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Small molecule inhibitor of HSP47 prevents pro-fibrotic mechanisms of fibroblasts *in vitro*

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Abstract

Excessive extracellular matrix deposition, in particular collagen, is an important cause of lung fibrosis. Heat shock protein 47 (HSP47), a collagen-binding protein, plays an important role in the intracellular processing of procollagen. A small molecule that blocks the collagen chaperone function of HSP47 has been reported as an HSP47 inhibitor. The aim of this study was to assess the effect of the HSP47 inhibitor on collagen synthesis and other fibrotic process *in vitro*. We evaluated collagen expression by western blot, and determined cell viability and migration by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and scratch test, respectively, in human and mouse lung fibroblasts. Treatment of lung fibroblasts with HSP47 siRNA decreased collagen type I expression. Similarly, the HSP47 inhibitor decreased collagen type I expression in transforming growth factor beta 1 (TGF- β 1)-treated lung fibroblasts in a dose-dependent manner. The inhibitor also decreased the viability and cell migration ability of

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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TGF- β 1-treated lung fibroblasts. Overall, we demonstrated that HSP47 is a potential therapeutic target for pulmonary fibrosis. The small molecule HSP47 inhibitor may mediate antifibrotic effects by suppressing the overexpression of collagen, and inhibiting the viability and migration of fibroblasts. Further research is needed to clarify the therapeutic potential of this HSP47 inhibitor for pulmonary fibrosis.

Keywords

antifibrotic drug; collagen; heat shock protein 47; pulmonary fibrosis

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a form of chronic, progressive, fibrosing interstitial pneumonia that is characterized by progressive worsening of dyspnea and lung function, and poor prognosis [1]. Fibroblasts, the cells primarily responsible for building and maintaining the extracellular matrix (ECM), play an important part in the maintenance of tissue integrity [2]. However, increased synthesis and deposition of ECM, which mainly comprises collagen type I and IV, is a driving cause of fibrosis in experimental and human lung disease [3,4].

Heat shock protein 47 (HSP47) is a collagen-binding, stress-inducible protein that has a specific role in the intracellular processing of procollagen as a collagen-specific molecular chaperone [5]. We previously reported that HSP47 expression is upregulated and associated with collagen synthesis in experimental pulmonary fibrosis models and pulmonary fibrotic disorders in human [6–13]. Furthermore, we reported that the antifibrotic drug pirfenidone can inhibit HSP47 and collagen type I expression in experimental pulmonary fibrosis models [7,12,13]. Our previous findings indicated that HSP47 could be a therapeutic target for collagen-related diseases such as pulmonary fibrosis. In fact, transfection with HSP47 siRNA attenuated experimental fibrosis in several organs, including the lung [14–19].

Thomson et al. developed an assay to screen small molecules from a large chemical library that inhibit the collagen chaperone function of HSP47. They identified four small molecules that inhibit the collagen chaperone function of HSP47, including methyl 6-chloro-2-oxo-2,3-dihydro-1,2lambda~4~,3-benzodithiazole-4-carboxylate (HSP47 inhibitor), without affecting HSP47 synthesis [20]. The aim of this study was to reveal the effects of this small molecule HSP47 inhibitor on collagen synthesis in an experimental pulmonary fibrosis model *in vitro*.

MATERIALS AND METHODS

Reagents

The HSP47 inhibitor was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture

Normal human adult lung fibroblasts (NHLF) (LONZA, Walkersville, MD, USA) were cultured in fibroblast basal medium (LONZA) supplemented with 2% fetal bovine serum (LONZA, Walkersville, MD, USA), human recombinant fibroblast growth factor (1.0 µg/mL), insulin (5 mg/mL), gentamicin, and amphotericin B at 37 °C in a humidified atmosphere with 5% CO₂. Normal mouse lung fibroblasts (MLg) (ATCC, Manassas, VA, USA) were grown in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO₂. All experiments were performed after 3–5 cell passages.

