreover, celastramycin treatment significantly upregulated the eNOS (endothelial NO synthase) levels, which increase NO production in PAECs (Online Figure IVC).

Again, a cell variability assay confirmed that celastramycin exerts antiproliferative effects on PAH-PASMCs (Figure 1F). We also found that celastramycin minimally induced apoptosis, assessed by staining with Annexin V or TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) in PAH-PASMCs (Figure 1G and Online Figure V). Thus, celastramycin is a novel drug that inhibits PAH-PASMC proliferation and induces apoptosis in a dose-dependent manner without harmful effects on normal PASMCs or cardiomyocytes.

### Development of Celastramycin Analogues and Their Structure-Activity Correlation

To determine the antiproliferative structure of the celastramycin molecule, we developed 25 analogs and examined their antiproliferative effects on PAH-PASMCs. First, we synthesized 8 analogs with different lengths of alkyl chain (R; Figure 2A). These analogs had strong antiproliferative effects on PAH-PASMCs (Figure 2B). Interestingly, their antiproliferative effects depended on the length of R (Figure 2C). In contrast, substitution of R with a chlorine atom (Figure 2D) completely abolished the antiproliferative effects (Figure 2B). Next, we introduced a single modification in the basal common structure, e (Figure 2E), which slightly attenuated the antiproliferative effects on PAH-PASMCs (Figure 2B). Moreover, excessive modification of the basic common structure (Figure 2F) completely abolished the antiproliferative effects on PAH-PASMCs (Figure 2B). These results suggest the crucial role of the basal common structure for the antiproliferative effects on PAH-PASMCs. Importantly, celastramycin analogs with the basal common structure inhibited proliferation in different PAH-PASMC cell lines in a dose-dependent manner (Online Figure VI). Finally, we evaluated the levels of cytosolic ROS after treatment with the 25 analogs and found that the analog b significantly reduced ROS in PAH-PASMCs (Figure 2G). Because we previously confirmed that intracellular ROS in PAH-PASMCs is increased compared with control PASMCs19 and higher levels of cytosolic ROS are mechanistically involved in the proliferation of PAH-PASMCs,19,27,28 we used the analog b in the following experiments in vivo and in vitro.

### Celastramycin Improves Mitochondrial Energy Metabolism in PAH-PASMCs

Abnormal activation of HIF-1α (hypoxia-inducible factor 1α) in normoxia is well known in PAH-PASMCs, which augment transcription of many genes promoting proinflammatory signals, impaired oxidative glucose metabolism, and the shift to aerobic glycolysis.29 Interestingly, celastramycin treatment reduced HIF-1α mRNA (HIF1A) and increased glucose transporter 1 mRNA (SLC2A1) in PAH-PASMCs compared with vehicle controls (Figure 3A, Online VIIA). Importantly, HIF-1α was upregulated in PAH-PASMCs compared with control PASMCs, and celastramycin treatment significantly reduced protein levels of HIF-1α in PAH-PASMCs compared with vehicle controls (Figure 3A). Additionally, celastramycin significantly reduced downstream PDK1 (pyruvate dehydrogenase lipoamide kinase isozyme 1), which inactivates PDH (pyruvate dehydrogenase) to convert pyruvic acid to acetyl CoA (coenzyme A), and DRP1 (dynamin-1-like protein) that promotes mitochondrial fission, both of which were upregulated in PAH-PASMCs compared with control PASMCs (Figure 3A). Moreover, celastramycin significantly reduced protein levels of Keap1 (Kelch-like ECH-associated protein 1), which suppresses Nrf2 (nuclear factor erythroid 2-related factor 2), in both PAH-PASMCs and control PASMCs (Figure 3B). Consistently, celastramycin increased protein levels of Nrf2 in nuclear extracts in PAH-PASMCs compared with vehicle controls (Figure 3B). Indeed, celastramycin upregulated the expression of Nrf2 (NFE2L2), a master regulator of cellular response against oxidative stress, and its downstream genes, NAD(P)H quinone dehydrogenase-1 (NQO1), heme oxygenase-1 (HMOX1), GCLC, and SOD2 in PAH-PASMCs compared with vehicle controls (Figure 3C, Online Figure VIII). Moreover, celastramycin significantly increased SOD2 in total cell lysates compared with vehicle controls (Online Figure VIIIC). Thus, we next focused on the role of celastramycin in altering the redox state in PAH-PASMCs. Indeed, we detected significantly higher levels of ROS in PAH-PASMCs compared with control PASMCs (Figure 3D). However, celastramycin treatment significantly reduced cytosolic ROS in PAH-PASMCs assessed by staining with CellROX and 2,7-dichlorodihydrofluorescein compared with vehicle controls (Figure 3D, Online Figure VIII). Consistently, NADPH oxidase activity was significantly higher in PAH-PASMCs compared with control PASMCs at baseline, which was significantly reduced by the celastramycin treatment (Figure 3E). Here, it has been demonstrated that NADPH oxidase regulates the activities of Nrf2 in several cell lines.30,31 Conversely, Keap1-Nrf2 pathway regulates the cytosolic ROS production through inhibition of NADPH oxidase.32 Indeed, celastramycin significantly reduced Keap1 and increased Nrf2 in PAH-PASMCs (Figure 3B). Thus, these reports and our data suggest that celastramycin downregulates Keap1 and upregulates Nrf2, contributing to the inhibition of NADPH oxidases in PAH-PASMCs. Next, to evaluate the antioxidative capacity of celastramycin, we evaluated levels of glutathione and oxidized glutathione (GSSG). To elicit antioxidative effects, glutathione is converted to GSSG, and only free glutathione has antioxidant effects. In contrast, GSSG lacks antioxidant functions and is a byproduct of the scavenging activity of glutathione. Thus, glutathione/GSSG ratio is important in assessing the total capacity of cytosolic ROS removal. Here, glutathione/GSSG ratio was significantly downregulated in PAH-PASMCs compared with control PASMCs, both of which were significantly increased by celastramycin treatment (Figure 3F). In contrast, celastramycin treatment significantly increased mitochondrial ROS (mROS) in PAH-PASMCs assessed by MitoSOX staining compared with vehicle controls (Figure 3G). Here, it is well known that dysregulated mitochondrial function and ATP production cause a decrease in the production of mROS in PAH-PASMCs.33 Thus, we next examined the role of celastramycin on mitochondrial functions in PAH-PASMCs.
Using a Seahorse XF24-3 apparatus, which provides information on mitochondrial functions through real-time measurements of oxygen consumption rate (OCR; a marker of oxidative phosphorylation) and extracellular acidification rate (ECAR; a surrogate for glycolysis), we evaluated the effects of celastramycin treatment on control PASMCs and PAH-PASMCs (Figure 3H). OCR reflects the mitochondrial respiration rate and energy production, while ECAR the rate of glycolysis. Here, we observed significantly lower levels of ATP production, maximal respiration, and OCR/ECAR ratio in PAH-PASMCs compared with control PASMCs, which were significantly increased by the celastramycin treatment.

