Potential novel targets: Protease-activated receptors in idiopathic pulmonary fibrosis
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Chapter 3

Pharmacological targeting of protease activated receptor-2 affords protection from bleomycin-induced pulmonary fibrosis

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ABSTRACT

Background
Idiopathic pulmonary fibrosis is the most devastating diffuse fibrosing lung disease which remains refractory to therapy. Protease activated receptor (PAR)-2 is an important mediator of fibrosis and PAR-2 deficiency limits bleomycin-induced pulmonary fibrosis. Here, we addressed whether PAR-2 deficiency persistently reduces bleomycin-induced pulmonary fibrosis or merely delays disease progression and whether pharmacological PAR-2 inhibition limits experimental pulmonary fibrosis.

Methods
Bleomycin was instilled intranasally into wild-type or PAR-2 deficient mice in the presence/absence of a specific PAR-2 antagonist. Fibrosis was assessed by hydroxyproline analysis, immunohistochemistry and western blot for fibrotic markers expression.

Results
Pulmonary fibrosis was consistently reduced in PAR-2 deficient mice throughout the fibrotic phase as evident from reduced Ashcroft scores (29%) and hydroxyproline levels (26%) at day 28. Moreover, P2pal-18S inhibited PAR-2-induced pro-fibrotic responses in both murine and primary human pulmonary fibroblasts (p<0.05). Treatment with P2pal-18S reduced the severity and extent of fibrotic lesions in lungs of bleomycin-treated wild-type but did not further reduce fibrosis in PAR-2 deficient mice. Importantly, P2pal-18S treatment starting even 7 days after the onset of fibrosis still as effectively limits pulmonary fibrosis as when treatment was started together with bleomycin instillation (p<0.05).

Conclusions
Overall, PAR-2 contributes to the progression of pulmonary fibrosis and targeting PAR-2 may be a promising therapeutic strategy for treating pulmonary fibrosis.
INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is lethal diffuse fibrosing lung disease with a 5-year mortality rate greater than 50%, which exceeds many types of cancers. IPF comprises a group of conditions characterized by interstitial fibroblast proliferation, alveolar destruction and excessive extracellular matrix (ECM) synthesis and accumulation. Studies exploring the mechanisms that are crucially involved in the development of IPF identified several possible targets for therapeutic interventions. Among those, protease-activated receptors (PARs) are key candidates, as these receptors mediate the cellular effects of coagulation factors and play central roles in influencing inflammatory and fibrotic responses.

PARs are seven-transmembrane G protein-coupled receptors which are activated by proteolytic cleavage by serine proteases. PAR-2 is one of four members of the PAR family that is widely expressed in many different cell types like (among others) fibroblasts and epithelial cells. Proteases such as trypsin, factor (F)VIIa, FXa, mast cell tryptase or matriptase cleave the N-terminal extracellular domain of PAR-2, thereby revealing a novel tethered ligand that binds to PAR-2 and activates its transmembrane signaling to intracellular G proteins. Importantly, activated PAR-2 mediates a number of pathophysiological pathways involved in acute/chronic inflammatory and fibrotic diseases of the joints, skin, brain, lung and gastrointestinal tract.

With respect to lung injury and pulmonary fibrosis, there is increasing evidence that PAR-2 is a critical contributor in the pathogenesis of IPF. Increased PAR-2 expression has been detected in the lungs of patients with IPF and a recent study proposed that the PAR-2/FVIIa axis may contribute to the development and/or progression of IPF. This study indeed provided in vitro evidence that FVIIa exerts pro-fibrotic effects in human fibroblasts by specifically activating PAR-2. In line, the prototypical PAR-2 agonist tryptase stimulates the growth of human lung fibroblasts and potentiate extracellular matrix production in a PAR-2-dependent manner. Moreover, PAR-2 expression significantly correlates with the extent of honeycombing and PAR-2 activation has been shown to be highly relevant to the progression of pulmonary fibrosis in an experimental animal model of bleomycin-induced injury. Indeed, genetic ablation of PAR-2 in mice affords protection from pulmonary fibrosis, as evident from a reduction in the extent and severity of fibrotic lesions and diminished collagen expression. In addition, treatment of pulmonary fibrosis bearing rats with diallylsulfide results in amelioration of collagen production and alveolar epithelial cell apoptosis through the involvement of PAR-2.
Notwithstanding the overwhelming amount of data supporting an important role of PAR-2 in pulmonary fibrosis, some controversy has also emerged over the topic. Indeed, using a similar model of bleomycin-induced pulmonary fibrosis as in the study mentioned above, edema and hydroxyproline levels were not different between wild type and PAR-2 deficient mice in a study by Su and colleagues. Also, a recent study showed that protein and mRNA expression levels of PAR-2 in IPF patients were not different from controls, and it has been claimed that it is doubtful whether blocking PAR-2 would serve as an effective treatment strategy for IPF. It is thus fair to state that, despite intriguing data supporting a role for PAR-2 in pulmonary fibrosis, its potential clinical relevance remains controversial.