For the collagen synthesis assay, we plated NHLF and MLg (2×10^5 cells) in 6-well plates, in a final volume of 2 mL. After 24 h, we stimulated NHLF and MLg with transforming growth factor beta 1 (TGF-β1; 5 ng/mL) (WAKO, Osaka, Japan) and HSP47 inhibitor (5 µM, 10 µM, and 25 µM), dimethyl sulfoxide (DMSO; 5 µg/mL) (WAKO, Osaka, Japan) as a negative control. The cells were used to evaluate the effect of the HSP47 inhibitor on collagen synthesis by western blotting, MTT assay, and scratch test. All assays were repeated at least four times.

RNA interference

Double-stranded siRNAs targeting human HSP47 (Stealth Select pre-designed siRNA SERPINH1 hss101423), mouse HSP47 (Silencer Select pre-designed siRNA SERPINH1 s63466), and scramble siRNA (Silencer Select Negative Control #2 siRNA) were purchased from Thermo Fisher Scientific. NHLF and MLg (2×10^5 cells) were cultured in 6-well plates and were transfected 24 h later with 3 µL siRNA oligomers (20 µM) diluted in 150 µL Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific) and mixed with 3 µL of transfection reagent (RNAiMAX, Thermo Fisher Scientific) pre-diluted in 150 µL Opti-MEM. After 10 min, the transfection complexes were added to the cells at a final volume of 2.5 mL medium. The scramble siRNA was used as a control. After 24 h, we extracted total proteins from the cells to evaluate the effect of HSP47 siRNA on collagen synthesis by western blot. Experiments were performed in quadruplicate for all conditions.

Western blotting

We collected and lysed the cells in 100 µL of cold RIPA buffer (Thermo Fisher Scientific) 24 h after treatment. The protein samples were centrifuged at $24,000 \times g$ for 15 min at 4 °C in a precooled centrifuge. After discarding the pellet, we mixed 10 µg of each sample with an equal volume of 2× Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA), for a final volume of 20 µL. We boiled each protein sample at 95 °C for 5 min. Next, the samples were subjected to electrophoresis on 10% Mini-PROTEAN TGX Gel (Bio-Rad) at 200 V for 30 min in Tris/Glycine/SDS buffer (Bio-Rad). The proteins were transferred onto membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad), blocked in Tris-buffered saline (TBS) with 0.05% TWEEN 20 (TBS-T) and 5% bovine serum albumin (pH 7.4). We incubated the membranes with an anti-HSP47 polyclonal antibody (PA5-27832, Thermo Fisher Scientific) diluted in TBS-T (1:1,000 dilution) or an anti-collagen I polyclonal antibody (PA5-29569, Thermo Fisher Scientific) diluted in TBS-T (1:5,000 dilution) overnight at 4 °C after 1 h at room temperature. After several

washes with TBS-T, the membranes were incubated with an anti-mouse IgG secondary antibody (1:1,000 dilution; HRP31466, Thermo Fisher Scientific) 1 h at room temperature. Following incubation and several washes with TBS-T, the membranes were visualized with a chemiluminescence Clarity Western ECL Substrate kit (Bio-Rad). The intensity of the anti-HSP47 and anti-collagen I signals were normalized to those of a mouse anti-GAPDH loading control antibody (1:1,000 dilution; MA5-5738, Thermo Fisher Scientific) and an anti- β -actin rabbit monoclonal antibody (1:1,000 dilution; #4970, Cell Signaling Technology, Danvers, MA, USA).

MTT assay

We dissolved 25 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) in 5 mL phosphate-buffered saline (PBS) solution. After the cells had undergone 24 h of experimental treatment in 6-well plates at a final volume 2.5 mL medium and MTT solution, we added 500 μ L MTT reagent to all wells and incubated for 30 min at 37 °C in a humidified atmosphere with 5% CO₂. After 30 min, the medium was removed, 600 μ L DMSO was added per well, and 100 μ L of each well was transferred to 96-well plates. We measured the absorbance of each well at 540 nm and the absorption spectra was recorded with a microplate reader (MULTISKAN FC, Thermo Fisher Scientific). In all experiments, the absorbance of DMSO was measured as a negative control.