Figure 2. Celastramycin analogs and structure-activity correlation. A, The chemical structures of 8 analogs, a–h, with different lengths of alkyl chain (R). B, The ratio of cell numbers after treatment with 25 analogs for 48 h compared with day 0 (1 μmol/L, n=8 each). Cell numbers were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. C, Structure-activity correlation between the length of the alkyl chain (R) and proliferation of pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension (PAH-PASMCs). D, The chemical structure of 1 analog, i, which substituted R into a chlorine atom. E, The chemical structures of 5 analogs, j–n, in which 1 or 2 branches in the basal common structure e were modified. F, The chemical structures of 11 analogs, o–y, in which the basal common structure e were excessively modified. G, The levels of reactive oxygen species (ROS) in PAH-PASMCs assessed by CellROX after treatment with the 25 analogs for 24 h (n=3 each). Data represent the mean±SEM. *P<0.05. Comparisons of parameters were performed with an unpaired Student t test or Dunnett test for multiple comparisons. Linear associations between 2 continuous variables were analyzed using a linear regression model.
Figure 3. Celastramycin (CEL)-mediated recovery of mitochondrial functions in pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension (PAH-PASMCs). A, Real-time polymerase chain reaction (RT-PCR) analysis of HIF-1α (hypoxia-inducible factor-1α; HIF1A) mRNA in control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=6) and quantification of HIF-1α, PDK1 (pyruvate dehydrogenase lipoamide kinase isozyme 1) and DRP1 (dynamin-1-like protein) in control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=6). B, Quantification of Keap1 (Kelch-like ECH-associated protein 1) in total cell lysate and Nrf2 (nuclear factor erythroid 2-related factor 2) in nuclear extract of control PASMCs and PAH-PASMCs after the treatment with CEL or vehicle for 24 h (n=6). C, RT-PCR analysis of Nrf2 (NFE2L2) mRNA in control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=6). D, Left, representative images of CellROX Deep Red fluorescence in control PASMCs and PAH-PASMCs. Nuclei were counterstained using DAPI (4',6-diamidino-2-phenylindole). Scale bars, 50 μm. Right, quantification of CellROX and 2,7-dichlorodihydrofluorescein (DCF) fluorescence intensity in control PASMCs and PAH-PASMCs after the treatment with CEL or control vehicle for 24 h (n=8 each). E, Quantification of NADPH oxidase activity in control PASMCs and PAH-PASMCs after the treatment with CEL or control vehicle for 24 h (n=8 each). F, Quantification of glutathione (GSH)/oxidized GSH (GSSG) ratio in control PASMCs and PAH-PASMCs after vehicle or CEL treatment for 4 h (n=8 each). G, Quantification of mitochondrial reactive oxygen species assessed by MitoSOX fluorescence intensity in control PASMCs and PAH-PASMCs after the treatment with CEL or control vehicle for 24 h (n=8 each). H, Quantification of the mitochondrial oxygen consumption rate (OCR) of control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=5). I, Quantification of the OCR of control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=5 each). Oligomycin inhibits ATP synthase (complex V), and the decrease in OCR followed by oligomycin correlates to the mitochondrial respiration associated with cellular ATP production. (Continued)
Figure 3 Continued. Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone is an uncoupling agent that disrupts the mitochondrial membrane potential. As a result, electron flow through the electron transport chain is uninhibited, and oxygen is maximally consumed by complex IV. Rotenone and antimycin A were injected to inhibit the flux of electrons through complex I and III, respectively, and thus shut down mitochondrial oxygen consumption. J, Left, representative images of control PASMCs and PAH-PASMCs labeled for mitochondria after the treatment with CEL or the control vehicle for 24 h. Nuclei were counterstained using DAPI. Scale bars, 20 μm. Right, quantification of mitochondrial fragmentation in control PASMCs and PAH-PASMCs labeled for mitochondria after the treatment with CEL or the control vehicle for 24 h. K, Representative transmission electron microscopy images of control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h. Scale bars, 2 μm. Data represent the mean±SEM. *P<0.05. Comparisons of parameters were performed with 2-way ANOVA, followed by Tukey honestly significant difference test for multiple comparisons. CON indicates control; ECAR, extracellular acidification rate; and Veh, vehicle.