In the present study, we addressed the controversy by first assessing whether PAR-2 deficiency limits bleomycin-induced pulmonary fibrosis or merely delays disease progression. We reaffirmed the importance of PAR-2 and subsequently evaluated the efficacy of pharmacological PAR-2 inhibition in pulmonary fibrosis.

**MATERIALS AND METHODS**

**Cells and Reagents**

Mouse embryonic NIH3T3 fibroblasts (American Type Culture Collection, Manassas, VA; CRL-1658) and primary human lung fibroblasts (derived from pulmonary control and IPF patient explants as described in; provided by INSERM U1152) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were grown at 37°C in an atmosphere of 5% CO₂. Unless indicated otherwise, cells were washed twice with PBS and serum-starved for 4 hours before stimulation. Human platelets were donated by a healthy volunteer and isolated as described before. Thrombin and trypsin were from Sigma (St-Louis, MO), whereas P2pal-18S (PAR-2 inhibitor; palmitate-RSSAMDENSEKKRKSAIK-NH₂) was from GL Biochem Ltd (Shanghai, China).

**Calcium Assay**

Calcium signaling responses were analyzed using the Fluo-4 Direct™ Calcium Assay Kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Cells were pre-incubated with or without 10 μM P2pal-18S and then challenged with PAR-1 agonist. Ca²⁺ flux was monitored for the indicated time points on a Bio-Tek HT Multi-Detection Microplate Reader (Winooski, United States).
Western Blot
Western blots were performed essentially as described before. In brief, cells were lysed in Laemmli lysis buffer and the lysates were incubated for 5 minutes at 95°C. Afterwards, protein samples were separated by 10% SDS gel electrophoresis and transferred to a PVDF membrane (Millipore, Billerica, MA). Membranes were blocked for 1 hour in 4% milk in TBST and incubated overnight with monoclonal antibodies against α-smooth muscle actin (a-SMA), GAPDH or collagen (all Santa Cruz, CA) at 4°C. All secondary antibodies were horseradish peroxidase (HRP)-conjugated from DakoCytomation (Glostrup, Denmark) and diluted according to the manufacturer’s instructions. Blots were imaged using Lumilight plus ECL substrate from Roche (Almere, The Netherlands) on an ImageQuant LAS 4000 biomolecular imager from GE Healthcare (Buckinghamshire, U.K). For quantification, densitometry was performed using ImageJ.

Animal Model of Pulmonary Fibrosis
Wild type C57Bl/6 mice were purchased from Charles River (Someren, Netherlands). PAR-2 deficient (PAR-2-/-) C57Bl/6 mice were originally provided by Jackson Laboratories (Maine) and bred at the animal care facility of the Academic Medical Center. All procedures were performed on ten-week-old mice in accordance with the Institutional Standards for Humane Care and Use of Laboratory Animals. Experiments were approved by the Animal Care and Use Committee of the Academic Medical Center (Amsterdam, Netherlands).

Bleomycin (Sigma, St-Louis, MO) was administered by intranasal instillation (1 mg/kg body weight in 45 μl saline) under anesthesia. In the PAR-2 pepducin dose finding experiment, animals were intranasally instilled with 2.5 or 10 mg/kg P2pal-18S half an hour before bleomycin administration and subsequently once daily until the end of the experiment. In the delayed treatment experiment, mice were treated once daily with 2.5 mg/kg pepducin starting one, three or seven-day after bleomycin instillation. In both experiments, 6% DMSO in saline was administered as a solvent control. Unless stated otherwise, mice were sacrificed 14 days after bleomycin instillation after which one lung was taken for histological analysis and one was homogenized.

Bronchoalveolar Lavage (BALF)
At 14 days after bleomycin instillation, the lungs were lavaged three times with 0.3 ml of saline by a 22-gauge Abbocath-Tcatheter into the trachea via a midline incision before animals were sacrificed. The BALF was centrifuged, and the supernatant was frozen at −70°C until use.
TGF-β ELISA
TGF-β1 was measured with the Mouse TGF-beta 1 DuoSet kit (R&D systems, UK) as per the manufacturer’s instructions.

