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RESEARCH ARTICLE

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Antiproliferative effect of selexipag active metabolite MRE-269 on pulmonary arterial smooth muscle cells from patients with chronic thromboembolic pulmonary hypertension

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Abstract

Chronic thromboembolic pulmonary hypertension (CTEPH) is a group 4 pulmonary hypertension (PH) characterized by nonresolving thromboembolism in the central pulmonary artery and vascular occlusion in the proximal and distal pulmonary artery. Medical therapy is chosen for patients who are ineligible for pulmonary endarterectomy or balloon pulmonary angioplasty or who have symptomatic residual PH after surgery or intervention. Selexipag, an oral prostacyclin receptor agonist and potent vasodilator, was approved for CTEPH in Japan in 2021. To evaluate the pharmacological effect of selexipag on vascular occlusion in CTEPH, we examined how its active metabolite MRE-269 affects platelet-derived growth factor-stimulated pulmonary arterial smooth muscle cells (PASMCs) from CTEPH patients. MRE-269 showed a more potent antiproliferative effect on PASMCs from CTEPH patients than on those from normal subjects. DNA-binding protein inhibitor (ID) genes *ID1* and *ID3* were found by RNA sequencing and real-time quantitative polymerase chain reaction to be expressed at lower levels in PASMCs from CTEPH patients than in those from normal subjects and were upregulated by MRE-269 treatment. *ID1* and *ID3* upregulation by MRE-269 was blocked by co-incubation with a prostacyclin receptor antagonist, and *ID1* knockdown by small interfering RNA transfection attenuated the antiproliferative effect of MRE-269. ID signaling may be involved in the antiproliferative effect of MRE-269 on PASMCs. This is the first study to demonstrate the pharmacological effects on PASMCs from CTEPH patients of a drug approved for the treatment of CTEPH. Both the vasodilatory and the antiproliferative effect of MRE-269 may contribute to the efficacy of selexipag in CTEPH.

KEYWORDS
CTEPH, ID1, PASMC, prostacyclin

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is classified as group 4 pulmonary hypertension (PH) and is characterized pathologically by nonresolving thromboembolism resulted in the formation of fibrous tissues causing vascular occlusive lesions in the proximal and distal pulmonary arteries.[1–4] Continuous elevation of pulmonary arterial pressure (PAP) by pulmonary vascular occlusion causes right ventricular hypertrophy leading to right heart failure.[5] The fibrous tissues in the proximal and distal pulmonary arteries are considered to cause increasing PAP via the direct effect of obstructing blood flow as well as the indirect effect of leading to vascular remodeling by activating bioactive substances such as thrombin and by increasing shear stress.[6,7] Pulmonary vascular remodeling mainly results from excessive proliferation of smooth muscle cells and also includes contributions from endothelial cells, adventitial fibroblasts, and the accumulation of circulating inflammatory cells.[7–11] The small vessel vasculopathy of CTEPH is histopathologically similar to that observed in pulmonary arterial hypertension (PAH). However, the details of the pathogenesis of the vascular remodeling in CTEPH are still unclear.

For symptomatic relief and long-term survival, pulmonary endarterectomy (PEA) is currently recommended in the international guidelines if the lesions are surgically accessible. In recent years, there has been a series of reports from Japanese centers on the efficacy and safety of refined balloon pulmonary angioplasty (BPA) for inoperable CTEPH and similar results were also reported from European centers.[12–15] Based on these reports, recent international guidelines recommend consideration of BPA to patients with inoperable CTEPH or residual PH after PEA.[1,5] In spite of recent remarkable advances in surgical/interventional therapy, some patients are ineligible for PEA/BPA or have symptomatic residual PH after the operation. For these patients, effective medical therapy is needed.

Riociguat, a soluble guanylate cyclase stimulator, became the first drug approved for the treatment of CTEPH in 2013 and is used worldwide. In June 2021, selexipag (Uptravi; 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-*N*-(methylsulfonyl)acetamide; also known as NS-304 or ACT-293987), an orally available and potent prostacyclin receptor (IP) agonist with a nonprostacyclin structure,[16] was approved for the treatment of CTEPH in Japan.[17] In randomized, placebo-controlled, double-blind clinical trials, selexipag significantly improved pulmonary vascular resistance (PVR) and other clinical parameters, such as 6-min walk distance, Borg Dyspnea Scale score, and World Health Organization functional class, in patients with inoperable CTEPH or postoperative/postinterventional residual PH.

Selexipag reduces right ventricular systolic pressure and attenuates pulmonary vascular remodeling in concert with reducing proliferative vascular smooth muscle cells in monocrotaline or Sugen 5416/hypoxia-induced PH in rats.[18–20] Selexipag is rapidly absorbed after oral administration and hydrolyzed to its active metabolite MRE-269 ({4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid; also known as ACT-333679). MRE-269 has more potent activity and a longer half-life than selexipag; therefore, it is considered to be the major contributor to the pharmacological activity of the drug.[16] MRE-269 has a potent vasodilating effect on pulmonary artery from rats and humans and inhibits cell proliferation in pulmonary arterial smooth muscle cells (PASMCs) from normal subjects.[21–24] An antiproliferative effect of MRE-269 has also been demonstrated in PASMCs obtained from PAH patients.[25,26] However, there are no reports on the pharmacological effects of drugs approved for the treatment of CTEPH on cells that participate in vascular remodeling in CTEPH. In this study, therefore, we investigate the effect of MRE-269 on the proliferation of PASMCs from CTEPH patients and the molecular mechanisms underlying the antiproliferative effect of MRE-269.

METHODS

Cell isolation and culture

PASMCs were isolated as previously described from endarterectomized tissue removed during PEA from three patients with CTEPH.[6] All experiments were carried out after approval by the Institutional Review Board of the National Hospital Organization Okayama Medical Center (approval number, H23-RINKEN-30). Written informed consent was obtained from each patient before the procedure. PASMCs from CTEPH patients (CTEPH PASMCs) and normal subjects (normal PASMCs; Lonza; catalog number, CC-258; lot numbers, 0000578443, 0000658401, and 0000669096) were cultured on collagen type I-coated dishes (Iwaki/AGC Techno Glass Co., Ltd.) in Dulbecco's modified Eagle medium (DMEM) (low glucose [1 g/L]; Gibco/Thermo Fisher Scientific) containing 10% (v/v) fetal bovine serum (FBS) with 1% (v/v) penicillin-streptomycin (Gibco/Thermo Fisher Scientific). PASMCs were incubated in a humidified 5% CO_2 atmosphere at 37°C. After reaching confluence, the cells were subcultured by trypsinization with TrypLE Express (Gibco/Thermo Fisher Scientific).

Cell images were acquired with an Olympus CKX41 microscope (Olympus).

Reagents

MRE-269 was synthesized at Nippon Shinyaku Co., Ltd. RO1138452 was from MedChemExpress.