(Circle 3I). Furthermore, PAH-PASMCs showed significantly increased glycolysis compared with control PASMCs, which was slightly reduced by the celastramycin treatment (Online Figure IX). Thus, we next examined the morphologies of mitochondria in PAH-PASMCs after celastramycin treatment for 24 hours. Importantly, celastramycin treatment showed increased mitochondrial networks assessed using a MitoTracker in PAH-PASMCs compared with vehicle controls (Figure 3J, Online Figure X). Additionally, celastramycin treatment showed increased mitochondrial networks assessed by transmission electron microscopy in PAH-PASMCs compared with vehicle controls (Figure 3K, Online Figure XI). Consistently, celastramycin treatment significantly reduced the levels of DRP1 (dynamin-1-like protein) that promotes mitochondrial fission (Figure 3A). Moreover, celastramycin increased the expressions of genes for mitochondrial fusion, such as mitofusin 1 (MFN1), mitofusin 2 (MFN2), and mitochondrial elongation factor (MIEFI) and reduced the expressions for mitochondrial fission, such as DRP1 (DNM1L), mitochondrial fission 1 protein (FIS1), and optic atrophy type 1 (OPA1) in PAH-PASMCs compared with vehicle controls (Online Figure XlIlD). Additionally, celastramycin significantly upregulated the expressions of genes for mitochondrial biogenesis, such as PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1-α, PPARGC1A), mitochondrial transcription factor A (TFAM), and nuclear respiratory factor 1 (NRF1) and genes for mitochondrial function, such as peroxisome proliferator-activated receptor-α (PPARα, PPARD) and β (PPARA, PPARD) in PAH-PASMCs compared with vehicle controls (Online Figure XlIlE). These results suggest that celastramycin treatment affects the balance between mitochondrial biogenesis and functions, resulting in increased mitochondrial networks and mROS levels in PAH-PASMCs.

Celastramycin-Mediated Changes in Metabolomics in PAH-PASMCs

It has been reported that circulating metabolites are dramatically altered in patients with PAH.14 Additionally, metabolic profiles predicted the long-term prognosis of patients with PAH, suggesting that metabolic changes could be important modifiers of disease progression.14 Based on the celastramycin-mediated recovery in mitochondrial function, we hypothesized that celastramycin may change metabolic profiles in PAH-PASMCs and their secreted proteins. Thus, we performed metabolomic analyses to evaluate the metabolic changes in PAH-PASMCs by the treatment with celastramycin. Here, we used a broad metabolomics platform to analyze >400 metabolites in total cell lysates of PAH-PASMCs and compared the changes after the treatment with celastramycin (Online Figure XIIA). Interestingly, we found a dramatic change in several metabolites by celastramycin treatment (Online Figure XIIIB). In agreement, celastramycin treatment increased the levels of metabolite in the tricarboxylic acid cycle, such as succinic acid, suggesting that celastramycin upregulates mitochondrial respiration in PAH-PASMCs (Online Figure XIIC). Here, we have measured the activities of the enzymes that regulate the levels of succinic acid (succinyl-CoA synthetase and succinate dehydrogenase) in PAH-PASMCs. Interestingly, celastramycin significantly increased both enzymes (Online Figure XIIC). However, the extents of the increases were higher for succinyl-CoA synthase (1+140%) than for SDHA (succinate dehydrogenase complex subunit A); +20%; Online Figure XIIIC). Thus, the increased levels of succinic acid by celastramycin can be explained, at least in part, by the increased ratio of succinyl-CoA synthetase/SDHA. Additionally, we further performed analyses of cytokines/chemokines and growth factors in conditioned medium from control PASMCs and PAH-PASMCs under treatment with celastramycin (Online Figure XIIID). Importantly, celastramycin treatment significantly reduced the secretion of cytokines/chemokines and growth factors from PAH-PASMCs, which were elevated compared with control PASMCs at baseline (Online Figure XIIID). These results suggest that celastramycin changes the cell metabolism and reduces inflammatory cytokines/chemokines and growth factors in PAH-PASMCs.