Scratch assay

Cell migration was determined through the scratch assay. Briefly, we culture NHLF and MLg cells (2×10^5 cells) in 2 mL of respective medium in 6-well plates until they reached 95% confluency. Then, we scratched the wells with a sterile pipette tip and washed twice with PBS. We added fresh medium to the wells and stimulated the cells with TGF- β 1 (5 ng/mL) plus HSP47 inhibitor (25 μ M) or DMSO (5.5 μ g/mL) as a negative control. We obtained cell migration images by phase-contrast microscopy (OLYMPUS CLX53 and cellSens Standard, Olympus, Tokyo, Japan) and assessed the scratch areas with BZ-X700 and BZ-X Analyzer (Keyence, Osaka, Japan). The wound healing area was determined as a percentage of the damaged area 24 h after scratching.

Statistical analysis

The data are expressed as the median and interquartile range or mean and standard error. All measurements were replicated for at least four times. We performed univariate (Dunnett's test and Wilcoxon rank-sum test) analyses to identify differences between groups. All P-values were two-sided and considered statistically significant when less than 0.05. All statistical analyses were performed with JMP Pro (version 14.0.0; SAS Institute, Cary, NC, USA).

RESULTS

Collagen type I and HSP47 expression in lung fibroblasts after transfection with HSP47 siRNA

To confirm the association between HSP47 and collagen type I expression in lung fibroblasts, we treated human and mouse fibroblasts with HSP47 siRNA. Figure 1 shows

a representative decrease in the expression of HSP47 and collagen type I determined by western blot after transfection of NHLF and MLg with HSP47 siRNA compared with siRNA scramble. These results suggest that collagen type I synthesis is related to the expression and/or function of HSP47.

Collagen type I and HSP47 expression in lung fibroblasts after treatment with the HSP47 inhibitor

Western blot analysis revealed a dose-dependent decrease in the expression of collagen type I in TGF- β 1-treated NHLF (Figure 2a) and MLg (Figure 2b) cells after treatment with increasing concentrations of HSP47 inhibitor. In contrast, HSP47 expression remained unaltered upon treatment with HSP47 inhibitor. These results suggest that the HSP47 inhibitor suppresses the production of collagen type I in lung fibroblasts.

Lung fibroblast viability after treatment with the HSP47 inhibitor

To evaluate the effect of the HSP47 inhibitor on fibroblast viability, we performed an MTT assay. The HSP47 inhibitor suppressed the viability of TGF- β -stimulated NHLF and MLg in a dose-dependent manner (Figure 3).

HSP47 inhibitor-induced effects on migration of lung fibroblasts

The HSP47 inhibitor (25 μ M) significantly impaired wound healing by TGF- β 1-treated NHLF (Figure 4 a, b) and MLg (Figure 4c, d). These results indicate that the HSP47 inhibitor negatively affects the migration of lung fibroblasts.

DISCUSSION

In the present study, we demonstrated that a small molecule compound with an inhibitory effect on HSP47 ability to bind to collagen can also prevent collagen synthesis, cell viability, and migration of lung fibroblasts. This is the first study showing the antifibrotic effects of this small molecule *in vitro*, with implications for lung fibrosis *in vivo*.

We previously established that HSP47 is associated with collagen synthesis, and is upregulated in experimental pulmonary fibrosis models and pulmonary fibrotic disorders in humans [6,8–11,21]. We have also reported that pirfenidone inhibits HSP47 and collagen type I expression in experimental pulmonary fibrosis models [7,12,13]. However, another study showed that pirfenidone reduces HSP47 mRNA, but has no impact on HSP47 protein levels in fibroblasts *in vitro* [22]. These results indicate that pirfenidone does not adequately disrupt HSP47 expression to hold therapeutic potential for pulmonary fibrosis in a clinical setting.

HSP47 has a role as a collagen-specific molecular chaperone in the intracellular processing of procollagen [5]. In the present study, we confirmed that HSP47 plays a pivotal role in the production of collagen type I in lung fibroblasts. Our results indicate that inhibition of HSP47 function or expression affects the synthesis of collagen type I, which is one of the main ECM components in lung fibrosis. Furthermore, this finding suggests that the HSP47 inhibitor may be a candidate antifibrotic drug for the prevent lung fibrosis mechanisms, such

as that in IPF. Next, we showed that the small molecule HSP47 inhibitor could suppress the expression of collagen type I without affecting HSP47 synthesis, in TGF- β 1-treated lung fibroblasts. These results suggest that the HSP47 inhibitor impaired collagen expression by disrupting the collagen chaperone function of HSP47, which is consistent with the findings of a previous study [20].