Bromodeoxyuridine (BrdU) uptake cell proliferation assays

The BrdU uptake cell proliferation assay was performed using cell proliferation enzyme-linked immunosorbent assay, BrdU (chemiluminescent) kits (Roche Holding AG). Briefly, PASMCs between four and eight passages were plated on 96-well culture plates at 3×10^3 cells/well and allowed to adhere overnight in culture medium. Growth arrest was then achieved by incubation in starvation medium (DMEM containing 0.1% FBS) for 48 h. Subsequently, the cells were incubated in starvation medium containing human recombinant platelet-derived growth factor-BB (PDGF; Sigma-Aldrich; 10 ng/mL) and BrdU (10 μmol/L) with or without serial concentrations of MRE-269 for 24 h. For the control, cells were incubated in starvation medium containing BrdU and 0.1% dimethyl sulfoxide (DMSO; Nacalai Tesque) with PDGF. After incubation, the medium was removed and the incorporated BrdU was assessed according to the manufacturer's protocol.

RNA-seq analysis

PASMCs were plated on collagen type I coated six-well culture plates at 1×10^5 cells/well and allowed to adhere overnight in culture medium. For analyzing gene expression under growth conditions, total RNA was extracted from the attached cells the next day using the NucleoSpin RNA Kit (Takara Bio) according to the manufacturer's protocol. For analyzing the effect of MRE-269 on gene expression, cell growth was arrested by incubation in starvation medium for 48 h. The cells were then incubated for 24 h in starvation medium containing 0.1% DMSO (as vehicle), PDGF (10 ng/mL) with 0.1% DMSO (as PDGF), or PDGF (10 ng/mL) with 1 μmol/L MRE-269 (as PDGF + MRE-269). After incubation, the medium was removed and total RNA was extracted from the cells using the NucleoSpin RNA Plus XS Kit (Takara Bio). The preparation of libraries and RNA sequencing were performed by Relixa Inc. Bioanalyzer quality control analysis was performed (RNA integrity number score > 9). Libraries were then prepared using the NEBNext Ultra™ Directional RNA Library Prep Kit for Illumina (New England Biolabs) and sequenced on a NovaSeq. 6000 (Illumina) to obtain an average of 26.7 million uniquely mapped reads for each sample. Quality control of the resulting sequence data was performed using FastQC version 0.11.7 (distributed at https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmed and filtered reads were aligned to the hg38 reference genome using Trimmomatic version 0.38[27] and Hisat2 version 2.1.0.[28] featureCounts version 1.6.3[29] was employed to quantify the raw expression count of the genes and calculate transcripts per kilobase million values.

Real-time quantitative polymerase chain reaction (RT-qPCR assay)

For analysis of the mode of action of MRE-269, total RNA was extracted from PASMCs using the RNeasy Mini Kit (Qiagen). Reverse transcription of messenger RNA (mRNA) into complementary DNA was performed using the PrimeScript™ RT reagent Kit (Takara Bio) according to the manufacturer's protocol. mRNA expression levels were quantified using TaqMan primer/probe sets (Supporting Information: Table 1) for the target with a LightCycler 480 System (Roche Holding AG) and normalized to the housekeeping control gene *ACTB*.

Small interfering RNA (siRNA) transfection

Silencer-select predesigned *ID1*-targeted siRNA (s555488), *ID3*-targeted siRNA (s7110), and scrambled control siRNA were purchased from Thermo Fisher Scientific. Normal PASMC-3 cells on 96-well culture plates or glass-bottom chamber slides (ibidi GmbH) were washed with starvation medium and transfected with siRNA by using Lipofectamine RNAiMAX (Thermo Fisher Scientific). After a 48-h incubation, cells were used for cell proliferation assay, RT-qPCR assay or immunofluorescence staining.

Western blot analysis

Normal PASMC-3 cells were plated on 60-mm cell culture dishes at 2.5×10^5 cells/dish and allowed to adhere overnight in culture medium, then cell growth was arrested by incubation in starvation medium for 48 h. Total cell lysates were prepared with RIPA lysis

buffer (Nacalai Tesque) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (Tocris Biosciences). Lysate samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with antibody specific for ID1 (Proteintech), ID3 (Abcam) or β-actin (Sigma-Aldrich). Antibodies were detected with the appropriate horseradish peroxidase-linked secondary antibodies (GE Healthcare) and membranes were developed with the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Images were acquired with the ChemiDoc Touch imaging system (Bio-Rad Laboratories).

Immunofluorescence staining

PDGF-stimulated normal PASMC-3 cells transfected with each siRNA and treated with MRE-269 or 0.1% DMSO were stained with Alexa-488 conjugated antibody specific for BrdU (Abcam) or proliferating cell nuclear antigen (PCNA; Cell Signaling Technologies) according to the manufacturer's protocol. Nuclear morphology was visualized by staining with Hoechst 33342 (Dojindo). Fluorescence images were acquired with a BZ-X800 fluorescence microscope, (Keyence) and merged using ImageJ.

Statistical analysis

For gene expression and cell proliferation experiments, data were expressed as the mean ± standard error of the mean (SEM) and figures were drawn with GraphPad Prism 6 (GraphPad Software). Statistical analysis of the fold changes of the mRNA expression of genes was performed by Student's *t* test for two groups and by Tukey's test for more than three groups. In the PDGF-induced cell proliferation assay, the effects of multiple doses of MRE-269 were analyzed by Dunnett's test. The effects of MRE-269 with control or gene-specific siRNAs were analyzed by Tukey's test. All statistical analyses were performed with SAS System Version 9.3 (SAS Institute Inc.) and EXSUS Version 8.1.0 (CAC Croit Corporation). A p value of less than 0.05 was considered statistically significant. For RNA-seq analysis, the raw count data were normalized by the Tag Count Comparison (TCC) R package[30] to detect differentially expressed genes (DEGs) between two groups, then gene ontology pathway enrichment analysis was performed with the web tool for the gene ontology database DAVID (Database for Annotation, Visualization, and Integrated Discovery, 2021 released[31,32]) at http://david.abcc.ncifcrf.gov/.

RESULTS

CTEPH PASMCs express IP at levels similar to those of normal PASMCs

CTEPH PASMCs were isolated from the lungs of three patients with CTEPH who underwent PEA. Preoperative mean PAP ranged from 38 to 50 mmHg, and PVR ranged from 6.9 to 9.7 Wood units (Table 1). No patients had received any PH-targeted drugs. Sex-matched PASMCs from normal subjects were used as the control. In contrast to normal PASMCs, which are elongated with a small cell body (Figure 1a, left panel), CTEPH PASMCs included cells with several different shapes, such as large flattened endothelial-like cells and elongated smooth muscle-like cells (Figure 1a, right panel). The gene expression of the differentiated smooth muscle cell markers smooth muscle protein 22-alpha (transgelin; *TAGLN*) and calponin 1 (*CNN1*) were significantly lower in CTEPH PASMCs than in normal PASMCs (*TAGLN*, 0.34 ± 0.06 [$p < 0.05$]; *CNN1*, 0.09 ± 0.05 [$p < 0.05$]). The expression of another smooth muscle cell marker, alpha-smooth muscle actin (α-SMA; *ACTA2*), tended to be lower in CTEPH PASMCs (0.40 ± 0.11 [$p = 0.15$]) (Figure 1b).