Hydroxyproline Assay
Hydroxyproline analysis was performed by the hydroxyproline assay kit as per the manufacturer’s instructions (Sigma, Netherlands) and as described before27.

Histological Analysis
Histological examination was performed essentially as described before19. Briefly, the excised lung was fixed in formalin, embedded in paraffin and 4-μm-thick slides were subsequently deparaffinized, rehydrated and washed in deionized water. Slides were stained with hematoxylin and eosin (H&E) according to routine procedures. In H&E staining, the severity of fibrosis was assessed according to the Ashcroft scoring system28 using a 100× magnification. Two independent observers were blinded to the treatment group and an average of 10 fields of each lung section was selected and scored. The average Ashcroft score was calculated by averaging the individual field scores.

Statistics
Statistical analyses were conducted using GraphPad Prism version 5.00, (GraphPad software, San Diego, CA, USA). Data were expressed as means ± SEM. Comparisons between two conditions were analyzed using two tailed unpaired t-tests when the data where normally distributed, otherwise Mann-Whitney analysis was performed. P-values of less than 0.05 were considered significant.

RESULTS
PAR-2 deficiency attenuates bleomycin-induced pulmonary fibrosis
Although PAR-2 deficiency has been shown to afford protection from bleomycin-induced lung injury before, those studies used day 14 as the endpoint which may just point to a delay of the onset of fibrosis19,29. To determine whether PAR-2 deficiency indeed limits bleomycin-induced pulmonary fibrosis or just delays progression, we compared lungs from wild type and PAR-2 deficient mice at day 28 and 48 after the instillation of bleomycin. As shown in Figure 1A, bleomycin-induced inflammatory and fibrotic processes culminate in severe pulmonary fibrosis at day 28 in wild type mice. In contrast, lung tissue sections from PAR-2 deficient mice at day 28 showed significantly
Targeting PAR-2 in pulmonary fibrosis

reduced interstitial fibrosis, with diminished ECM deposition and less destruction of alveolar units (Figure 1B). Both wild type and PAR-2 deficient mice showed some degree of resolution by day 48 (Figure 1C and D). Quantification of the bleomycin-induced histological changes in wild type and PAR-2 deficient mice using the Ashcroft score shows that PAR-2 deficient mice develop less severe fibrotic lesions at day 28 as compared to wild type mice (Figure 1E and F). These observations are also supported by hydroxyproline levels, depicting collagen deposition is significantly reduced in PAR-2 deficient lungs at day 28 (Figure 1G and H). Overall, these results show that PAR-2 deficiency indeed provides protection against bleomycin-induced pulmonary fibrosis throughout the fibrotic stage and does not merely delay disease onset.

**P2pal-18S effectively inhibits PAR-2 mediated pro-fibrotic responses in fibroblasts**

Next, we determined the specificity of the observed effects in PAR-2 deficient mice by assessing whether pharmacological PAR-2 inhibition by P2pal-18S would reduce lung fibrosis as well. Before assessing the *in vivo* effect of P2pal-18S, we first determined its specificity for blocking PAR-2 dependent fibrotic responses *in vitro*. Although the specificity of P2pal-18S has been tested previously, we here use human platelets (not expressing PAR-2) to confirm that P2pal-18S only targets PAR-2 and does not interact with other PARs. As shown in Figure 2A, PAR-1 activation by thrombin induced Ca\(^{2+}\) influxes that peaked within 1 minute after stimulation, whereas pretreatment with 10 μM P2pal-18S did not interfere with this reaction. We next assessed whether P2pal-18S inhibits PAR-2 driven pro-fibrotic responses in fibroblasts. We show that trypsin induced fibroblast differentiation, as evident from increased \(\alpha\)-SMA expression and collagen production compared to vehicle treated cells. Trypsin-induced \(\alpha\)-SMA and collagen expression were clearly down-regulated by the treatment with 10 μM P2pal-18S (Figure 2B).

