

# Inhibition of Thrombin as a Novel Strategy in the Treatment of Scleroderma-Associated Interstitial Lung Disease

Galina S. Bogatkevich<sup>1</sup>, Kristin B. Highland<sup>1</sup>, Tanjina Akter<sup>1</sup>, Paul J. Nietert<sup>1</sup>, Ilia Atanelishvili<sup>1</sup>, Joanne van Ryn<sup>2</sup> and Richard M. Silver<sup>1</sup>

<sup>1</sup>*Medical University of South Carolina, Charleston,*

<sup>2</sup>*Boehringer Ingelheim GmbH & Co.KG, Biberach,*

<sup>1</sup>*USA*

<sup>2</sup>*Germany*

## 1. Introduction

Activation of the coagulation cascade leading to generation of thrombin has been extensively documented in various forms of lung injury including systemic sclerosis-associated interstitial lung disease (SSc-ILD). The molecular mechanisms underlying the pathogenesis and progression of lung fibrosis in SSc-ILD and in idiopathic pulmonary fibrosis (IPF) are not entirely clear. The conceptual process of fibrogenesis involves tissue injury and activation of the coagulation cascade, the release of various fibrogenic factors, and the induction of myofibroblasts culminating in enhanced extracellular matrix deposition. Cells with a myofibroblast phenotype appear in the early stages of fibrosis and are characterized by an increased proliferative capacity and abundant expression of  $\alpha$ -SMA, collagens and other extracellular matrix proteins (Hinz et al., 2007). Myofibroblasts can be cultured from bronchoalveolar lavage (BAL) fluid of SSc-ILD patients, and thrombin activity is also significantly greater in BAL fluid from SSc-ILD patients compared with healthy controls (Ludwicka et al., 1992; Ohba et al., 1994). Thrombin differentiates lung fibroblasts to a myofibroblast phenotype, increases lung fibroblast proliferation (Bogatkevich et al., 2001), and enhances the proliferative effect of fibrinogen on fibroblasts (Gray et al., 1993). Thrombin is also a potent inducer of fibrogenic cytokines, such as transforming growth factor- $\beta$  (TGF- $\beta$ ) (Bachhuber et al., 1997), connective tissue growth factor (CTGF) (Chambers et al., 2000; Bogatkevich et al., 2006), platelet-derived growth factor-AA (PDGF-AA) (Ohba et al., 1994), chemokines (Mercer et al., 2007), and ECM proteins such as collagen, fibronectin, and tenascin in various cells, including lung fibroblasts (Tourkina et al., 2001; Chambers et al., 1998; Armstrong et al., 1996).

Dabigatran is a selective direct thrombin inhibitor that reversibly binds to thrombin and prevents the cleavage of Arg-Gly bonds of fibrinogen needed for the formation of fibrin. Recently, we have demonstrated that binding of dabigatran to thrombin prevents cleavage of the extracellular N-terminal domain of the protease-activated receptor 1 (PAR-1), which is responsible for most profibrotic events induced by thrombin (Bogatkevich et al., 2009). In

the absence of dabigatran, thrombin binds to PAR-1, cleaves the peptide bond between residues Arg-41 and Ser-42, thereby unmasking a new amino terminus, SFLLRN, which then can bind to the second extracellular loop of PAR-1 and initiate receptor signaling (Macfarlane et al., 2001). Dabigatran-bound thrombin is unable to cleave and activate PAR-1. The aim of this chapter is to provide a molecular basis for therapeutic interventions in SSc-ILD by inhibition of thrombin.

## 2. Increased expression of thrombin and PAR-1 in SSc-ILD

Thrombin is a multi-functional serine protease and a key enzyme of blood coagulation, catalyzing the conversion of fibrinogen to fibrin. In addition to its essential role in coagulation, thrombin has several important functions at a cellular level, both in normal health and in multiple disease processes, including pulmonary fibrosis (Chambers, 2008). Our laboratory as well as others has demonstrated dramatically increased levels of thrombin in BALF from scleroderma patients with lung fibrosis and other fibrosing lung diseases (Ohba et al., 1994; Hernandez-Rodriguez et al., 1995). We have reported that BALF from normal subjects contains a low level of thrombin activity ranging from zero to 150 units per mg of BALF protein ( $48.6 \pm 8.7$  U/mg, mean  $\pm$  SEM). BAL fluids of SSc patients express up to 100-fold higher thrombin activity, ranging from 22 to 7,525 units per mg of BALF protein ( $699.9 \pm 201.1$  U/mg, mean  $\pm$  SEM;  $P < 0.001$ ) (Fig. 1A). Elevated levels of thrombin activity have been also observed in bleomycin-induced pulmonary fibrosis in mice (Howell et al., 2005). We found that the level of active thrombin in BAL fluid from bleomycin-treated mice was 35-fold higher ( $1.3 \pm 0.1$  ng/ml, mean  $\pm$  SEM) compared to that in control mice treated with saline ( $46.1 \pm 7.9$  ng/ml, mean  $\pm$  SEM;  $P < 0.01$ ) (Fig. 1B).

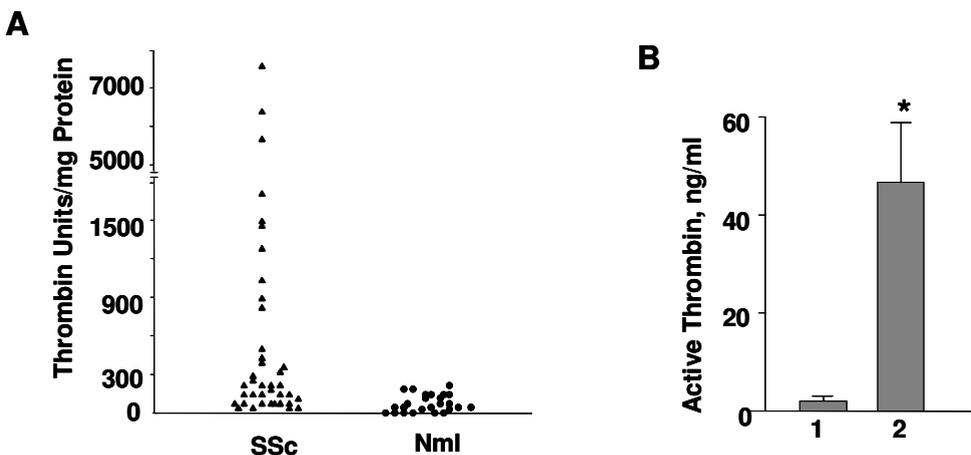


Fig. 1. (A) Thrombin levels in BALF of scleroderma patients (SSc, closed triangles,  $n = 42$ ) and normal subjects (Nml, closed circles,  $n = 27$ ). Active thrombin was measured by fluorometric method using a synthetic substrate Boc-Val-Pro-Arg-7-(4-methyl) coumarylamide, and expressed as units per mg of BALF protein. (B) Thrombin levels in BALF of mice treated with saline (1) and with bleomycin-induced pulmonary fibrosis (2).