Celastramycin Inhibits Inflammatory Signaling in PAH-PASMCs

Celastramycin was originally identified as a potent suppressor of immune deficiency pathways, which regulate Gram-positive bacterial infections via transcription factor NF-κB (nuclear factor κB)–like transcriptional factor.11,12 Inflammation and oxidative stress are closely connected by cytokines/chemokines and growth factors.15 Excessively augmented NF-κB expression is recognized in PAH-PASMCs and induces the transcription of many genes producing proinflammatory and proinflammatory signals and impaired mitochondrial metabolism.15 Consistent with this, knockdown of NF-κB by small interfering RNA (siRNA) significantly reduced PAH-PASMC proliferation compared with control siRNA (Figure 4A). Importantly, celastramycin treatment significantly reduced protein levels of NF-κB in the nuclear extracts of PAH-PASMCs compared with vehicle controls (Figure 4B). Moreover, phosphorylation of ERK (extracellular signal-regulated kinases) 1/2 in total cell lysates was significantly upregulated in PAH-PASMCs compared with control PASMCs, which was significantly reduced by celastramycin treatment (Figure 4C). Additionally, celastramycin significantly reduced gene expression levels of NF-κB (RELA) and Toll-like receptor 4 (TLR4) compared with vehicle controls (Figure 4D). These results suggest that celastramycin inhibits inflammation through suppression of TLR4-NF-κB signaling in PAH-PASMCs. BRD4 (bromodomain-containing
Figure 4. Celastramycin (CEL)-mediated inhibition of pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension (PAH-PASMC) proliferation. A, The ratio of cell numbers in PAH-PASMCs treated with control small interfering RNA (si-control) or si–NF-κB (nuclear factor κB) for 48 h (n=8 each). Cell numbers were measured by 3-[4,5-di-methylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. B, Quantification of NF-κB in nuclear extracts of control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=6). C, Quantification of phosphorylated ERK (extracellular signal-regulated kinase)1/2 and total ERK1/2 in total cell lysates of control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=6). D, Real-time polymerase chain reaction (RT-PCR) of NF-κB p65 (RELA) and toll-like receptor 4 (TLR4) mRNA in control PASMCs and PAH-PASMCs after the treatment with CEL or the control vehicle for 24 h (n=6). E, Quantification of BRD4 (bromodomain-containing protein 4) and survivin in total cell lysate and NFATc2 (nuclear factor of activated T cells 2) in nuclear extract of control PASMCs and PAH-PASMCs after the treatment with CEL or vehicle for 24 h (n=6). F, RT-PCR analysis of Keap1 (Kelch-like ECH-associated protein 1; KEAP1) mRNA in PAH-PASMCs after treatment with si-BRD4 or si-control for 48 h (n=6). G, Schematic representation of the molecular mechanisms promoting inflammation, oxidative stress, and mitochondrial dysfunction through activation of HIF-1α (hypoxia-inducible factor 1α) and NF-κB in PAH-PASMCs. Constitutively activated HIF-1α induces the transcription of many genes and dysregulation of mitochondrial energy metabolism, which promotes cell proliferation, apoptosis-resistance, survival, and stress resistance by targeting several downstream genes. CEL treatment downregulates HIF-1α and NF-κB and upregulates Nrf2 (nuclear factor erythroid 2-related factor 2) in PAH-PASMCs. These effects result in decreased reactive oxygen species (ROS) and inflammation with recovered mitochondrial energy metabolism, leading to inhibition of excessive proliferation in PAH-PASMCs. Data represent the mean±SEM. *P<0.05. Comparisons of parameters were performed with 2-way ANOVA, followed by Tukey honestly significant difference test for multiple comparisons. CON indicates control; TCA, tricarboxylic acid; and Veh, vehicle.
protein 4) is an epigenetic reader that binds to acetylated histone tails and other proteins to regulate transcription of genes involved in many cellular functions, such as cell cycle, apoptosis, and inflammation. Here, protein levels of BRD4 in PAH-PASMCs was significantly upregulated compared with control PASMCs, which was significantly reduced by celastramycin treatment (Figure 4E). Additionally, celastramycin treatment significantly reduced protein levels of survivin and translocation of NFATc2 (nuclear factor of activated T cells 2) to the nucleus, both of which are downstream of BRD4 and regulate the cell cycle in PAH-PASMCs (Figure 4E). Here, based on the previous reports, we hypothesized that celastramycin-mediated downregulation of BRD4 may have effects on Keap1-Nrf2 signaling. Indeed, inhibition of BRD4 by siRNA significantly reduced the expression of Keap1 (Figure 4F). Altogether, celastramycin reverses altered mitochondrial metabolism and reduces inflammation and ROS production through changes in HIF-1α, NF-κB, and Nrf2, leading to inhibition of excessive proliferation in PAH-PASMCs (Figure 4G). Recently, there is mounting evidence that dysfunctional DNA-damage response mechanisms promote resistance to apoptosis and proliferative phenotype in PAH-PASMCs. Interestingly, we found that DNA damage was significantly increased in PAH-PASMCs compared with control PASMCs, both of which were significantly reduced by celastramycin treatment in a dose-dependent manner (Online Figure XIII A). Consistently, protein levels of γH2AX were significantly reduced by celastramycin treatment (Online Figure XIII B). Indeed, PAH is characterized by elevation of circulating cytokines (eg, IL-6) that promotes DNA damage\(^\text{22}\) and oxidative stress that induces DNA damage through DNA base oxidation and deamination. Thus, celastramycin-mediated anti-inflammatory and antioxidative effects may have alleviated the DNA damage especially in PAH-PASMCs. Next, we further evaluated the enzyme implicated in DNA repair, PARP-1 (poly[ADP-ribose] polymerase-1), in control PASMCs and PAH-PASMCs. However, celastramycin did not have any significant effects on the protein levels of PARP-1 (Online Figure XIII B). Altogether, celastramycin may play as a modulator of DNA damage with anti-inflammatory and anti-oxidative effects on PAH.

Zinc Finger C3H1 Domain-Containing Protein–Mediated Inhibition of BRD4 and HIF-1α by Celastramycin Treatment

A recent study clearly demonstrated that ZFC3H1 (zinc finger C3H1 domain-containing protein; encoded by ZFC3H1) is a binding partner of celastramycin. Moreover, ZFC3H1 plays a crucial role in the degradation of nuclear RNAs, such as mRNAs, ribosomal RNAs, and noncoding RNAs and thus regulates multiple intracellular signaling pathways. Here, we hypothesized that celastramycin-mediated inhibitory effects on transcriptional modulators could be regulated by its binding partner, ZFC3H1. Indeed, inhibition of ZFC3H1 by siRNA significantly reduced the mRNA expression and protein levels of BRD4 (Figure 5A). Additionally, inhibition of ZFC3H1 by siRNA significantly reduced the expression of HIF-1α and its downstream DRP1 (Figure 5B). Moreover, inhibition of ZFC3H1 induced downregulation of Keap1 and resultant upregulation of Nrf2 and SOD2 (Figure 5C). In contrast, we overexpressed ZFC3H1 in PAH-PASMCs using a ZFC3H1-encoding plasmid. Importantly, overexpression of ZFC3H1 significantly upregulated BRD4, HIF1α, DRP1, and Keap1 (Figure 5D through 5F). Altogether, celastramycin-mediated multiple effects can be explained by the inhibition of ZFC3H1, leading to suppression of BRD4 and HIF-1α (Figure 5G). However, celastramycin significantly increased the expression of PGC1α and its downstream signaling, TFAM, PPARA (peroxisome proliferator-activated receptor-α), and PPARD (peroxisome proliferator-activated receptor-δ) in PAH-PASMCs (Online Figure VII E), which upregulate the expression of mitofusin 2 and mitochondrial biogenesis. Here, we have demonstrated that AMPK (AMP-activated protein kinase) plays a crucial role against the development of PAH. Additionally, AMPK contributes to the activation of PGC1α and mitochondrial biogenesis, and mROS are a physiological activator of AMPK signaling. Thus, we consider that celastramycin-mediated upregulation of mROS may have activated AMPK and downstream PGC1α in PAH-PASMCs. Indeed, celastramycin treatment significantly increased the phosphorylation of AMPK, which increases the expression of PGC1α, especially in PAH-PASMCs (Online Figure VIII F). Altogether, celastramycin-mediated multiple effects are based on its inhibitory effects on its binding partner, ZFC3H1 (Figure 5G).