Bleomycin administration is known to increase PAR-2 agonist expression in BALF which may lead to fibroblast differentiation or ECM synthesis. We next thus assessed the inhibitory effect of P2pal-18S against BALF induced pro-fibrotic effects on murine fibroblasts. NIH3T3 cells seeded in BALF of wild type mice treated with bleomycin showed increased expression of \(\alpha\)-SMA and collagen as compared to cells seeded in BALF derived from saline-treated mice, while preincubation with P2pal-18S partially decreased this effect (Figure 2C). Finally, we determined whether P2pal-18S also limits PAR-2 induced fibrotic responses on primary human lung fibroblasts. We stimulated...
fibroblasts isolated from nonfibrotic controls and IPF patients with trypsin in the absence or presence of P2pal-18S. IPF-derived fibroblasts exhibited high α-SMA and collagen expression persistently (Figure 2D and E showing three independent fibroblast isolations) while control fibroblasts only showed increased α-SMA and collagen expression after treated with trypsin. The expression of α-SMA and collagen were reduced by P2pal-18S in both control and IPF fibroblasts (Figure 2F and G). Overall these in vitro data show that P2pal-18S effectively antagonizes PAR-2-mediated profibrotic effects in both murine and human fibroblasts.

P2pal-18S limits the development of pulmonary fibrosis in the bleomycin model

After having shown that P2pal-18S inhibits PAR-2-induced fibroproliferative responses in fibroblasts, we set out to examine whether P2pal-18S would effectively limit fibrosis in the bleomycin model. To this end, mice were intranasally instilled with different concentrations of P2pal-18S, after which the extent and severity of fibrosis was determined at day 14. As shown in Figure 3A, bleomycin instillation resulted in extensive fibrotic foci accompanied by increased deposition of ECM. Both 2.5 mg/kg and 10
mg/kg P2pal-18S treatment significantly reduced the severity of regional interstitial fibrosis (compare Figures 3B and C with 3A). Quantification of the bleomycin-induced histopathological changes in the different groups using the Ashcroft score shows that P2pal-18S treatment results in less severe fibrotic lesions (Figure 3D). We also analyzed total collagen accumulation in the lung and, as shown in figure 3E, bleomycin-induced
collagen levels were attenuated by P2pal-18S treatment, although the 10 mg/kg dose did not reach statistical significance.

We next assessed TGF-β1 levels in lung homogenates as TGF-β1 is a key profibrotic cytokine and its gene and protein expression levels are known to be increased by bleomycin\textsuperscript{31}. As shown in Figure 3F, TGF-β1 levels increased in bleomycin-instilled mice, while the 2.5 mg/kg dose of P2pal-18S significantly attenuated the TGF-β1 increase by about 50%. The 10 mg/kg dose of P2pal-18S also reduced TGF-β1 level but this reduction again did not reach statistical significance.

Delayed treatment with P2pal-18S effectively limits pulmonary fibrosis progression in the bleomycin model

After having established that long-term treatment with P2pal-18S effectively limits bleomycin-induced pulmonary fibrosis, we assessed whether therapeutic modes of

![Image](https://via.placeholder.com/150)

**Figure 3.** P2pal-18S treatment attenuates bleomycin-induced pulmonary fibrosis. (A-C) Representative H&E stained lung tissue sections obtained 14 days after bleomycin instillation in saline treated mice (A) and mice treated with 2.5 mg/kg (B) or 10 mg/kg P2pal-18S (C). (D) Quantification of pulmonary fibrosis in control and P2pal-18S treated mice using the Ashcroft score. Data are expressed as mean ± SEM (n=8 per group). Total collagen content (E) and TGF-β1 levels (F) in lung homogenates of the different groups of mice obtained 14 days after saline or bleomycin instillation. Data are expressed as mean ± SEM (n=8 per group; * P<0.05; **P<0.01).
administration would still be effective. P2pal18 treatment (2.5 mg/kg) was started either 1, 3 (preventive treatment; concurrent with the inflammatory phase) or 7 (therapeutic treatment; upon the establishment of lung fibrosis) days after bleomycin instillation and continued once daily till the end of the experiment. As shown in Figure 4A-C, histological analysis of lung slides from bleomycin-instilled mice not treated with P2pal-18S again showed severe fibrotic lesions (about 5 fold increase compared to lungs of non-bleomycin-instilled controls). Treatment with P2pal-18S in the preventive mode strongly inhibited bleomycin-induced pulmonary fibrosis, as evident from lower Ashcroft scores (Figure 4G) and reduced collagen accumulation (Figure 4H).