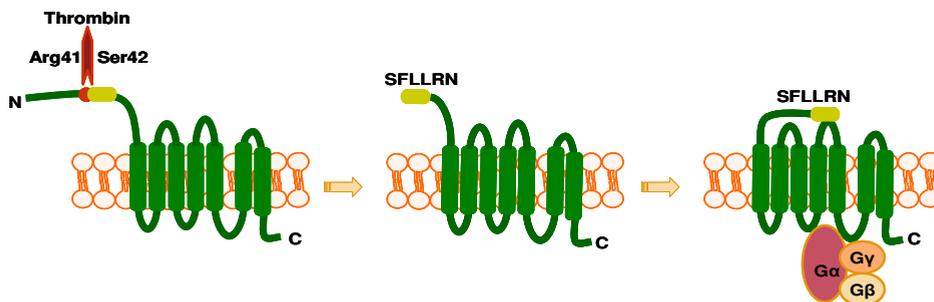


Fig. 2. Mechanism of PAR-1 activation. Proteolytic cleavage of the N-terminus results in the unmasking of a tethered ligand SFLLRN, which in turn interacts with extracellular loop-2 of the receptor and initiates cell signaling via activation of heterotrimeric G-proteins.

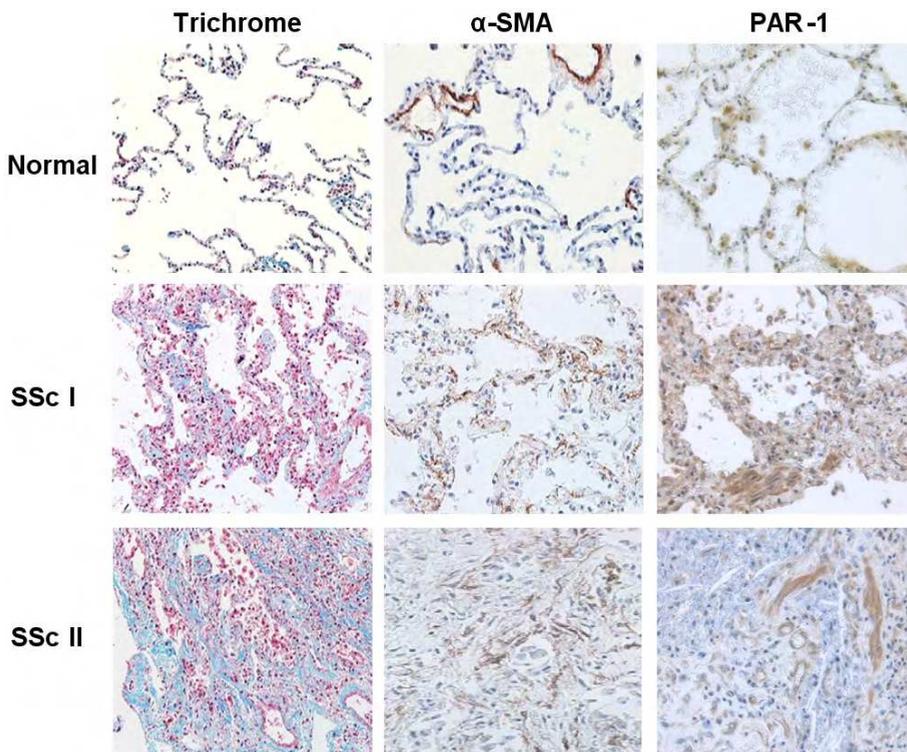


Fig. 3. PAR-1 and α-SMA expression is significantly increased in pulmonary fibrosis associated with scleroderma. Sections of normal and scleroderma (SSc) lung stained by trichrome, α-SMA antibody, and PAR-1 antibody; α-SMA and PAR-1 were visualized as brown color by diaminobenzidine (DAB) and counterstained with hematoxylin. Top panel represents normal lung tissue, middle panel (SSc I) represents lung tissue in early stage of lung involvement and bottom panel (SSc II) represents lung tissue with late stage of fibrosis.

The majority of the cellular responses to thrombin are mediated via the G protein-coupled PAR-1 receptor (Howell et al., 2002; Coughlin 1999). PAR-1 is activated by proteolytic cleavage of N-terminal domains, leading to the exposure of a new amino-terminus, a “tethered ligand” that in turn activates the receptor (Fig. 2). PAR-1 activation has been shown to be an important in the pathophysiology of various lung diseases, including SSc-ILD (Howell et al., 2002; Bogatkevich et al., 2005). PAR-1 is present in a variety of cell types, including leukocytes, platelets, T cells, endothelial cells, vascular smooth muscle cells and fibroblasts (Shrivastava et al., 2007). In the bleomycin model of pulmonary fibrosis, PAR-1 deficient mice show a significant reduction of inflammatory cells in BALF as compared with wild-type mice, and significant protection from lung fibrosis is seen in the PAR-1 deficient mice (Howell et al., 2005). The relative protection from pulmonary fibrosis observed in this model is a reduction in expression of two major fibrogenic growth factors, CTGF and transforming growth factor  $\beta$ - (TGF- $\beta$ ), as well as a reduction of the chemokine (C-C motif) ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP-1).

Elevated expression of PAR-1 has been shown in patients with IPF and in a murine model of bleomycin-induced lung fibrosis (Chambers, 2008; Howell et al., 2005). In previous studies we demonstrated that PAR-1 expression is also dramatically increased in lung tissue from scleroderma patients, mainly in lung parenchyma in context with myofibroblasts present in inflammatory and fibroproliferative foci (Bogatkevich et al., 2005). PAR-1 expression diminishes in the later stages of pulmonary fibrosis (Fig. 3) suggesting its important role in lung fibroblast activation during the early development of pulmonary fibrosis.

### 3. SSc-ILD, thrombin, and myofibroblasts

In the pathogenesis of pulmonary fibrosis in general and SSc-ILD in particular, lung fibroblasts undergo specific phenotypic modulation and develop cytoskeletal features similar to those of smooth muscle cells. These phenotypically altered, activated fibroblasts, or “myofibroblasts”, express a contractile isoform of actin ( $\alpha$ -smooth muscle actin,  $\alpha$ -SMA) and promote contractility of lung tissues. Myofibroblasts appear to be the principal mesenchymal cells responsible for tissue remodeling, collagen deposition, and the restrictive nature of the lung parenchyma associated with pulmonary fibrosis (Tomasek et al., 2002).