Celastramycin Ameliorates PH in Rodent Models

Based on the celastramycin-mediated inhibitory effects on PAH-PASMC proliferation, we performed in vivo experiments in rodent models of PH. First, we examined the effect of celastramycin in hypoxia-induced PH in mice (Figure 6 A). Daily administration of celastramycin using osmotic pumps during 21 days of normoxia or chronic hypoxia had no effect on body weight or blood pressure compared with vehicle controls (Figure 6B and 6C). Moreover, celastramycin treatment significantly reduced cytokines/chemokines and growth factors in the lungs, many of which were significantly increased under hypoxia compared with normoxic controls (Figure 6D, Online Table III). Importantly, celastramycin significantly reduced perivascular inflammation (Online Figure XIV) and muscularization of distal pulmonary arteries compared with vehicle controls (Figure 6E). Here, we hypothesized that celastramycin significantly accelerated PH development compared with vehicle controls (Figure 6F). Next, to further assess the therapeutic potential of celastramycin for PAH, we used a model of monocrotaline-induced PH in rats (Figure 7 A). In this monocrotaline-induced rat model, we started celastramycin treatment during the development of PH (prevention protocol). Daily administration of celastramycin for 3 weeks had no effect on body weight or food consumption compared with vehicle controls (Figure 7B). Again, consistent with the results in vitro, celastramycin treatment significantly reduced cytokines/chemokines and growth factors in the lung compared with vehicle controls (Figure 7C, Online Table III). Moreover, celastramycin significantly suppressed muscularization of distal pulmonary arteries compared with vehicle controls (Figure 7D). Consistently, celastramycin treatment...
Figure 5. ZFC3H1 (zinc finger C3H1 domain-containing protein)-mediated inhibition of BRD4 (bromodomain-containing protein 4) and HIF-1α (hypoxia-inducible factor 1α) by celastramycin treatment. A, Real-time polymerase chain reaction (RT-PCR) analysis and Western blotting of BRD4 in pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension (PAH-PASMCs) after treatment with si-ZFC3H1 or si-control for 48 h (n=6). B, RT-PCR analysis of HIF-1α (HIF1A) and dynamin-1-like protein (DNM1L) mRNA in PAH-PASMCs after treatment with si-ZFC3H1 or si-control for 48 h (n=6).C, RT-PCR analysis of Keap1 (Kelch-like ECH-associated protein 1; KEAP1), Nrf2 (nuclear factor erythroid 2-related factor 2; NFE2L2), and SOD2 (superoxide dismutase 2; SOD2) mRNA in PAH-PASMCs after treatment with si-ZFC3H1 or si-control for 48 h (n=6). D, RT-PCR analysis and Western blotting of BRD4 in PAH-PASMCs after the treatment with ZFC3H1 plasmid DNA or control plasmid DNA for 48 h (n=6). E, RT-PCR analysis of HIF-1α (HIF1A) and dynamin-1-like protein (DNM1L) mRNA in PAH-PASMCs after the treatment with ZFC3H1 plasmid DNA or control plasmid DNA for 48 h (n=6). F, RT-PCR analysis of Keap1 (KEAP1), Nrf2 (NFE2L2), and SOD2 (SOD2) mRNA in PAH-PASMCs after the treatment with ZFC3H1 plasmid DNA or control plasmid DNA for 48 h (n=6). G, Schematic representation of the molecular mechanisms of celastramycin-mediated effects on PAH-PASMCs. Abnormally activated HIF-1α and NF-κB (nuclear factor-κB) promotes inflammation, oxidative stress, and mitochondrial dysfunction, which induce excessive proliferation of PAH-PASMCs. Activation of NF-κB also increases production of cytokines/chemokines and growth factors, which recruit abundant inflammatory cells, leading to additional production of growth factors. Secreted growth factors activate NOX (NADPH oxidases), one of the main sources of intracellular reactive oxygen species (ROS), and induce production of cytotoxic ROS in PAH-PASMCs. Here, celastramycin directly binds to ZFC3H1, a zinc finger protein, which downregulates the expression of BRD4 and HIF-1α. Celastalamycin-mediated decrease in BRD4 downregulates NF-κB and Keap1, which suppress inflammation and activate Nrf2, leading to upregulation of antioxidants, such as NAD(P)H quinone dehydrogenase-1 (NQO1), superoxide dismutase 2 (SOD2), heme oxygenase-1 (HMOX1), and glutamate-cysteine ligase catalytic subunit (GCLC), resulting in inhibition of ROS production. Constitutively activated HIF-1α in PAH-PASMCs upregulates DRP1 (dynamin-1-like protein) and promotes mitochondrial fission and induces dysregulation of mitochondrial energy metabolism.
performed biochemical tests in Sugen/hypoxia-induced PH and RV failure in several different animal models. Finally, we evaluated the therapeutic potential of celastramycin for PAH, we used a third animal model of PH, in which rats were exposed to chronic hypoxia for 21 days in combination with injection of SU5416 (Sugen/hypoxia model; Figure 8A). In this Sugen/hypoxia rat model, we started celastramycin treatment after the development of PH (treatment protocol). Daily administration of celastramycin for 14 days had no effect on body weight or food consumption compared with vehicle controls (Figure 8B). Protein levels of inflammatory cytokines (eg, IL-2, IL-6) in the lungs were significantly reduced by celastramycin treatment in the Sugen/hypoxia rat model (Figure 8C, Online Figure XV, Online Table III). Moreover, celastramycin significantly suppressed muscularization of distal pulmonary arteries compared with vehicle controls (Figure 8D). Additionally, celastramycin treatment was associated with a marked reduction in proliferation and a trend for increased apoptosis in the distal pulmonary arteries in rats (Online Figure XVI). Consistently, celastramycin treatment significantly reduced RVSP and RV hypertrophy compared with vehicle controls (Figure 7E). Importantly, celastramycin treatment significantly improved hemodynamic parameters, such as RV diastolic diameter, RV fractional area change, pulmonary artery acceleration time, tricuspid annular plane systolic excursion, and cardiac output as determined by echocardiography (Figure 8F). In contrast, celastramycin treatment did not change left ventricular diastolic diameter or left ventricular ejection fraction (Figure 8G). Again, we used a Seahorse XF24-3 apparatus to evaluate the mitochondrial function in cardiomyocytes using neonatal rat cardiomyocytes (Online Figure XVIIA). Importantly, we observed higher levels of ATP production, maximal respiration, and OCR/ECAR ratio in celastramycin-treated neonatal rat cardiomyocytes compared with vehicle controls in vitro (Online Figure XVIIIB and XVIIIC). Moreover, celastramycin increased the expressions of genes for mitochondrial fusion, such as MFN1 and MFN2, reduced the expressions for mitochondrial fission, such as FIS1 and mitochondrial fission 2 protein (Mff), and upregulated the expressions of genes for mitochondrial biogenesis, such as TFAM in neonatal rat cardiomyocytes, compared with vehicle controls (Online Figure XVIIID). Here, the metabolic status of neonatal rat cardiomyocytes shifted from aerobic to energetic by the OCR/ECAR analysis (Online Figure XVE). Indeed, celastramycin treatment significantly reduced mean pulmonary arterial pressure and increased cardiac output (CO), resulting in the significant reduction on total pulmonary vascular resistance compared with vehicle controls (Figure 8H). Altogether, celastramycin treatment significantly improved exercise capacity and increased treadmill walking distance (Figure 8I). These results suggest that celastramycin suppresses inflammation in the lungs and improves systemic metabolism, ameliorating PH and RV failure in seven different animal models. Finally, we performed biochemical tests in Sugen/hypoxia-induced PH model in rats after the treatment with celastramycin or vehicle. Importantly, there was no significant change in the functions of liver and kidney and hematologic profiles after the celastramycin treatment (Online Table IV).