The expression of prostanoid receptor family genes in normal PASMCs and CTEPH PASMCs was assessed by RT-qPCR (Figure 1c). Among the members of the prostanoid receptor family, IP (*PTGIR*) was highly expressed in both normal PASMCs (1.99 ± 0.38) and CTEPH PASMCs (1.60 ± 0.77). The thromboxane receptor (TP; *TBXA2R*) and the prostaglandin E1 receptor (EP1; *PTGER1*) were both more highly expressed in CTEPH PASMCs (*TBXA2R*, 0.77 ± 0.18; *PTGER1*, 0.24 ± 0.06) than in normal PASMCs (*TBXA2R*, 0.43 ± 0.05; *PTGER1*, 0.09 ± 0.04). However, there were no significant differences in the gene expression of prostanoid receptor family members between normal PASMCs and CTEPH PASMCs. These results suggest that selexipag exerts its pharmacological effect on CTEPH PASMCs via IP, as it does in normal PASMCs.

MRE-269 suppresses PDGF-induced proliferation of CTEPH PASMCs in a dose-dependent manner

PDGF is a potent inducer of vascular smooth muscle cell proliferation and angiogenesis. High deposition of PDGF has previously been observed in distal pulmonary arteries of CTEPH patients, and the expression of platelet-derived growth factor receptor and the proliferative response to PDGF are enhanced in cells derived from CTEPH patients.[33]

TABLE 1 Demographics of normal subjects and CTEPH patients and the hemodynamics parameters of CTEPH patients.

Cell line	Lot number	Age (years)	Sex	Diagnosis	mPAP (mmHg)	PVR (Wood units)	PH targeted drugs
normal PASMC-1	0000578443	52	Female	–	–	–	–
normal PASMC-2	0000658401	51	Female	–	–	–	–
normal PASMC-3	0000669096	51	Male	–	–	–	–
CTEPH PASMC-1	–	75	Female	CTEPH	50	9.7	–
CTEPH PASMC-2	–	51	Female	CTEPH	46	9.0	–
CTEPH PASMC-3	–	57	Male	CTEPH	38	6.9	–

Abbreviations: CTEPH, chronic thromboembolic pulmonary hypertension; mPAP, mean pulmonary arterial pressure; PASMC, pulmonary arterial smooth muscle cells; PH, pulmonary hypertension; PVR, pulmonary vascular resistance.

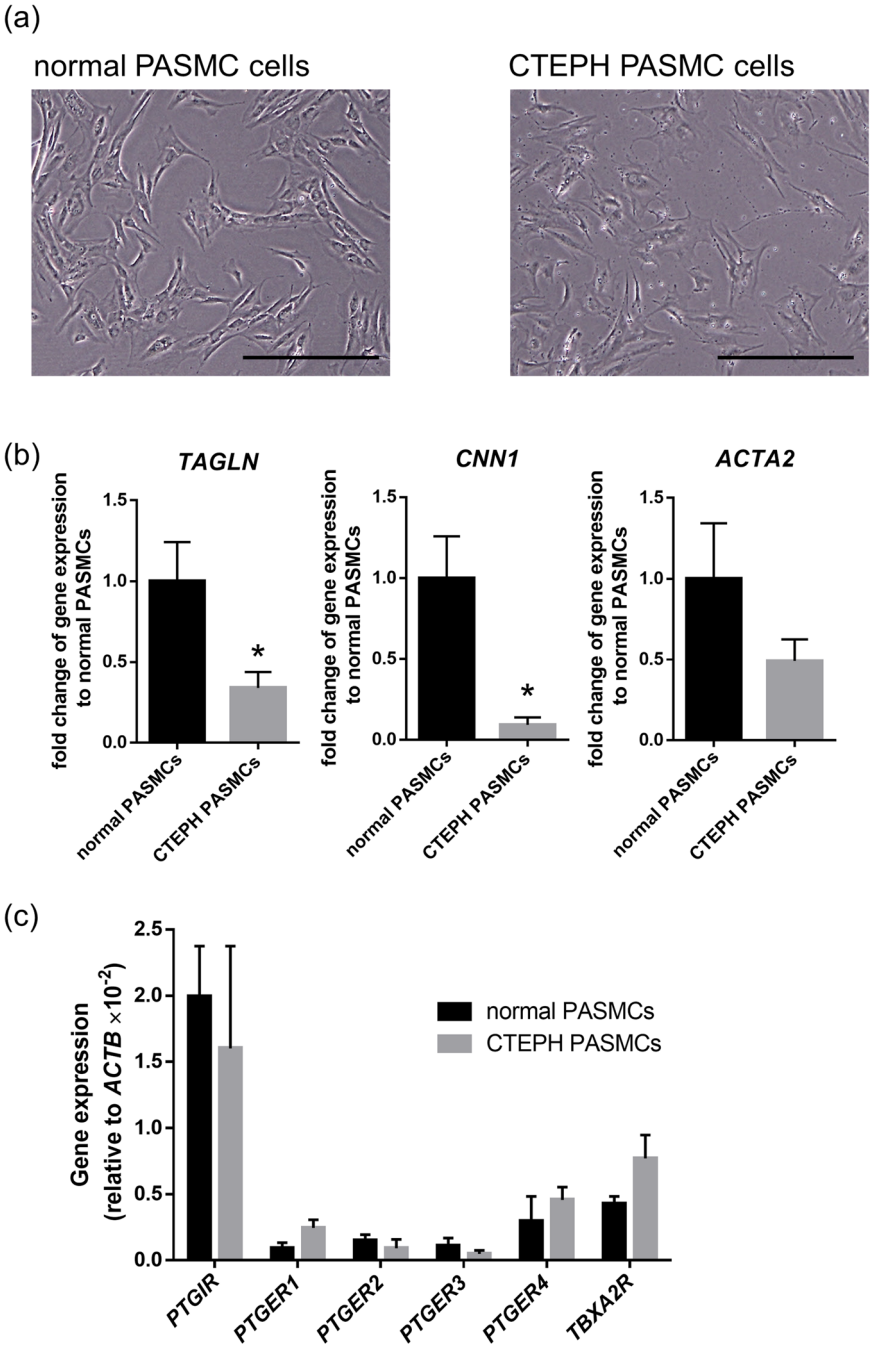


FIGURE 1 Morphological characteristics and identification of CTEPH (chronic thromboembolic pulmonary hypertension) pulmonary arterial smooth muscle cells (PASMCs). (a) Representative cell morphology of normal PASMCs (left panel) and CTEPH PASMCs (right panel); scale bar = 0.5 mm. (b) Messenger RNA (mRNA) expression levels of *TAGLN*, *CNN1,* and *ACTA2* in normal PASMCs and CTEPH PASMCs were determined by real-time quantitative polymerase chain reaction (RT-qPCR). Mean ± standard error of the mean (SEM) of fold change of gene expression over normal PASMCs is shown ($n = 3$). *$p < 0.05$ versus normal PASMCs (Student's t test). (c) mRNA expression levels of prostanoid receptor family genes in normal PASMCs and CTEPH PASMCs were determined by RT-qPCR and normalized to *ACTB* as the internal control. Data are the mean ± SEM, $n = 2$ or 3.