We have demonstrated that myofibroblasts are present in the BALF of SSc patients and that myofibroblasts cultured from SSc BALF express more collagen I, III, and fibronectin than normal lung fibroblasts (Ludwicka et al., 1992). Myofibroblasts from BALF also show a greater proliferative response upon exposure to TGF- $\beta$  and PDGF when compared to normal lung fibroblasts. Several groups of investigators have demonstrated a correlation between fibrosis and  $\alpha$ -SMA expressing myofibroblasts in a number of different tissues (Tomasek et al., 2002; Zhang et al., 1994; Walker et al., 2001). Myofibroblasts isolated from various fibrotic tissues, including lungs, are thought to be the primary source of collagen and other ECM proteins (Tomasek et al., 2002; Zhang et al., 1994). Studies in animals employing the bleomycin-induced model of pulmonary fibrosis have identified myofibroblasts to be the primary source of increased collagen expression and a major source of cytokines and chemokines as well (Zhang et al., 1996; Vyalow et al., 1993).

The precise source(s) of myofibroblasts is still not well known. Relative contributions from circulating mesenchymal stem cells or from local trans-differentiation of epithelial cells to fibroblasts have been reported (Hinz et al., 2007). It has become generally accepted that lung

fibroblasts may differentiate to a myofibroblast phenotype under the influence of local growth factors and cytokines, such as TGF- $\beta$ , endothelin-1, and thrombin (Hinz et al., 2007; Bogatkevich et al., 2001; Shi-Wen et al., 2004). Interestingly, thrombin itself has been demonstrated to induce the secretion of TGF- $\beta$  and endothelin-1 (Shi-Wen et al., 2004; Bachhuber et al., 1997).

#### **4. Thrombin induces differentiation of lung fibroblasts to a myofibroblast phenotype resistant to apoptosis while inducing apoptosis of alveolar epithelial cells**

Our previous studies demonstrated that thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the PAR-1/PKC $\epsilon$ -dependent pathway (Bogatkevich et al., 2001; 2003; 2005). We previously reported that thrombin induces resistance to FasL-induced apoptosis in normal lung fibroblasts and that a similar level of resistance to FasL is observed in SSc lung fibroblasts *de novo* (Bogatkevich et al., 2005). Interestingly, we also found that thrombin-induced resistance to apoptosis is not specific only for FasL. We showed that thrombin also induces resistance to other apoptotic factors such as camptothecin and ceramide, and that SSc lung fibroblasts are also resistant to apoptosis induced by these stimuli. Because of the involvement of Fas-FasL pathway in various pulmonary disorders, we have selected this molecule for further investigations of apoptosis. We demonstrated that FasL induces apoptosis in normal lung fibroblasts in a dose-dependent manner and that this effect is inhibited by thrombin, as well as by overexpression of constitutively-activated PAR-1 or constitutively-activated PKC $\epsilon$  (Bogatkevich et al., 2005).

The activation of Akt in different cell lines is necessary for promotion of cell survival and protection from apoptosis. We observed that thrombin induced sustained phosphorylation of Akt at Ser-473 in lung fibroblasts. Phosphorylation of Akt occurred within 10 min of thrombin treatment, reaching a maximum at 30 min and decreasing after 2 hours (Fig. 4 A). Basal levels of total Akt were similar in lung fibroblasts and in A549 alveolar epithelial cells (AEC), and thrombin induced Akt phosphorylation only in lung fibroblasts and not in AEC (Fig.4B) at any time point.

Activation of the thrombin receptor PAR-1 is known to mediate apoptosis of intestinal epithelial and lung epithelial cells (Suzuki et al, 2005; Ando et al., 2007). We incubated human A549 AEC and mouse primary AEC with thrombin to determine if thrombin induces apoptosis in these cells. After 24 hours of incubation with thrombin (1U/ml), A549 cells demonstrated 4.2 times and AT2 cells demonstrated 3.45 times more DNA fragments when compared to control cells, consistent with thrombin induction of apoptosis of these different AEC types (Fig. 4C).

The best recognized hallmark of both early and late stages of apoptosis is the activation of cysteine aspartate-specific proteases, caspases. Upon activation, the caspases cleave specific substrates and thereby mediate many of the typical biochemical and morphological changes in apoptotic cells, such as cell shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebs (Kohler et al., 2002). Caspase-3 is activated during most apoptotic processes and is believed to be the main executioner caspase. We observed that thrombin activates caspase-3 in AEC, but not in lung fibroblasts (Fig. 4D)