Figure 4. Delayed P2pal-18S treatment effectively attenuates bleomycin-induced pulmonary fibrosis. (A-F) Representative H&E stained lung tissue sections obtained 14 days after bleomycin instillation in wild type control treated mice (A), wild type mice treated with 2.5 mg/kg P2pal-18S from day 1 (B), day 3 (C) or day 7 (D) after bleomycin instillation, PAR-2-/- control treated mice (E) and PAR-2-/- mice treated with 2.5 mg/kg P2pal-18S (F). (G) Quantification of pulmonary fibrosis in delayed P2pal-18S treated wild type mice using the Ashcroft score. (H) Total collagen content in lung homogenates from the different groups of WT mice obtained 14 days after saline or bleomycin instillation. (I) Quantification of pulmonary fibrosis in PAR-2-/- mice treated with or without P2pal-18S using the Ashcroft score (Note that the wild type untreated controls are the same as depicted in panel G, as the experiments were performed at the same time.) (J) Total collagen content in lung homogenates from PAR-2-/- mice and PAR-2-/- mice treated with P2pal-18S obtained 14 days after bleomycin instillation. The wild type controls are the same as depicted in panel H. Data are expressed as mean ± SEM (n=8 per group; * P<0.05**P<0.01).
Interestingly, similar findings were obtained when P2pal-18S was administered in the therapeutic mode (compare Figure 4A and D). Indeed, P2pal-18S treatment starting on day 7 reduced the bleomycin-induced increase in Ashcroft score (although not statistically significant) and collagen deposition (Figure 4G and H).

To corroborate the specificity of P2pal-18S in an in vivo setting, we analyzed the effect of P2pal-18S treatment (2.5 mg/kg) in bleomycin-treated PAR-2 deficient mice. As shown in figure 4E and F, PAR-2 deficient mice developed less fibrosis as compared to wild type mice. More importantly, P2pal-18S treatment did not further reduce fibrosis and collagen deposition in the PAR-2 deficient mice (Figure 4I and J), suggesting that P2pal-18S limits pulmonary fibrosis by specifically targeting PAR-2.

**DISCUSSION**

IPF is a progressive lung disorder for which very few therapeutic options are available\(^1,3\). It is often suggested as an uncontrolled wound healing response, in which multiple effectors are tightly involved. PAR-2, as a critical receptor that orchestrates a diverse range of signaling pathways, plays an important role in mediating pro-fibrotic effects in fibroblasts and in preclinical experimental animal models\(^7,19,32-34\), although there is still some controversy regarding the role of PAR-2 in pulmonary fibrosis. In the present study, we aimed to address the controversy and provide several lines of evidence to support the crucial role of PAR-2 in the progression of pulmonary fibrosis.

Bleomycin-induced pulmonary fibrosis is stably formed at around day 14, which is consequently frequently used as endpoint in literature\(^19,27,35\). However, the fibrotic stage induced by bleomycin peaks at around 3–4 weeks and the reduction in fibrosis observed in PAR-2 deficient mice at day 14\(^19,29\) may thus only indicate a delay in the onset of fibrosis instead of reduced progression. To address this concern, we increased the follow-up time, and show that the severity of fibrosis as well as collagen accumulation are still significantly reduced in PAR-2 deficient mice compared with wild type mice 28 days after bleomycin instillation. Furthermore, both wild type and PAR-2 deficient mice enter the resolution phase of fibrosis by day 48, indicating the peak of fibrosis has passed. By using different endpoints of the murine bleomycin model, we provide evidence that PAR-2 deficiency provides persistent protection along the development of pulmonary fibrosis, suggesting PAR-2 is indeed an interesting target for therapeutic intervention.
P2pal-18S is a cell-penetrating lipopeptide “pepducin” antagonist of PAR-2 that efficiently blocks PAR-2-dependent inflammatory responses in mouse models. We confirm and extend these findings by showing that P2pal-18S effectively blocks PAR-2 driven pro-fibrotic responses in vitro. Indeed, pre-incubation of murine fibroblasts with P2pal-18S blocks trypsin-induced ERK phosphorylation, reduces proliferation and migration and diminishes \( \alpha \)-SMA and collagen expression (Figure 2). Moreover, P2pal-18S significantly inhibits collagen expression not only in control fibroblasts but also in IPF-derived human fibroblasts. IPF-derived human fibroblasts actually exhibit a more differentiated and fibrotic myofibroblast phenotype, as evident from elevated basal expression levels of both \( \alpha \)-SMA and collagen, which may explain the reduced responsive to the trypsin stimulation as compared to control fibroblasts. As opposed to complete inhibition of trypsin-induced signaling, P2pal-18S did only partly block ECM deposition induced by BALF from bleomycin-treated wild type mice. This suggests that BALF partially exerts pro-fibrotic effects through PAR-2 activation but that BALF also contains additional pro-fibrotic components. Overall, these data show that P2pal-18S efficiently block PAR-2-dependent fibrotic responses in vitro.