We assessed the effect of MRE-269, an active metabolite of selexipag, on PDGF-induced proliferation in PASMCs from CTEPH patient 1 (CTEPH PASMC-1 cells) because these cells showed the most potent proliferative response to PDGF stimulation. To compare the pharmacological effect of MRE-269 on normal PASMCs, the antiproliferative effect of MRE-269 on PASMCs from normal subject 3 (normal PASMC-3 cells), which had the most potent proliferative response to PDGF, was assessed. The cell cycle was synchronized by incubating the cells in serum-starved medium for 48 h, then PDGF-induced cell proliferation was measured by the incorporation of BrdU into the DNA of mitotic cells after incubation in PDGF-containing medium with or without MRE-269. MRE-269 suppressed the PDGF-induced proliferation of normal PASMC-3 cells at a concentration of 3 μmol/L (to 49.9 ± 9.5%; $p < 0.05$), and its IC_{50} value against normal PASMC-3 cells was 3.67 μmol/L (95% confidence interval [CI], 1.31–10.25). The numbers of BrdU and PCNA-positive cells were significantly decreased in normal PASMC-3 cells treated with 3 μmol/L MRE-269 (Supporting Information: Figure). MRE-269 also suppressed the protein expression of a cell proliferation marker, cyclin D1. The reduction of the incorporation of BrdU indicated an antiproliferative effect of MRE-269 on normal PASMC-3 cells. MRE-269 significantly suppressed the PDGF-induced proliferation of CTEPH PASMC-1 cells at concentrations of 0.01 μmol/L (to 64.2 ± 5.0%; $p < 0.01$) and higher, and its IC_{50} value against CTEPH PASMC-1 cells was 0.07 μmol/L (95% CI, 0.03–0.20) (Figure 2). These results suggest that MRE-269 has a more potent antiproliferative effect on PASMCs from CTEPH patients than on those from normal subjects.

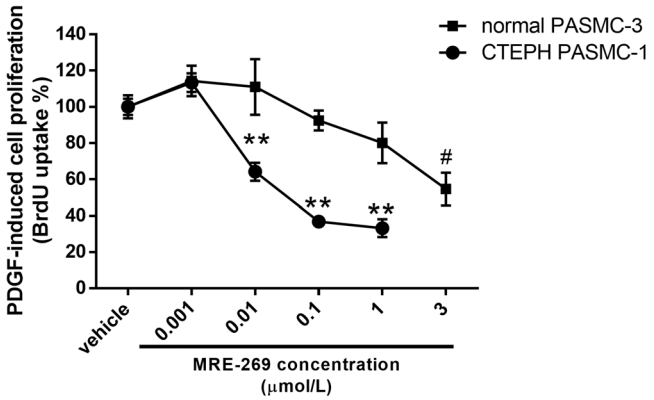


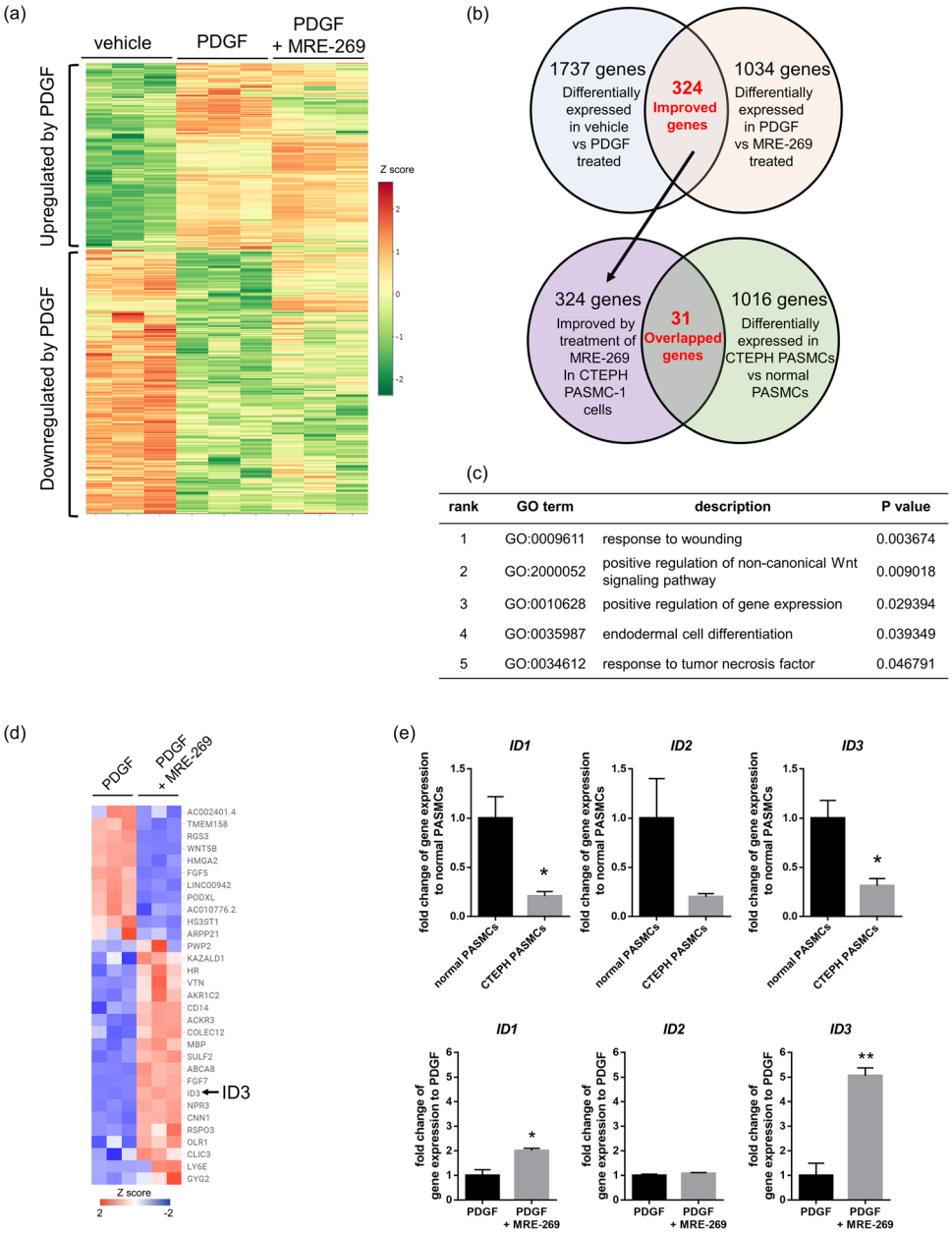
FIGURE 2 Effect of MRE-269 on platelet-derived growth factor (PDGF)-induced cell proliferation. Relative amounts of bromodeoxyuridine taken up by PDGF-stimulated chronic thromboembolic pulmonary hypertension (CTEPH) pulmonary arterial smooth muscle cells (PASMC)-1 and normal PASMC-3 cells treated with 0.1% dimethyl sulfoxide (vehicle) or the indicated concentration of MRE-269 is shown. Data are the mean ± standard error of the mean, $n = 4$. $^{**}p < 0.01$ versus vehicle for CTEPH PASMC-1 cells, $\#p < 0.05$ versus vehicle for normal PASMC-3 cells, (Dunnett's test).