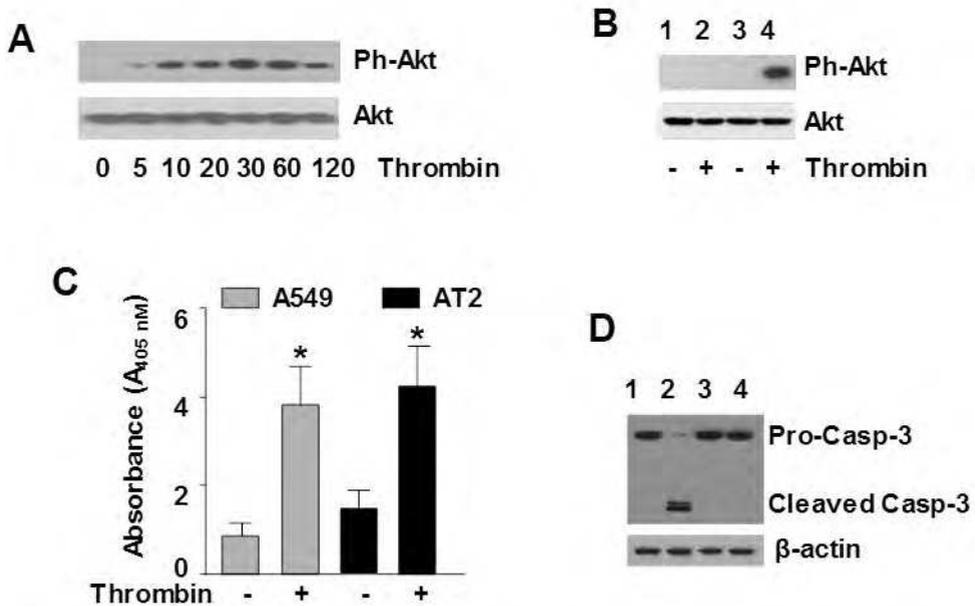


Fig. 4. Distinct effects of thrombin in lung fibroblasts and AEC. (A) Time course of thrombin-induced Akt Phosphorylation was determined in lung fibroblasts treated with 1U/ml thrombin for various time points. (B) AEC (lanes 1 and 2) and lung fibroblasts (lanes 3 and 4) were incubated with or without thrombin for 30 minutes. Cell extracts were immunoblotted with phospho-Akt or total Akt antibody (Cell Signaling Technology). (C) Thrombin-induced apoptosis of human SAEC and mice AT2 cells. Each bar represents the mean  $\pm$  SD of duplicate determinations in 3 experiments. \*Statistically significant differences between cells stimulated with thrombin and dabigatran versus cells stimulated with thrombin ( $p < 0.05$ ). (D) Western blot analysis of caspase-3 expression in AEC and lung fibroblasts. Confluent cultures of AEC (lanes 1 and 2) and lung fibroblasts (lanes 3 and 4) were incubated with or without thrombin for 24 hours. Cell were collected with lysis buffer, subjected to SDS-polyacrylamide gels, and analyzed by immunoblotting using anti-caspase-3 antibodies from Cell Signaling Technology. Expression of  $\beta$ -actin is shown to confirm the equal loading of protein. Note that caspase-3 antibody recognizes full length pro-caspase-3 (35kDa) and the fragment (17 kDa) from caspase-3 resulting from cleavage.

### 5. Effects of direct thrombin inhibitor dabigatran on aec and lung fibroblasts *in vitro*

Thrombin is a well-known mitogen and has been shown to induce human lung fibroblast proliferation. We measured the effect of dabigatran on thrombin-induced lung fibroblast proliferation using a quick cell proliferation assay. This method is based on cleavage of a tetrazolium salt, WST-1, to formazan by cellular mitochondrial dehydrogenases. Expansion of the number of viable cells results in an increase in the activity of the mitochondrial dehydrogenases leading to an increase in the amount of formazan dye detected by

spectrometry. Basal levels of viable lung fibroblasts were in a range of between 0.38 and 0.51 OD. Thrombin increased fibroblast proliferation 1.8-fold within 24 hours. The direct thrombin inhibitor, dabigatran, itself had no significant effect on lung fibroblast proliferation, yet dabigatran significantly inhibited thrombin-induced proliferation of lung fibroblasts. Neither thrombin nor dabigatran affected AEC cell proliferation (Fig. 5A). Dabigatran, however, inhibited thrombin-induced apoptosis of AEC A549 (Fig. 5B).

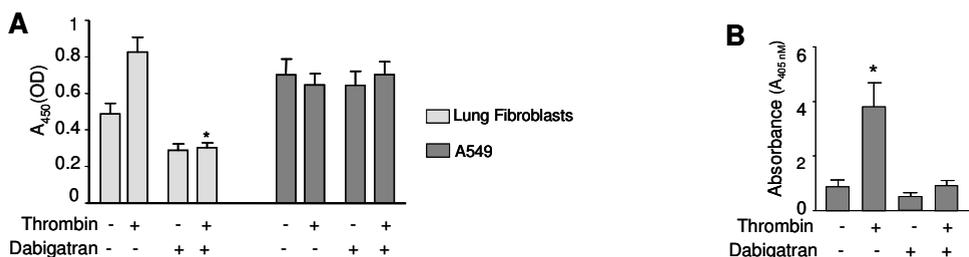


Fig. 5. (A) Dabigatran inhibits thrombin-induced lung fibroblasts proliferation and has no effects on the proliferation of AEC. (B) Dabigatran inhibits thrombin-induced apoptosis of AEC. The *asterisk* represents statistically significant ( $p < 0.05$ ) differences between cells stimulated with thrombin *versus* cells stimulated with thrombin and dabigatran.

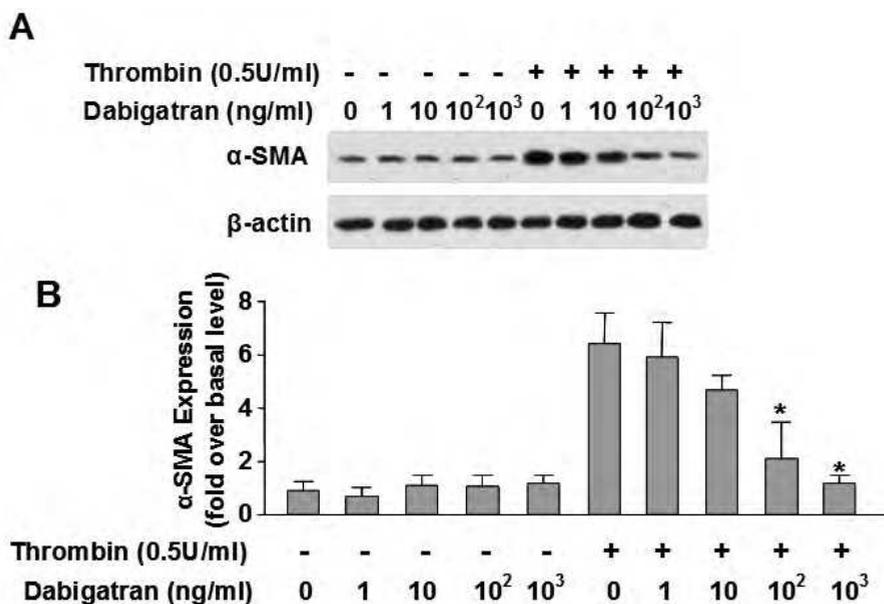


Fig. 6. (A) Dabigatran inhibits  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expression in lung fibroblasts. (B) The images were scanned and analyzed with NIH Imaging software. Densitometric analysis of immunoblots from 3 independent experiments is presented. The *asterisk* represents statistically significant differences ( $p < 0.05$ ) between cells stimulated with thrombin and dabigatran *versus* cells treated with thrombin alone.

The appearance of myofibroblasts in areas of active fibrosis strongly suggests that myofibroblasts are key contributors to the pathogenesis of SSc-ILD. Lung fibroblasts from SSc-ILD patients express abundant and highly organized  $\alpha$ -SMA (Bogatkevich et al., 2001). Moreover, thrombin receptor PAR-1 and  $\alpha$ -SMA co-localize in lung tissue in early stages of lung fibrosis (see Fig. 3). In contrast, normal lung fibroblasts contain relatively small amounts of  $\alpha$ -SMA which is not fully organized. Previously we reported that within 24 hours of exposure thrombin increases the amount of highly organized  $\alpha$ -SMA in normal lung fibroblasts (Bogatkevich et al., 2001 and 2003). Although dabigatran had no effect on the basal level of  $\alpha$ -SMA in normal lung fibroblasts, dabigatran significantly decreased thrombin-induced  $\alpha$ -SMA expression in a dose-dependent manner (Fig. 6).