We could also observe that the HSP47 inhibitor could prevent TGF- β 1-stimulated fibroblast viability. Fibroblasts are the main cells responsible for building and maintaining the ECM, which makes them essential for tissue integrity maintenance [2]. Tissue fibrosis is typically characterized by the hyperproliferation of fibroblasts and excessive ECM synthesis and secretion [23]. In fibrotic disorders, fibroblasts display pathological proliferation, viability, motility, contractile function, and connective tissue production [24–26]. Furthermore, pathological fibroblast function is promoted by TGF- β , the most potent profibrotic cytokine [27,28]. Altogether, these results indicate that the HSP47 inhibitor could attenuate lung fibrosis by preventing fibroblast cell viability.

We also demonstrated that the HSP47 inhibitor could reduce cell migration ability of TGF- β 1-treated lung fibroblasts. Tissue repair after injury or disease can be understood as a spectrum that ranges from overhealing – as in hypertrophic scarring, keloids, and organ fibrosis – to functional tissue regeneration such as fetal wound healing [23,29]. In fibrotic diseases, abnormal ECM dynamics and organization are major drivers of cell dysfunction and, ultimately, organ failure [30]. Furthermore, TGF- β is a multifunctional growth factor that exerts pleiotropic effects on wound healing by regulating cell viability, migration, and ECM production [31]. TGF- β 1-induced migration of fibroblasts is associated with excessive wound healing. In this study, the HSP47 inhibitor suppressed the migration ability of TGF- β 1-stimulated fibroblasts, indicating that it may have antifibrotic effects. Our results suggest that this small molecule HSP47 inhibitor may be able to mediate antifibrotic effects by suppressing the overexpression of collagen, the viability, and migration of fibroblasts.

In conclusion, the present study demonstrates that HSP47 is a potential therapeutic target for pulmonary fibrosis. The small molecule HSP47 inhibitor may have antifibrotic effects based on its ability to prevent collagen overexpression, and inhibit the viability and migration of fibroblasts *in vitro*. One limitation of the present study is that it was solely performed *in vitro*. An *in vivo* study demonstrating the effects of the HSP47 inhibitor on pulmonary fibrosis, and establishing the optimal therapeutic dosage and administration method, is still necessary to comprehensively assess the potential of the inhibitor as a therapeutic agent for fibrosis.

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Highlights

- HSP47 plays an important role in the intracellular processing of procollagen.
- We evaluated the impact of a small molecule inhibitor of HSP47 in lung fibroblasts.
- The HSP47 inhibitor suppressed collagen type I expression in human and mouse cells.
- The HSP47 inhibitor suppressed the viability and migration of lung fibroblasts.
- We demonstrate the therapeutic potential of the HSP47 inhibitor for lung fibrosis.

Figure 1. HSP47 siRNA inhibits collagen type I and HSP47 expression in lung fibroblasts. Western blot analysis of the expression of collagen type I (COL1) and HSP47 after transfection of NHLF (a) and MLg (b) with HSP47 siRNA.

Figure 2. The HSP47 inhibitor suppressed collagen type I, but not HSP47, protein expression. Western blot analysis of the expression of collagen type I (COL1) and HSP47 in TGF- β 1-treated NHLF (a) and MLg (b) after treatment with increasing doses of the HSP47 inhibitor.

Figure 3. Treatment with the HSP47 inhibitor suppresses fibroblast proliferation.

Effects of the HSP47 inhibitor on the proliferation of NHLF (a) and MLg (b). The points on the graph represent the ratios of the absorbances at 540 nm of cells stimulated with TGF- β 1 in the presence and absence of the HSP47 inhibitor. The dots represent the mean and the bars indicate the standard error. * $P < 0.05$.

Figure 4. Migration of human and mouse fibroblasts is inhibited by the HSP47 inhibitor. Representative images of HSP47 inhibitor impact on the migration of NHLF (a) and MLg (c). All images were acquired at $\times 40$ magnification. Quantification of NHLF (b) and MLg (d) wound healing in the presence and absence of the inhibitor. The box plot displays the median and interquartile range, and the bars indicate the highest and lowest values in the range of $\pm 1.5 \times$ the interquartile range. * $P < 0.05$.