MRE-269 upregulates the expression of DNA-binding protein inhibitor (ID) family members in CTEPH PASMCs

To investigate the signaling pathways responsible for the antiproliferative effect of MRE-269 on CTEPH PASMCs, we performed RNA sequencing to identify transcriptional changes caused by MRE-269 in PDGF-stimulated CTEPH PASMC-1 cells. CTEPH PASMC-1 cells were treated with vehicle, PDGF or PDGF plus MRE-269 for 24 h and their gene expression was analyzed using RNA sequencing. The resulting heat map shows the gene expression changes induced by PDGF stimulation compared with vehicle (Figure 3a). The gene signature of CTEPH PASMC-1 cells treated with PDGF plus MRE-269 was considerably different from that of PDGF-stimulated cells. 1737 DEGs were identified in PDGF-stimulated cells (false discovery rate [FDR] cutoff = 0.1; $\log_2$ fold change [$\log_2$FC] cutoff = 1) compared with vehicle-treated cells (Figure 3b). Of the 1737 DEGs, 324 had their expression significantly improved in cells treated with PDGF plus MRE-269 (FDR cutoff = 0.1; $\log_2$FC cutoff = 1) compared with PDGF-stimulated cells.

We additionally analyzed the transcriptional differences between normal PASMCs and CTEPH PASMCs to identify the signaling pathways involved in the potent pharmacological response of CTEPH PASMCs to MRE-269. The gene expression of PASMCs from the three normal subjects and three CTEPH patients (donor demographics are shown in Table 1) was analyzed using RNA sequencing. 1016 DEGs were identified in CTEPH PASMCs (FDR cutoff = 0.1; $\log_2$FC cutoff = 1) compared with normal PASMCs. Among the 324 genes whose expression was significantly changed by MRE-269 treatment of CTEPH PASMC-1 cells, we identified 31 in common with the 1016 DEGs (Figure 3b). The top five differentially regulated pathways (Gene Ontology terms) were the response to wounding, positive regulation of the noncanonical Wnt signaling pathway, positive regulation of gene expression, endodermal cell differentiation, and response to tumor necrosis factor (Figure 3c). Among these 31 genes, 11 were increased in CTEPH PASMCs and downregulated by MRE-269, while 20 were decreased in CTEPH PASMCs and upregulated by MRE-269 (Figure 3d).

DNA-binding protein inhibitor-3 (ID3) is a member of the ID transcription factor family, which is downstream of the bone morphogenetic protein receptor (BMPR) signaling pathway. The ID transcription factor family consists of four members, ID1, ID2, ID3, and ID4.[34] ID1 and ID3 are well studied and are upregulated by BMP stimulation in PASMCs.[35–37] ID2 is downregulated in pulmonary vascular



rank	GO term	description	P value
1	GO:0009611	response to wounding	0.003674
2	GO:2000052	positive regulation of non-canonical Wnt signaling pathway	0.009018
3	GO:0010628	positive regulation of gene expression	0.029394
4	GO:0035987	endodermal cell differentiation	0.039349
5	GO:0034612	response to tumor necrosis factor	0.046791

FIGURE 3 MRE-269 upregulates the expression of DNA-binding protein inhibitor (ID) family members in chronic thromboembolic pulmonary hypertension (CTEPH) pulmonary arterial smooth muscle cells (PASMCs). (a) Heat map of $\log_2$ expression of 1737 genes identified as differentially expressed in platelet-derived growth factor (PDGF)-stimulated versus non-stimulated CTEPH PASMC-1 cells (false discovery rate cutoff = 0.1; $\log_2$ fold change cutoff = 1) and their behavior after treatment with MRE-269. Genes were clustered using the complete agglomeration method on the Euclidean distance between their centered and scaled expression levels. The average color is white, $\log_2$ expression above average is red, and $\log_2$ expression below average is green. (b) Venn diagrams showing overlap between genes whose expression was significantly improved by treatment of CTEPH PASMC-1 cells with MRE-269 and differentially expressed in CTEPH PASMCs versus normal PASMCs. (c) List of the top five Gene Ontology terms. Analysis performed on the 31 genes in common with the 1016 differentially expressed genes. (d) Heat map of $\log_2$ expression. The average color is white, $\log_2$ expression above average is red, and $\log_2$ expression below average is blue. (e) Messenger RNA (mRNA) expression levels of *ID1*, *ID2,* and *ID3* in normal PASMCs and CTEPH PASMCs is determined by real-time quantitative polymerase chain reaction (RT-qPCR). The mRNA level was normalized to *ACTB* as the internal control. Mean ± standard error of the mean (SEM) of fold change of gene expression over normal PASMCs are shown ($n = 3$). *$p < 0.05$ versus normal PASMCs (Student's t test). (f) mRNA expression levels of PDGF-stimulated CTEPH PASMC-1 cells (PDGF) and MRE-269-treated PDGF-stimulated CTEPH PASMC-1 cells (PDGF + MRE-269) were determined by RT-qPCR and normalized to *ACTB* as the internal control. Mean ± SEM of fold change of gene expression over PDGF is shown ($n = 3$). *$p < 0.05$, **$p < 0.01$ versus PDGF (Student's t test).