Contractile phenotype is another characteristic feature of myofibroblasts. Contractile forces of the myofibroblast are generated by  $\alpha$ -SMA, which is extensively expressed in stress fibers and by large fibronexus adhesion complexes connecting intracellular actin with extracellular fibronectin fibrils (Gabbiani, 2003). Fibroblasts cultured in collagen gel matrices provide an *in vitro* model of fibrocontractility and fibrosing diseases such as scleroderma and IPF (Grinnell, 1999). When cultured within collagen gels fibroblasts recognize collagen fibers leading to contraction of the gels. This is believed to reflect the *in vivo* phenomenon of wound contraction and extracellular remodeling in connective tissue. In lung fibrosis it might also reflect the pathologic stiffness observed in SSc-ILD and other restrictive lung diseases. We previously observed that thrombin induces collagen gel contraction by normal lung fibroblasts in a dose-dependent manner with a maximal effect at 0.5 U/ml (Bogatkevich et al., 2001). To further investigate the effects of dabigatran on collagen gel contraction we used floating and fixed collagen gel assays with normal and SSc lung fibroblasts treated with and without thrombin or dabigatran for 48 hours. Contraction of floating collagen gels is considered to resemble more closely the initial phase of wound contraction and reflects the induction of the myofibroblast phenotype by various growth factors (Grinnell, 1999; Shi-Wen et al., 2004). In contrast, attached or fixed collagen gels serve as a model of the late phase of excessive scarring observed in contractures and reflect the direct ability of proteins to enhance contraction of already formed  $\alpha$ -SMA through mechanical stress. SSc lung fibroblasts inherently contain higher levels of  $\alpha$ -SMA and readily contracted both floating and fixed collagen gels (Fig. 7). Dabigatran significantly reduced collagen gel contraction by SSc lung fibroblasts and  $\alpha$ -SMA in both floating and fixed collagen gels; however, thrombin only slightly induced  $\alpha$ -SMA and did not significantly affect collagen gel contraction by SSc lung fibroblasts.

We observed notable differences for floating and fixed collagen gels seeded with normal lung fibroblasts when stimulated with thrombin. Thrombin strikingly contracted floating collagen gels within 48 hours in a similar manner as within 24 hours; in contrast, thrombin only slightly affected fixed collagen gels. Similarly,  $\alpha$ -SMA was induced to a much higher extent by thrombin in floating gels as compared to fixed gels. In contrast, dabigatran inhibited collagen gel contraction and  $\alpha$ -SMA not only in floating but also in fixed collagen gels, thus blocking differentiation to a myofibroblast phenotype, as well as reversing the already existing myofibroblast phenotype.

Normal lung fibroblasts naturally produce collagen type I and CTGF in very low concentrations. Thrombin and the PAR-1 selective activating peptide PARI-AP notably increased the production of both of these proteins within 48 hours. Pre-treatment of lung fibroblasts with dabigatran (1 $\mu$ g/ml) prevented the accumulation of collagen type I and CTGF induced by thrombin, but not by PARI-AP (Fig. 8A).

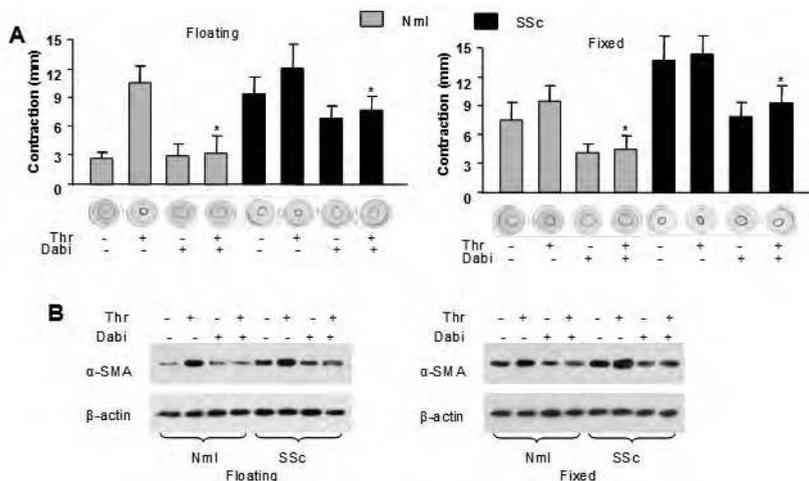


Fig. 7. (A) Inhibition of floating and fixed collagen gel contraction by dabigatran in lung fibroblasts. Data are presented as mean values ± SD of three experiments. The asterisk represents statistically significant differences ( $p < 0.05$ ) between cells stimulated with thrombin and dabigatran versus cells treated with thrombin alone. (B)  $\alpha$ -SMA expression in floating and fixed collagen gels

Lung fibroblasts from SSc-ILD patients express considerably higher levels of  $\alpha$ -SMA, CTGF, and collagen type I when compared with normal lung fibroblasts. To establish whether dabigatran would interfere with the expression of these markers of fibrogenesis, we incubated SSc lung fibroblasts with dabigatran (1  $\mu$ g/ml) for 24, 48, 72, and 96 hours. We observed that the addition of dabigatran for 72 and 96 hours reduced the levels of  $\alpha$ -SMA and CTGF; however, treatment of cells with dabigatran for 48 and 24 hours had little or no effect (Fig. 8B). In contrast, the level of collagen type I was not significantly affected by dabigatran even after 72 hours. Yet after 96 hours of incubation with dabigatran SSc lung fibroblasts expressed significantly less collagen type I. To investigate whether, in addition to  $\alpha$ -SMA expression,  $\alpha$ -SMA organization would also be affected by dabigatran in SSc lung fibroblasts, we performed fluorescence microscopy studies. We observed that prolonged incubation of SSc lung fibroblasts with dabigatran indeed results in decreased  $\alpha$ -SMA expression and organization (Fig. 8C). Over-production of collagen with increased expression of CTGF is considered to be a molecular hallmark of fibrosis (Grotendorst et al., 2004). Thrombin increases the expression of collagen type I and CTGF (Chambers et al., 1998 and 2000, Bogatkevich et al., 2006). Importantly, we have demonstrated that dabigatran restrains thrombin-induced accumulation of collagen type I and CTGF in human lung fibroblasts. Although we observed that incubation of SSc-ILD fibroblasts with dabigatran for 24 hours had no effect, upon longer exposure to dabigatran (72 hours) considerable inhibition of CTGF and  $\alpha$ -SMA expression/organization was observed. Even longer exposure (96 hours) was required for dabigatran to significantly decrease collagen type I, suggesting that down-regulation of collagen by dabigatran in SSc lung fibroblasts occurs after inhibition of CTGF and  $\alpha$ -SMA. It was reported that CTGF induces collagen I by stimulating transcription and promoter activity (Chujo et al., 2005; Gore-Hyer et al., 2002),

and diminishing CTGF by small interfering RNA lowers collagens I and IV in rats (Luo et al., 2008). Similarly, the inhibition of  $\alpha$ -SMA by the NH<sub>2</sub>-terminal peptide of  $\alpha$ -SMA results in reduction of collagen gene expression (Hinze et al., 2002). Therefore, our data suggest that dabigatran may contribute to collagen type I down-regulation secondarily via reduced expression of CTGF and  $\alpha$ -SMA.