**Discussion**

In this study, we demonstrated that celastramycin inhibits PAH-PASMC proliferation by the suppression of inflammation and oxidative stress and ameliorates PH in 3 different animal models. These concepts are based on the following findings: (1) we selected celastramycin as a compound that inhibits cell proliferation dose-dependently with small effects on control PASMCs, (2) celastramycin treatment increased genes for mitochondrial biogenesis and function, leading to improved mitochondrial energy metabolism and networks, (3) celastramycin significantly diminished nuclear translocation of HIF1-α and NF-κB and reduced cytokines/chemokines and growth factors, and (4) celastramycin inhibited PASMC proliferation and ameliorated PH in different animal models.

**Identification of Celastramycin as a Novel Drug for PAH**

There are 3 main types of drugs for treating PAH, all of which aim to dilate the pulmonary arteries.3 However, these treatments cannot stop or reverse this aggressive disease, although they help to slow its progression. Thus, patients with advanced PAH and RV failure require lung transplantation, and some patients die even after the vasodilator therapy with these drugs.4 As an additional strategy for PAH, effective treatment that achieves reverse remodeling of pulmonary arteries is warranted. Thus, we focused on the inhibition of PAH-PASMC proliferation to discover a novel drug for PAH. PAH-PASMCs have special characteristics in terms of proliferative and antiapoptotic features in common with cancer cells.6 The development of academic drug discovery is in response to the fact that the discovery of new drugs has come to a standstill in the pharmaceutical industry. Under these situations, the DDI was founded in Japan to promote an environment in which academic drug discovery can be performed. Using these platforms, we were able to promote drug discovery based on a clinical perspective and the knowledge of the pathogenesis of PAH. When we consider the pathological findings of PAH, actively proliferative cell components, and occlusion of the distal pulmonary arteries, it is evident that treatment with pulmonary vasodilators is useful only in patients with mild progression. In the present study, we performed phenotypic screening and discovered compounds with antiproliferative effects on PAH-PASMCs and selected the compounds specific for the cells responsible for the disease. Then, we developed 25 analogs of the hit compound to determine the lead compound and finally selected celastramycin b with antioxidant effects for in vivo treatment. Finally, celastramycin was effective in 3 animal models of PH with no apparent side effects.
Figure 6. Celastramycin (CEL) ameliorates hypoxia-induced pulmonary hypertension (PH) in mice. A, Schematic protocols for CEL administration to hypoxia-induced PH in wild-type mice, in which 10 mg/(kg·d) CEL or control vehicle was administered using an osmotic pump during the 3 wk of hypoxic exposure (10% O₂). B, The time-course of body weight from the starting point of administration of CEL or control vehicle under normoxia (21% O₂, n=8 each) or hypoxia (10% O₂, n=14 each) for 3 wk. C, Systolic blood pressure of hypoxia-induced PH mice and control mice measured by tail-cuff systems after the treatment with CEL or control vehicle for 3 wk (n=6 each). D, Levels of cytokines/chemokines and growth factors in the lungs after the treatment with CEL or vehicle under normoxia or hypoxia (10% O₂) for 3 wk (n=6 each). E, Muscularization of the distal pulmonary arteries (PA) with a diameter of 20–70 µm after the treatment with CEL or control vehicle under normoxia (n=8 each) or hypoxia (n=14 each). Scale bar, 25 µm. F, Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy (RVH) in wild-type mice after the treatment with CEL or control vehicle under normoxia (n=8 each) or hypoxia (10% O₂, n=14 each) for 3 wk. RVH denotes the ratio of the right ventricle to the left ventricle plus septum (RV/LV+S). Data represent the mean±SEM. *P<0.05. Comparisons of means between 2 groups by the bootstrap method. The multiplicity of the testing was adjusted by the Holm method. αSMA indicates alpha smooth muscle actin; EM, Elastica-Masson; F, fully muscularized vessels; FGF, fibroblast growth factor; G-CSF, granulocyte-colony stimulating factor; IFNγ, interferon γ; MCP, monocyte chemotactic protein; M-CSF, macrophage colony-stimulating factor; MIP, macrophage inflammatory protein; MO-KC, mouse keratinocyte-derived chemokine; N, nonmuscularized vessels; P, partially muscularized vessels; PDGF-BB, platelet derived growth factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF, tumor necrosis factor; and VEGF, vascular endothelial growth factor.
This means that the antiproliferative strategy for PASMCs may be a novel therapeutic strategy to treat patients with PAH; celastramycin can be a possible drug for PAH. In the previous article, we have already discovered sanguinarine that reduces selenoprotein P expression and PASMC proliferation and ameliorates PH in mice and rats. In the present study,
Figure 8. Celastramycin (CEL) ameliorates sugen/hypoxia-induced pulmonary hypertension in rats. A, Schematic protocols for CEL administration to the Sugen/hypoxia rat model, in which rats were exposed to chronic hypoxia (10% O₂) for 3 wk in combination with the VEGF (vascular endothelial growth factor) receptor blocker SU5416 (20 mg/kg, subcutaneous injection) followed by daily administration of 3 mg/kg body weight CEL or control vehicle by intraperitoneal injection for 2 wk. B, The time-course of body weight changes from the starting point of SU5416 injection for 5 wk in Sugen/hypoxia rats and control rats (n=12 each). C, Levels of IL (interleukin)-2 and IL-6 in the lungs of Sugen/hypoxia rats and control rats after the treatment with CEL or control vehicle for 2 wk (n=6 each). D, Left, Representative pictures of distal pulmonary arteries (PAs). Middle, muscularization of the distal PAs with a diameter of 50–100 μm after the treatment with CEL or vehicle control in rats (n=12 each). Right, medial wall thickness of the distal PAs in rats (n=12 each). Scale bar, 50 μm. E, Right ventricular (RV) systolic pressure (RVSP) and RV hypertrophy (RVH; n=12 each). RVH denotes the ratio of the RV to the left ventricle plus septum (RV/LV+S). F, Left, Representative echocardiographic images illustrating RV diastolic diameter (RVDd), RV fractional area change (RVFAC), PA acceleration time (PAAT), and tricuspid annular plane systolic excursion (TAPSE). Right, Echocardiographic measurement of RVDd, RVFAC, PAAT, TAPSE, and cardiac output (CO; n=12 each). G, Echocardiographic measurement of LV diastolic diameter (LVDd) and LV ejection fraction (LVEF; n=12 each). H, Mean PAs pressure (mPAP) and total pulmonary resistance (TPR) in rats (n=12 each). I, Walking distance assessed by treadmill test (n=12 each). Data represent the mean±SEM. *P<0.05. Comparisons of means between 2 groups by the bootstrap method. The multiplicity of the testing was adjusted by the Holm method. αSMA indicates alpha smooth muscle actin; EM, Elastica-Masson; F, fully muscularized vessels; N, nonmuscularized vessels; P, partially muscularized vessels; and PAH, pulmonary arterial hypertension.
we used our different original library and selected celastramycin by rigorous screening to verify its safety on normal cells, which implies that celastramycin has different effects on PAH-PASMCs compared with the effects of sanguinarine-mediated inhibition of selenoprotein P.