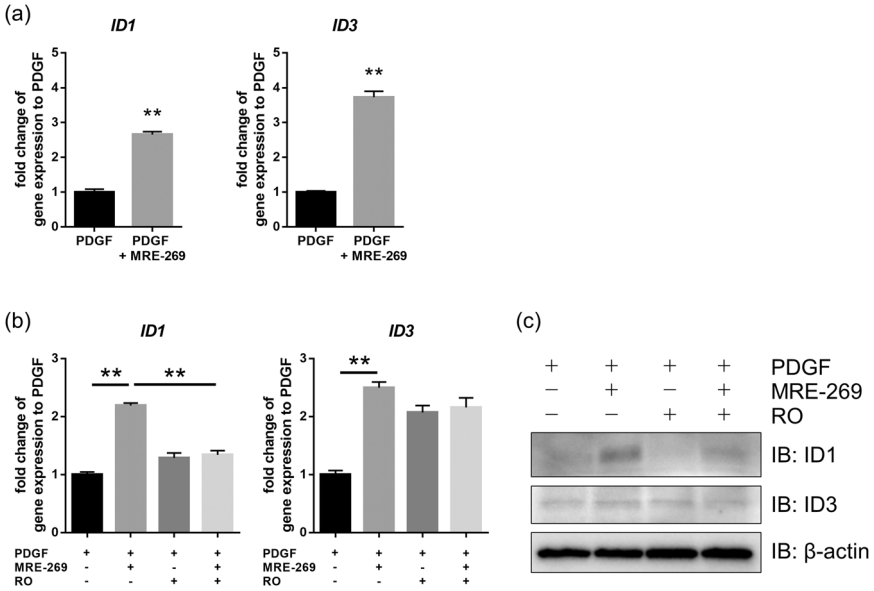


FIGURE 4 Effect of MRE-269 on the gene expression of DNA-binding protein inhibitor (ID) family members. (a) Messenger RNA (mRNA) expression levels of *ID1* and *ID3* in platelet-derived growth factor (PDGF)-stimulated normal pulmonary arterial smooth muscle cells (PASMC)-3 cells (PDGF) and in MRE-269-treated PDGF-stimulated normal PASMC-3 cells (PDGF + MRE-269) were determined by real-time quantitative polymerase chain reaction (RT-qPCR) and normalized to *ACTB* as the internal control. Mean ± standard error of the mean (SEM) of fold change of gene expression to PDGF is shown ($n = 3$). **$p < 0.01$ versus PDGF (Student's *t* test). (b) mRNA expression levels of *ID1* and *ID3* in PDGF-stimulated normal PASMC-3 cells incubated with or without MRE-269 (1 μmol/L) or RO1138452 (RO; 3 μmol/L) were determined by RT-qPCR and normalized to *ACTB* as the internal control. Mean ± SEM of fold change of gene expression over PDGF is shown ($n = 3$). **$p < 0.01$ (Tukey's test). (c) Protein expression of ID1 and ID3 in PDGF-stimulated normal PASMC-3 cells incubated with or without MRE-269 or RO1138452 were determined by western blot analysis. β-Actin was used as an internal control.

cells in a PAH animal model.[38] The role of ID4 in the pathology of PAH or CTEPH has not yet been reported. Therefore, we investigated the change in gene expression of *ID1*, *ID2,* and *ID3* by using RT-qPCR. In agreement with the results of RNA sequencing, *ID1* and *ID3* showed significantly lower expression in CTEPH PASMCs than in normal PASMCs (*ID1*, to 0.21 ± 0.05 [$p < 0.05$]; *ID3*, to 0.31 ± 0.13 [$p < 0.05$]; Figure 3e). *ID2* was also expressed at lower levels in CTEPH PASMCs. Treatment of PDGF-stimulated CTEPH PASMC-1 cells with MRE-269 caused significant upregulation of *ID1* and *ID3* compared with vehicle treatment (*ID1*, to 2.01 ± 0.09 [$p < 0.05$]; *ID3*, to 5.06 ± 0.32 [$p < 0.05$]; Figure 3f). The expression of *ID2* was not affected. These results demonstrate that MRE-269 altered *ID1* and *ID3* gene expression in PDGF-stimulated CTEPH PASMCs.

MRE-269 upregulates the expression of ID family members via IP

Regardless of the state of gene expression under unstimulated conditions, the fold change of induction of *ID1* and *ID3* by MRE-269 was similar between normal PASMC-3 cells and CTEPH PASMC-1 cells. Therefore, we used normal PASMC-3 cells to investigate whether the upregulation of ID family members contributes to the antiproliferative effect of MRE-269. To confirm the effect of MRE-269 on *ID1* and *ID3* expression, we assessed its effect on normal PASMCs. The gene expression of *ID1* was significantly upregulated by incubation of PDGF-stimulated normal PASMC-3 cells with MRE-269 (to 2.65 ± 0.08 [$p < 0.01$]), as was that of *ID3* (to 3.73 ± 0.17 [$p < 0.01$]) (Figure 4a). Co-incubation with MRE-269 and RO1138452, an IP antagonist, almost completely blocked the MRE-269-induced upregulation of *ID1* (Figure 4b). *ID3* gene expression in the co-incubation with MRE-269 and RO1138452 was also lower than that with MRE-269 alone. Consistent with the results of gene expression, the protein expression of ID1 was also upregulated by incubation with MRE-269, and this upregulation was blocked by co-incubation with MRE-269 and RO1138452. The protein expression of ID3 was moderately upregulated by incubation with MRE-269 (Figure 4c).

Knockdown of ID1 expression attenuates the antiproliferative activity of MRE-269

The transfection of *ID1*- or *ID3*-targeted siRNA into normal PASMC-3 cells reduced the gene expression of

ID1 (to 0.40 ± 0.06) or *ID3* (to 0.04 ± 0.004) at 3 days after transfection (Figure 5a). PDGF-induced cell proliferation was not significantly affected by knockdown of *ID1* or *ID3* (*ID1*-targeted siRNA, 147.5 ± 4.2% [$p = 0.07$]); *ID3*-targeted siRNA, 133.5 ± 22.4% [$p = 0.20$]) (Figure 5b). The antiproliferative effect of MRE-269 was significantly attenuated in normal PASMC-3 cells transfected with *ID1*-targeted siRNA compared to normal PASMC-3 cells transfected with control siRNA (from 53.8 ± 7.2% to 91.5 ± 1.8% [$p < 0.01$]). MRE-269 had a moderate antiproliferative effect on normal PASMC-3 cells transfected with *ID3*-targeted siRNA (to 69.0 ± 4.0% [$p = 0.12$]) (Figure 5c,d). These results demonstrate that the upregulation of ID family members by MRE-269 via IP contributes to its antiproliferative effect on PASMCs.

DISCUSSION

In this study, we found that MRE-269, the active metabolite of selexipag, suppressed the PDGF-induced proliferation of CTEPH PASMCs at concentrations of 0.01 μmol/L and higher, and promoted the gene expression of *ID1* and *ID3*. CTEPH PASMC-1 cells were chosen to assess the antiproliferative effect of MRE-269 and analyze the change in gene expression because they had the highest responsiveness to PDGF stimulation. However, MRE-269 also showed an antiproliferative effect on CTEPH PASMC-3 cells at concentrations in a similar range (data not shown). The antiproliferative effect of MRE-269 was similar between the two CTEPH PASMCs and did not depend on responsiveness to PDGF stimulation or the hemodynamics of the patients. In a clinical study, the maximum plasma concentration of MRE-269 reached about 0.02 μmol/L after repeated administration of selexipag at a dose of 0.6 mg twice a day for 8 days, and its terminal elimination half-life ($t_{1/2}$) was about 10.53 h in normal adults.[39] Therefore, the antiproliferative effect of MRE-269 may contribute to its therapeutic benefit in the treatment of CTEPH.

The PDGF-induced proliferation of CTEPH PASMCs was suppressed by MRE-269 at lower concentrations than with normal PASMCs. The antiproliferative effect of MRE-269 on CTEPH PASMCs may be more potent than on normal PASMCs. However, the absolute BrdU uptake values were different between the cell lines. This limits our ability to compare the potency of the antiproliferative effect of drugs between cell lines, and further work is needed to confirm the potency of the antiproliferative effect of MRE-269 on CTEPH PASMCs.