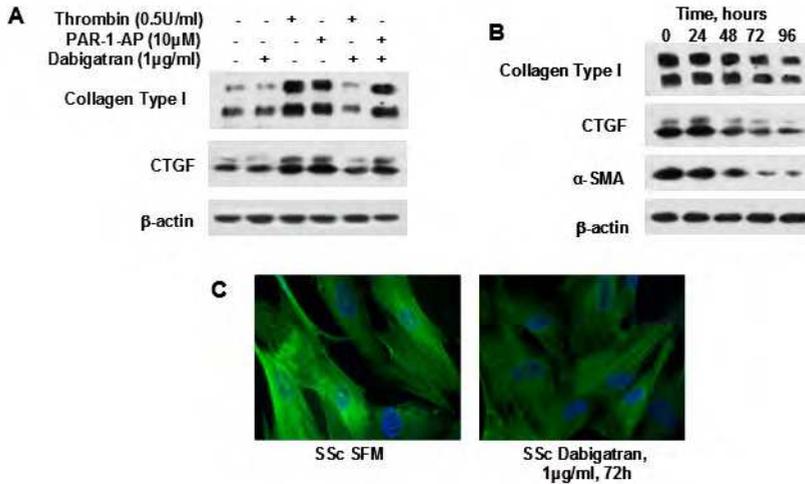


Fig. 8. (A) Dabigatran inhibits thrombin- but not PAR-1-AP-induced collagen type I and CTGF. (B) Dabigatran inhibits collagen type I, CTGF, and  $\alpha$ -SMA expression in scleroderma lung fibroblasts. (C) Dabigatran inhibits  $\alpha$ -SMA expression and organization in scleroderma lung fibroblasts. The experiments were repeated three times in three different cell lines and representative immunoblots and images are presented.

## 6. *In vivo* antifibrotic effects of direct thrombin inhibition with dabigatran

Since dabigatran restrained important *in vitro* profibrotic events in lung fibroblasts, we reasoned that dabigatran would diminish bleomycin-induced pulmonary fibrosis. For *in vivo* studies we employed dabigatran etexilate, the oral prodrug of dabigatran. The prodrug does not have antithrombin activity; however, after oral administration dabigatran etexilate is rapidly converted by ubiquitous esterases to the active moiety, dabigatran (Sorbera et al., 2005, Wiene et al., 2007). Drugs administered during the early phase of tissue injury act predominantly as anti-inflammatory agents and should be considered as “preventive treatment”, whereas “true” antifibrotic agents might be effective irrespective of timing, particularly if administered during the “fibrotic” or later phase of the model (Moeller et al., 2008). To distinguish between anti-inflammatory and antifibrotic drug effects, we compared the effect of oral administration of dabigatran etexilate on the day of bleomycin instillation (day 1) and on day 8 after bleomycin instillation in mice.

In control mice that received saline or saline and dabigatran etexilate, lung histology was characterized by alveolar structures composed of septa, vascular components, and connective tissue. Alveolar septa were thin allowing maximum air to occupy the lung. Lung tissue isolated from bleomycin-treated mice demonstrated extensive peribronchial and

interstitial infiltrates of inflammatory cells (H&E staining), thickening of the alveolar walls, and multiple focal fibrotic lesions with excessive amounts of ECM protein shown by trichrome differential staining (Fig. 9A). By contrast, significantly fewer cellular infiltrates, decreased thickness of alveolar septa, and reduced accumulation of ECM proteins were all noted in mice treated with dabigatran etexilate. Importantly, such beneficial effects of dabigatran etexilate on bleomycin-induced pulmonary fibrosis were observed not only in mice receiving dabigatran etexilate beginning on the same day as bleomycin (day 1), but were seen also in mice that received dabigatran etexilate beginning on day 8 after bleomycin administration (Fig. 9A). The overall level of fibrotic changes was quantitatively assessed based on the Ashcroft scoring system (Ashcroft et al., 1988). The score in mice treated with bleomycin + placebo was nearly 9-fold higher compared to control mice ( $5.76 \pm 1.64$  and  $0.65 \pm 0.7$  respectively). Fibrosis in mice treated with dabigatran etexilate beginning on day 1 after bleomycin instillation was significantly reduced (2.8-fold,  $p < 0.05$ ) when compared to the bleomycin + placebo group, suggesting an anti-inflammatory effect of dabigatran (Fig. 9B).

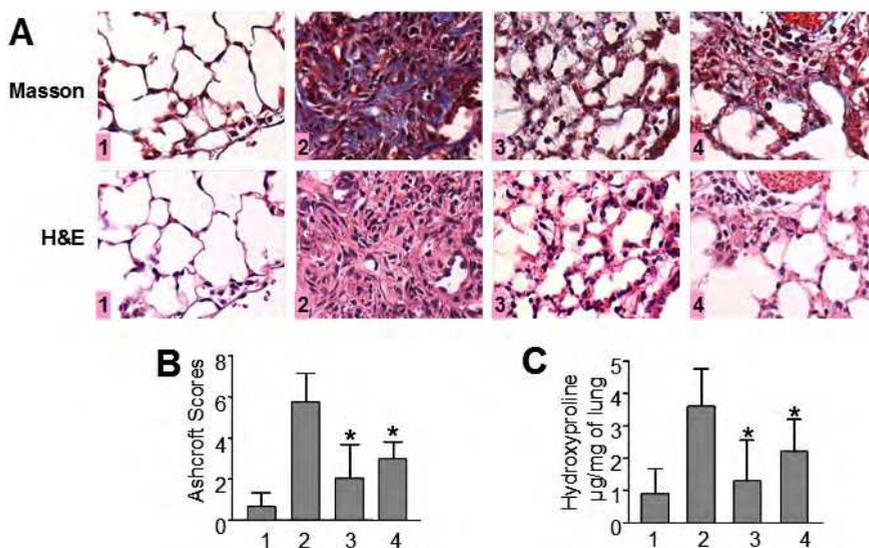


Fig. 9. Effect of dabigatran etexilate on bleomycin-induced pulmonary fibrosis. (A) Representative histological findings of lung inflammation and fibrosis. 1 - control (saline + placebo or saline + dabigatran), 2 - bleomycin + placebo, 3 - bleomycin + dabigatran etexilate (day 1), 4 - bleomycin + dabigatran etexilate (day 8),  $n = 40$  (10 mice per group) (B) Quantitative evaluation of fibrotic changes (Ashcroft scores). (C) Collagen lung content measured by hydroxyproline assay,  $n = 32$  (8 mice per group). Values are the mean  $\pm$  SD. \* =  $P < 0.05$  versus bleomycin + placebo-treated mice.