**ZFC3H1-Mediated Anti-Inflammatory Effects by Celastramycin**

In the present study, celastramycin inhibited NF-κB nuclear translocation and exerted anti-inflammatory effects on PAH-PASMCs in vitro and in rodent models of PH in vivo. Celastramycin is a benzoyl pyrrole-type compound originally discovered as a new antibiotic in the extract of *Streptomyces MaB-QuH-8* from the plants of the Celastraceae in 2002.49 Then, after searching for natural substances that regulate innate immunity using an ex vivo Drosophila culture system, celastramycin was identified as a potent suppressor of immune deficiency pathways, which regulate Gram-positive bacterial infections via transcription factor NF-κB-like transcriptional factor.11,12 Thus, NF-κB-related signal transduction pathways seem to be a target for celastramycin in mammalian cells.13 Inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are involved in NF-κB signaling and play crucial roles in the development of PAH.50–52 Thus, the anti-inflammatory effect of celastramycin is one of its main mechanisms of action. A recent study identified that ZFC3H1, an uncharacterized zinc finger protein, is a binding partner of celastramycin, which blocks the formation of the NF-κB transcription complex.13 Moreover, ZFC3H1 links ATP-dependent RNA helicase MTR4 with polyadenylate-binding nuclear protein 1 in the poly(A) tail exosome targeting connection, which plays a crucial role in the degradation of nuclear RNAs.40,41 Exosomes are involved in the processing of a wide range of RNAs, including mRNAs, ribosomal RNAs, and noncoding RNAs,42 and have been shown to regulate the production of cytokines and other inflammatory proteins.53 Thus, celastramycin may modulate poly(A) tail exosome targeting connections by binding to ZFC3H1, resulting in changes in the degradation of RNAs with inflammatory effects. Indeed, celastramycin-mediated multiple effects were due to the inhibition of ZFC3H1, leading to suppression of BRD4 and HIF-1α.

**Celastramycin Reduces Cytosolic ROS in PAH-PASMCs**

There is mounting evidence of the role of oxidative stress in the pathogenesis of PAH.15 In the present study, celastramycin significantly reduced cytosolic ROS in PAH-PASMCs. A possible mechanism of this celastramycin-mediated reduced cytosolic ROS is the upregulation of Nrf2, which is essential for oxidative and electrophilic stress responses, and resultant downregulation of NADPH oxidase activities.30,31 Here, it has been demonstrated that NADPH oxidase regulates the activities of Nrf2 in several cell lines.30,31 Conversely, Keap1-Nrf2 pathway regulates the cytosolic ROS production through inhibition of NADPH oxidases.21 Indeed, celastramycin treatment significantly reduced NADPH oxidase activity and Keap1 and upregulated downstream Nrf2 in PAH-PASMCs. Induction of the Nrf2 transcript is an effective approach for enhancing the activity of Nrf2, although Nrf2 activity is tightly regulated by proteasomal degradation via Keap1-mediated ubiquitination.54 Nrf2 enables adaptation to oxidants and electrophiles by stimulating the transcriptional activation of about 100 cytoprotective genes, including *GCLC*, which regulates glutathione biosynthesis, *HMOX1*, which catalytically degrades potentially toxic heme to biliverdin, and *NQO1*, which inhibits the formation of free radicals via the redox-cycling of quinones. Indeed, in the present study, celastramycin significantly upregulated mRNA levels of *GCLC*, *HMOX1*, and *NQO1* and protein levels of GSH in PAH-PASMCs. Consistently, activation of Nrf2 inhibited PASMC proliferation and ameliorated hypoxia-induced PH in mice.55 In contrast, celastramycin treatment significantly increased mROS in PAH-PASMCs. This is consistent with the higher levels of ATP production, maximal respiration, and OCR/ECAR ratio in celastramycin-treated cells. Dysregulated mitochondria in PAH-PASMCs show lower mROS production compared with normal PASMCs, and an increase in mROS causes apoptosis in PAH-PASMCs.56–58 These data indicate that the celastramycin-mediated reduction in ROS (eg, Nrf2-mediated ROS scavengers, NADPH oxidase-derived ROS reduction) was significant as compared to the celastramycin-mediated increase in mitochondrial ROS production. In total, celastramycin reduced the levels of cytosolic ROS (assessed by 2,7-dichlorodihydrofluorescein and CellROX) through significant upregulation of Nrf2 (ROS scavengers), downregulation of NADPH oxidases, and slight increase in mitochondrial ROS (by increased mitochondrial respiration and ATP production) in PAH-PASMCs.