Abnormalities of coagulation and the fibrinolytic system are found in CTEPH patients, and occlusion of pulmonary arteries with thromboemboli eventually induces vascular remodeling. Factors associated with inflammation or coagulation, such as C-reactive protein and thrombin, induce the proliferation of CTEPH PASMCs and endothelial dysfunction.[6,8,40] The pharmacological effect of MRE-269 on PASMC proliferation and endothelial dysfunction induced by inflammation or coagulation factors needs further investigation.

Even though CTEPH PASMCs included not only elongated smooth-muscle-like cells but also large flattened endothelial-like cells and the gene expression of smooth muscle markers was low, the gene expression of vascular endothelial markers, such as vascular endothelial cadherin (VE-cadherin; *CDH5*), vascular endothelial growth factor receptor 2 (VEGFR2; *KDR*) and tyrosine-protein kinase receptor (Tie-2; *TIE2*), in CTEPH PASMCs were at the same level as in normal PASMCs, and there were no significant differences between them in the RNA-seq analysis (*CDH5*, 1.31 ± 1.20-fold compared with normal PASMCs [$p = 0.82$]; *KDR*,1.47 ± 0.68-fold [$p = 0.54$]; *TIE2*, 1.04 ± 0.81-fold [$p = 0.97$]; Student's *t* test). The reduction of the expression of differentiated smooth muscle cell markers may indicate that the CTEPH PASMCs had shifted to a secretory type. These multimorphological and immature phenotypes are consistent with the phenotypes of cells from CTEPH patients observed in a previous study.[8]

The gene expression of prostanoid receptors was not significantly different between CTEPH PASMCs and normal PASMCs; however, TP and EP1 were expressed at higher levels in CTEPH PASMCs. TP and EP1 signaling counteracts IP signaling. Prostacyclin-induced NO release inhibits the activation of EP1 and TP in rat mesenteric artery,[41] and an imbalance of thromboxane A_2 and prostacyclin secretion in the pulmonary artery is proposed to be involved in the development of PH.[42] Further study is needed to validate the prostanoid receptor gene expression profile and its contribution to the pathogenesis of CTEPH. However, the high selectivity of MRE-269 for IP compared with other prostanoid receptors may be beneficial for the treatment of CTEPH.[16]

The PDGF-induced proliferation of CTEPH PASMCs was suppressed by MRE-269 at lower concentrations than with normal PASMCs. We hypothesize that the genes that play important roles in the high responsiveness of CTEPH PASMCs to treatment with MRE-269 are probably among the genes which are expressed at lower levels in CTEPH PASMCs and which are upregulated by MRE-269 treatment. *ID1* and *ID3* were identified by RNA-sequencing and RT-qPCR as genes which were expressed at lower levels in CTEPH PASMCs and upregulated by MRE-269 treatment. ID family proteins are transcription factors regulated by bone