Interestingly, fibrosis in mice that received dabigatran beginning on day 8 after bleomycin instillation was also significantly reduced (1.9-fold,  $p < 0.05$ ) compared to the bleomycin + placebo group, suggesting that in addition to anti-inflammatory properties dabigatran demonstrated a strong anti-fibrotic effect.

The most profound development of fibrosis in the bleomycin model is observed by day 21; therefore, we determined the effect of dabigatran on collagen accumulation in lungs 21 days

after bleomycin treatment. To quantify collagen accumulation within the lungs we employed hydroxyproline assay, which is based on colorimetric measurements of hydroxyproline in lung hydrolysates reflecting total collagen in lung tissue. We observed that dabigatran etexilate did not affect the basal level of hydroxyproline (data not shown). However, dabigatran etexilate significantly lowered hydroxyproline in bleomycin-treated mice by 64% and by 39% when administrated beginning on day 1 and day 8, respectively (Fig. 9C). From these studies, we conclude that dabigatran etexilate downregulates lung collagen by exerting an antifibrotic effect via thrombin inhibition.

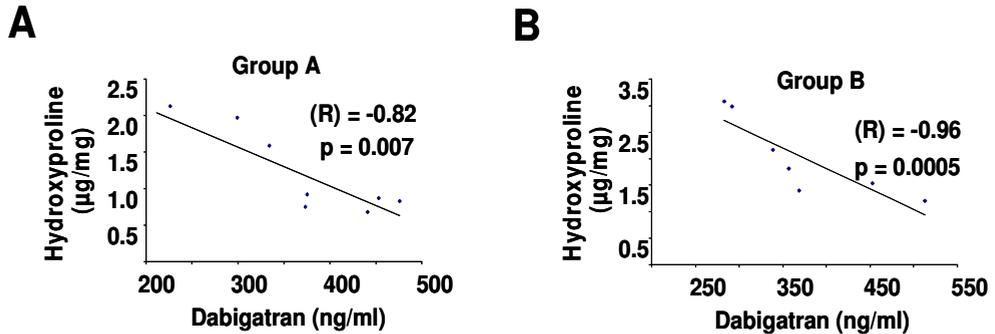


Fig. 10. Negative correlation of collagen content in lung tissue versus dabigatran levels in plasma on day 1 (Group A) and day 8 (Group B), respectively.

The association between hydroxyproline levels in lung tissue and dabigatran concentrations in plasma was tested using the Spearman rank correlation test. The average plasma concentration of dabigatran obtained from mice fed with dabigatran chow was  $342.1 \pm 90.0$  ng/mL ( $n=21$ ). We observed a strong negative correlation of hydroxyproline versus dabigatran plasma levels (Fig. 10). There was no correlation between hydroxyproline and dabigatran plasma levels in control mice receiving saline and dabigatran chow (data not shown). This suggests that dabigatran affects collagen expression induced by tissue injury (fibrosis) while not interfering with basal levels of collagen in normal lung tissue.

The total nucleated cell count in BALF was markedly higher in the bleomycin-treated group on day 14 as compared to saline control animals (Table 1).

Bronchoalveolar lavage was performed on day 14 after bleomycin administration. Data are presented as mean  $\pm$  SD ( $n = 8$  mice per treatment group). \* =  $P < 0.01$  versus bleomycin + placebo-treated mice.

Dabigatran etexilate treatment starting on day 1 and day 8 significantly reduced total nucleated BAL cell counts from bleomycin-treated mice ( $p < 0.01$ ). Dabigatran etexilate alone did not affect BALF cell counts (data not shown). The percentage of BALF neutrophils was significantly decreased in bleomycin/dabigatran etexilate-treated mice when compared to bleomycin/placebo-treated mice ( $p < 0.01$ ). Total BALF protein was increased by 7.9-fold in bleomycin-placebo treated mice when compared to control and was significantly decreased by dabigatran treatment ( $p < 0.001$ ). On day 21 after bleomycin instillation there was notably fewer cells in BALF in mice treated with dabigatran etexilate when compared to placebo-treated mice. However, there were no significant differences in cell numbers among studied groups (data not shown).

Parameters	Control	Bleo/Placebo	Bleo/Dabigatran Day 1	Bleo/Dabigatran Day 8
Total Protein (mg/ml)	0.24±0.09	1.89±0.26	0.87±0.41*	0.89±0.36*
Total Cell (x10 <sup>5</sup> /ml)	8.0±3.5	41.9±17.0	18.2±7.5*	21.0±8.5*
Macrophages (%)	97.8±1.6	71.6±8.3	84.8±9.3*	79.4±9.0
Lymphocytes (%)	2.2±1.4	10.3±4.4	8.7±3.7	9.1±5.8
Neutrophils (%)	0	18.1±5.6	6.5±2.2*	11.5±5.4*