In line with a potential therapeutic role of PAR-2 in pulmonary fibrosis, we show that P2pal-18S limits bleomycin-induced pulmonary fibrosis even when administered at the fibrotic phase. Treatments during the first seven days after bleomycin administration are generally considered “preventive” whereas treatments starting at day 7 are considered “therapeutic.” After showing that once daily P2pal-18S treatment (2.5mg/kg) during the 14 days of the experiment significantly limited bleomycin-induced pulmonary fibrosis, we next showed that administration of P2pal-18S also effectively decreases the histological grade of pulmonary fibrosis and collagen contents in both a preventive (1 and 3 days delayed treatment) and therapeutic (7 days delayed treatment) mode. Importantly, the protective effect afforded by PAR-2 deficiency against bleomycin-induced pulmonary fibrosis was nearly identical to that observed in wild type mice treated with P2pal-18S. In PAR-2 deficient mice as well as in P2pal-18S treated mice, the severity of the fibrotic lesions assessed by the Ashcroft score was decreased by around 1 point. In line, quantitative measurement of collagen deposition in the lungs reduced by about 34% in PAR-2 deficient mice and 29% in P2pal-18S treated mice. Interestingly, the anti-fibrotic effect of P2pal-18S was lost in PAR-2/-/- mice, demonstrating that P2pal-18S is highly specific for PAR-2 in vivo. This is in accordance with a previous study showing that P2pal-18S significantly reduces mouse paw edema and inflammation in wild type but not PAR-2 deficient mice. Moreover, our in vitro data show that P2pal-
18S does not interfere with thrombin-induced calcium fluxes in human platelets. As platelets respond to thrombin through PAR-1 and/or PAR-4\textsuperscript{37}, these data indicate that P2pal-18S does not have cross-reactivity with other PARs. Together, our data indicate that P2pal-18S provides effective and specific pharmacologic inhibition of PAR-2 in an animal model of pulmonary fibrosis and that inhibition of PAR-2 may thus be a promising therapeutic strategy for treating pulmonary fibrosis, although future clinical studies are needed to confirm this notion.

It is noteworthy that, similarly as observed for targeting PAR-1 in pulmonary fibrosis\textsuperscript{27}, P2pal-18S at a dose of 2.5 mg/kg seemed to be more effective in reducing fibrosis than at a high dose of 10 mg/kg. Although we do not have a definite explanation for this finding, it is tempting to speculate that the phenomenon may be due to the fact that PAR-2, again similarly to PAR-1, acts both as a pro- and anti-inflammatory receptor in the respiratory system. In cultured human epithelial cells, activation of PAR-2 causes release of pro-inflammatory cytokines such as interleukin (IL)-8\textsuperscript{38}. PAR-2 stimulation is also required for serralyisin to activate the critical transcription factors for host inflammatory responses\textsuperscript{39}. On the other hand, PAR-2 activation enhances LPS-induced expression of the anti-inflammatory cytokines while suppressing gene expression of pro-inflammatory cytokines\textsuperscript{40} and PAR2-TLR signaling integration drives “customized” inflammatory responses\textsuperscript{41}. Therefore, the extent of PAR-2 inhibition may affect the balance of its pro- and anti-inflammatory properties. Alternatively, the slight differences observed between the 2.5 and 10 mg doses may simply be attributed to the variability within the analysis rather than any definable biological phenomenon.

Pharmacological PAR-2 inhibition may not just benefit pulmonary fibrosis due to the fact that PAR-2 influences a large range of pathophysiological pathways and indeed PAR-2 deficiency affords protection to, amongst others, renal fibrosis\textsuperscript{34}, heart failure\textsuperscript{42} and pulmonary hypertension\textsuperscript{43}. In line, PAR-2 inhibition reduced, again amongst others, pulmonary hypertension, acute biliary pancreatitis, and osteoarthritis\textsuperscript{44-45}. It is thus tempting to speculate that P2pal-18S may have an impact on a large plethora of other (PAR-2 dependent) disorders although this tantalizing hypothesis needs to be addressed experimentally.

In summary, our data endorse the importance of PAR-2 in mediating pro-fibrotic effects and identify the PAR-2 antagonist P2pal-18S as an effective inhibitor of bleomycin-induced pulmonary fibrosis. Thus, targeting PAR-2 signaling with P2pal-18S may offer a novel therapeutic option for patients with pulmonary fibrosis.
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