**Celastramycin Improves Mitochondrial Metabolism and Networks in PAH-PASMCs**

Celastramycin treatment increased OCR and OCR/ECAR ratio in PAH-PASMCs. The reason why the tricarboxylic acid cycle is upregulated may be that celastramycin reduces PDK1, which inactivates PDH to convert pyruvic acid to acetyl CoA. Additionally, celastramycin treatment ameliorated mitochondrial morphology. The mitochondrial network is fragmented in PAH-PASMCs,29 and this disruption is mechanistically related to imbalanced proliferation and apoptosis in PAH-PASMCs.29 Fragmentation of the mitochondrial network reflects, in part, increased fission.43 Fission creates smaller, more discrete mitochondria, which facilitates mitophagy, or accelerates cell proliferation.59 When mitochondria cannot divide, mitosis does not proceed, and cells are arrested in the G2-M phase of the cell cycle.7 Thus, it is conceivable that celastramycin normalized the balance between fission and fusion in PAH-PASMCs, resulting in increased mitochondrial network activity and decreased proliferation. A possible mechanism of the celastramycin-mediated alteration in this balance may be the downregulation of HIF-1α, which is excessively activated in PAH-PASMCs and is likely an upstream stimulus for impaired mitochondrial fusion and enhanced fission in PAH.29 Indeed, celastramycin treatment reduced the levels of DRP1, which promotes mitochondrial fission, in PAH-PASMCs. The reason for the celastramycin-mediated downregulation of HIF-1α may involve the suppression of NF-kB signaling. Indeed, NF-κB has been shown to directly affect HIF-1α expression at the HIF-1α promoter region, contributing to the regulation of basal levels of mRNA and protein. Additionally, NF-κB-mediated
downstream inflammatory cytokines also have direct effects on mitochondrial function in PAH-PASMCs.\(^\text{9,16}\) Furthermore, celastramycin-mediated upregulation of \(HMOX1\) should lead to altered mitochondrial energy metabolism. Indeed, it has been reported that \(HMOX1\) in the heart stimulates mitochondrial biogenesis via the induction of Nrf2 and its nuclear translocation. The number of mitochondria is regulated by mitochondrial biogenesis to meet the energy demands of the cell and compensate for cell damage.\(^\text{59}\) Thus, celastramycin may have altered the mitochondrial energy metabolism through the progression of mitochondrial biogenesis in PAH-PASMCs. Finally, celastramycin-mediated functional improvement of exercise capacity in the Sugen/hypoxia model indicated that celastramycin directly affects cardiac myocytes in addition to PASMCs in terms of mitochondrial biogenesis. We consider that the celastramycin-mediated metabolic changes may have contributed to the greater effects in Sugen/hypoxia-induced model rather than monocrotaline-induced model.

**Study Limitations**

There are several limitations to the present study. First, we mainly evaluated ROS levels (cytosolic and mitochondrial), inflammation, and mitochondrial energy metabolism, but there might be other mechanisms through which celastramycin suppresses cell proliferation. Indeed, celastramycin treatment significantly upregulated the eNOS levels, which increase NO production in PAECs. Nrf2 activators are in clinical trial for PH treatment and Nrf2 activation can increase NO and decrease superoxide generation.\(^\text{60}\) Thus, it is possible that the mechanism of action of celastramycin is actually via Nrf2-dependent NO upregulation, which could explain both the decreased RVSP and RV hypertrophy (both via direct effects of NO in cardiomyocytes and also in response to reduced RVSP) rather than an entirely PASMC-mediated effect. Moreover, new approaches for PAH therapy need to show benefit on the top of optimized treatment with currently approved therapy.\(^\text{20}\)

Thus, the combination of celastramycin with other drugs, such as sildenafil, should be examined in the future. Second, the cytokine data in 3 animal models are complex and difficult to clearly understand because of the different models with different mechanisms. Third, we were unable to use cardiomyocyte patients with PAH because it is difficult for us to perform primary culture of cardiomyocytes from patients with PAH.\(^\text{9}\)

Finally, we selected celastramycin from the 25 analogs, but other analogs might have better effects in vivo because some other analogs inhibited cell proliferation more strongly than celastramycin in vitro.

**Clinical Implications and Conclusions**

We found that celastramycin inhibits proliferation of PAH-PASMCs in a dose-dependent manner with small effect on control PASMCs via anti-inflammatory and antioxidant effects, accompanied by metabolic improvement. Consistently, celastramycin successfully ameliorated hypoxia-induced PH in mice, monocrotaline-induced PH in rats, and Sugen/hypoxia-induced PH in rats. In conclusion, we discovered a new antiproliferative compound, celastramycin, which is effective in rodent models of PH. Celastramycin could be a promising drug for the treatment of patients with PAH.

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**Disclosures**

None.

**References**


**Disclosures**

None.