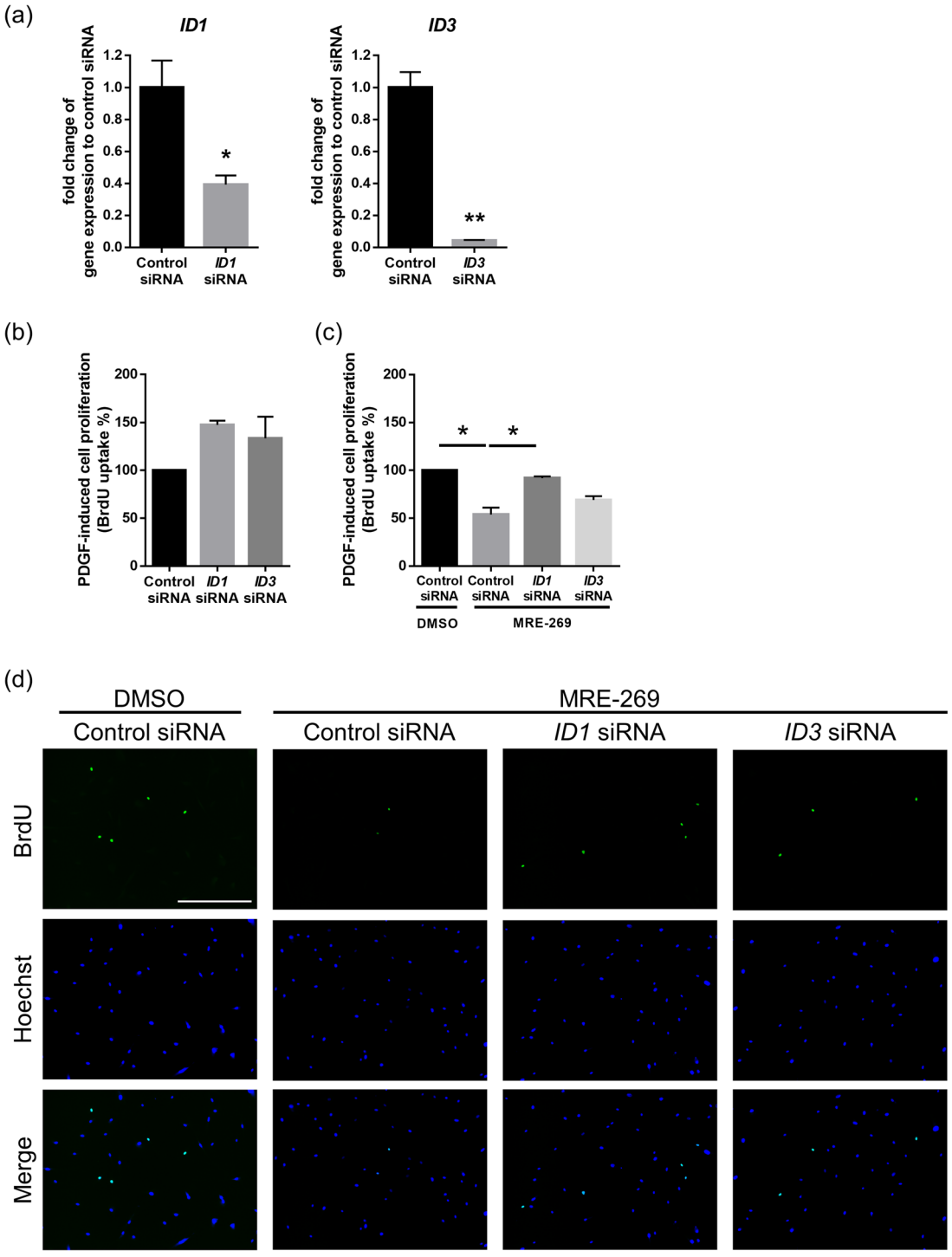


FIGURE 5 Effect of knockdown of DNA-binding protein inhibitor (ID) family members on the antiproliferative activity of MRE-269. (a) Messenger RNA expression levels of *ID1* and *ID3* in small interfering RNA (siRNA)-transfected normal pulmonary arterial smooth muscle cells (PASMC)-3 cells were determined by real-time quantitative polymerase chain reaction and normalized to *ACTB* as the internal control. Mean ± standard error of the mean (SEM) of fold change of gene expression over control siRNA-transfected cells is shown, $n = 3$. *$p < 0.05$, **$p < 0.01$ versus control siRNA (Student's t test). (b) Relative amounts of bromodeoxyuridine (BrdU) taken up by platelet-derived growth factor (PDGF)-stimulated normal PASMC-3 cells transfected with each siRNA. Data are the mean ± SEM, $n = 3$. (c) Relative amounts of BrdU taken up by PDGF-stimulated normal PASMC-3 cells transfected with each siRNA and treated with MRE-269 or 0.1% dimethyl sulfoxide (DMSO). Data are mean ± SEM, $n = 3$. *$p < 0.05$ (Tukey's test). (d) Representative immunofluorescence images of PDGF-stimulated normal PASMC-3 cells transfected with each siRNA and treated with MRE-269 or 0.1% DMSO stained with alexa-488 conjugated anti-BrdU antibody (top panels) and Hoechst 33342 (Hoechst; middle panels); scale bar = 0.5 mm. Merged images are shown in bottom panels.

morphogenetic proteins (BMPs) and are involved in cell differentiation and proliferation. The binding of BMPs to their specific type I and type II receptors leads to the phosphorylation of Smad1/5/9. Phosphorylated Smad proteins are translocated into the nucleus and regulate the expression of target genes, including ID family proteins. The upregulation effect of MRE-269 on the gene expression of *ID1* and *ID3* was also observed in normal PASMCs. This effect was through the activation of IP and contributed to the antiproliferative activity. IP is a G-protein-coupled receptor and stimulates the G protein alpha subunit. Like prostacyclin analogs, such as iloprost, beraprost and treprostinil, MRE-269 increases the concentration of intracellular cyclic AMP (cAMP) in normal PASMCs.[22] In addition, iloprost and dibutyryl AMP, a cAMP mimic, upregulate *ID1* expression in normal PASMCs.[43] *ID1* gene expression is probably controlled not only by BMPR signaling but also by the intracellular cAMP concentration because the promoter sequence of the *ID1* gene contains both a BMP-responsive element and a cAMP-response element.[35,44] Therefore, MRE-269 probably regulates *ID1* gene expression by increasing the intracellular cAMP concentration.

The antiproliferative effect of MRE-269 was significantly attenuated in normal PASMCs transfected with *ID1*-targeted siRNA. ID1 may make the main contribution to the antiproliferative effect of MRE-269 on PASMCs. Although the gene expression of *ID3* was upregulated by MRE-269 to a similar extent to that of *ID1* in both CTEPH PASMCs and normal PASMCs, the antiproliferative effect of MRE-269 on normal PASMCs transfected with *ID3*-targeted siRNA was not significantly attenuated. Apparently inconsistent results related to the role of *ID3* in vascular cell proliferation have been reported. Thus, lentiviral overexpression of *ID3* inhibits the growth of normal PASMCs.[37] In contrast, overexpression of *ID3* with a plasmid vector increases vascular endothelial cell growth and the numbers of vascular smooth muscle cells.[45] The inconsistency of these results may be caused by a difference in *ID3* function between normal PASMCs and other vascular cells or by differences in the condition of the cells. We did not observe inhibition of cell growth by knockdown of *ID3* in normal PASMCs.

We used normal PASMCs to investigate the contribution of the upregulation of ID family members to the antiproliferative effect of MRE-269 because of the limited availability of PASMCs from CTEPH patients. Further studies will be needed to confirm the contribution of the upregulation of ID family members to the antiproliferative effect of MRE-269 on CTEPH PASMCs.

Heterozygous germline mutation of *BMPR2*, a BMP-specific type II receptor, is the most common mutation in patients with heritable PAH. Although some groups have reported that BMPR2 mutations that are correlated with PAH are also detected in CTEPH patients,[46,47] other groups did not detect such mutations in CTEPH patients.[48,49] A large-scale study will be needed to validate the prevalence of BMPR2 mutations in CTEPH patients. Interestingly, downregulation of the expression of BMPR2 and its downstream signaling molecules, ID1 and ID3, has been detected in lung tissue from PAH patients without BMPR2 mutations and in PAH animal models.[50–52] These results suggest that a reduction in BMPR2 signaling may play an important role in the pathogenesis of PAH regardless of the presence or absence of BMPR2 mutations. We did not assess gene mutations or polymorphisms in the CTEPH PASMCs we used. However, the low gene expression of *ID1* and *ID3* observed in CTEPH PASMCs suggests that defects in BMP signaling may also be involved in the pathogenesis of CTEPH.

In conclusion, MRE-269 inhibited the PDGF-induced proliferation of CTEPH PASMCs at concentrations which can be reached by clinical doses, and upregulation of ID signaling by MRE-269 may contribute to this effect. As far as we know, this is the first in vitro study to demonstrate the pharmacological effects on CTEPH PASMCs of a drug approved for the treatment of CTEPH. In addition to its potent vasodilating effect, the antiproliferative effects of selexipag on PASMCs in vascular remodeling may contribute to its efficacy in inoperable CTEPH and postoperative residual PH.

AUTHOR CONTRIBUTIONS

Kazuya Kuramoto designed and performed the experiments, analyzed the data, and wrote the manuscript. Aiko Ogawa designed the experiments and wrote the manuscript. Kazuko Kiyama, Yuji Ohno, Chiaki Fuchikami, and Kyota Hayashi performed the experiments and provided technical support. Keiji Kosugi, Keiichi Kuwano, and Hiromi Matsubara conceived and supervised the research studies. All authors contributed to and discussed the results and critically reviewed the manuscript. All authors read and approved the final manuscript. The authors disclose receipt of financial support for the research, authorship, and/or publication of this article from Nippon Shinyaku Co., Ltd.

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CONFLICT OF INTEREST STATEMENT

Kazuya Kuramoto, Yuji Ohno, Chiaki Fuchikami, Kyota Hayashi, Keiji Kosugi, and Keiichi Kuwano are employees of Nippon Shinyaku Co., Ltd. Aiko Ogawa received lecture fees from Bayer Yakuhin, Pfizer Japan, Nippon Shinyaku, and Actelion Pharmaceuticals Japan outside the submitted work. Kazuko Kiyama has no conflicts of interest to disclose. Hiromi Matsubara received lecture fees from Bayer, Pfizer Japan, Nippon Shinyaku, Janssen Pharmaceutical (Actelion Pharmaceuticals), GlaxoSmithKline, and Kaneka Medix outside the submitted work.

DATA AVAILABILITY STATEMENT

The RNAseq data set is available online from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE221511. All other data are available from the authors upon reasonable request.

ETHICS STATEMENT

The study was reviewed and approved by National Hospital Organization Okayama Medical Center (Okayama, Japan) and Nippon Shinyaku Co., Ltd (Kyoto, Japan).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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