Table 1. Analysis of bronchoalveolar lavage fluid

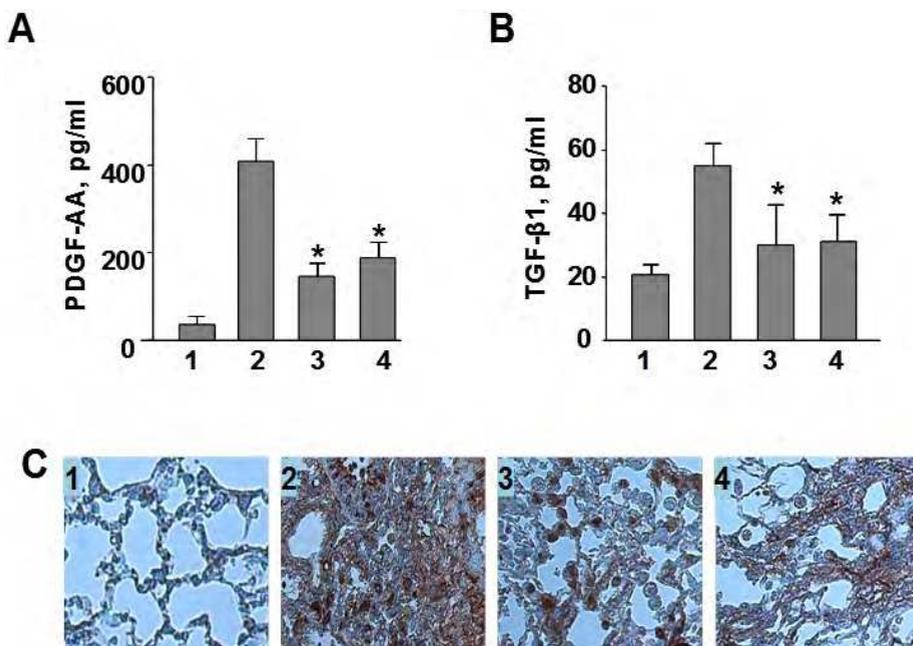


Fig. 11. Effect of dabigatran etexilate on PDGF-AA (A) and TGF-β1 (B) levels in BALF. (C) TGF-β1 expression in lung tissue. 1 - control (saline/placebo), 2 - bleomycin/placebo, 3 - bleomycin/dabigatran etexilate (day 1), 4 - bleomycin/dabigatran etexilate (day 8), n = 24 (6 mice per group). Values are the mean ± SD. \* = P < 0.01 versus bleomycin/placebo-treated mice.

We observed that PDGF-AA was up-regulated (11.3-fold) when compared to controls in bleomycin-placebo-treated mice. Dabigatran etexilate significantly reduced PDGF-AA by 65% and 54% when initiated on day 1 and day 8, respectively (p<0.01) (Fig. 11A). The level of TGF-β1 in bleomycin-placebo-treated mice was 2.7-fold higher compared to controls (saline/placebo- and saline/dabigatran-treated mice). Dabigatran etexilate significantly

reduced TGF- $\beta$ 1 concentrations from  $54.9 \pm 6.1$  pg/ml in bleomycin/placebo-treated mice to  $29.9 \pm 9.3$  and  $31.1 \pm 8.7$  pg/ml when administered beginning on day 1 and day 8, respectively ( $p < 0.01$ , Fig. 11B). TGF- $\beta$ 1 expression was also assessed by immunohistochemistry in lung tissue. In this analysis, TGF- $\beta$ 1 was not detectable in lung from control mice, whereas it was strongly expressed in fibrotic areas of lung tissue from mice treated with bleomycin plus placebo (Fig. 11C). Dabigatran etexilate visibly reduced TGF- $\beta$ 1 expression when used as either early or late treatments. Similar to TGF- $\beta$ 1, CTGF and  $\alpha$ -SMA were not detectable in lung tissue from control mice, with the exception of  $\alpha$ -SMA expression in smooth muscle cells located in and around blood vessels and airways (Fig. 12). However, CTGF and  $\alpha$ -SMA were each strongly upregulated in the lungs of bleomycin-treated mice. Dabigatran etexilate reduced this expression of CTGF and  $\alpha$ -SMA in lung tissue, when administered beginning on either day 1 or day 8 of bleomycin treatment.

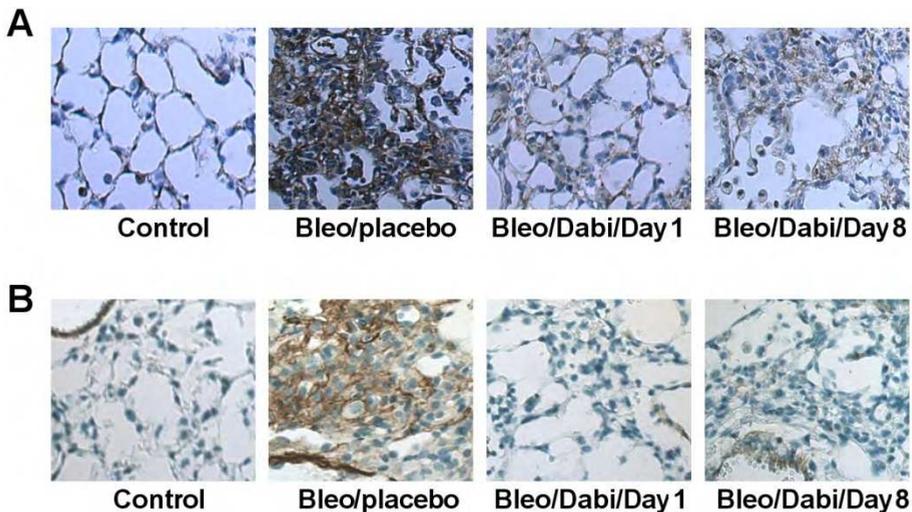


Fig. 12. Immunohistochemical evaluation for CTGF (A) and  $\alpha$ -SMA (B) expression in lung tissue.

In this and all other experiments within the study we found that both early and late treatments with dabigatran etexilate were able to inhibit bleomycin-induced pulmonary fibrosis; however, the inhibition of pulmonary fibrosis was more profound with early administration. The efficacy of early treatment with dabigatran etexilate was higher because it targeted the inflammatory stage of fibrosis, while later treatment was introduced at the time of a more developed degree of lung injury. The role of inflammation in the pathogenesis of progressive pulmonary fibrosis remains a controversial issue. Bleomycin causes a severe acute inflammatory response followed by chronic inflammation and fibrosis. It has been shown that the degree of inflammation in bleomycin-induced lung injury is associated with the intensity of fibrosis (Moore & Hogaboam, 2008).

The concentration of dabigatran etexilate used in these experiments yielded plasma levels that are slightly higher than those achieved in patients treated with dabigatran etexilate for various thrombotic diseases ( $\sim 180$  ng/ml peak levels achieved with 150 mg twice daily dose) (Van Ryn et al., 2010). The dose used in this study resulted in a  $\sim 2$ -fold elevation of the aPTT and  $\sim 10$ -fold elevation of the TT (Fig. 13A).

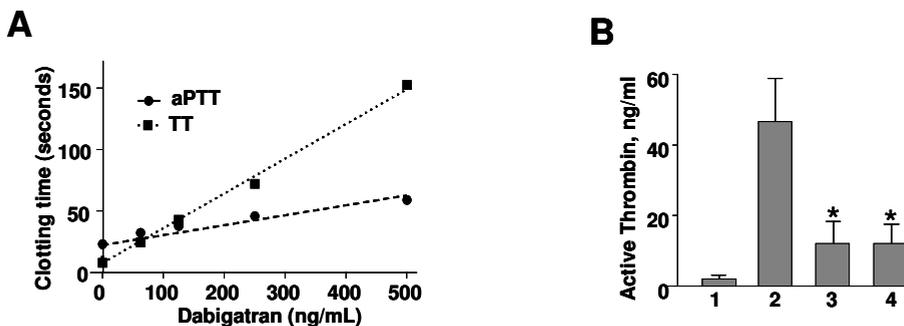


Fig. 13. (A) Effect of increasing concentrations of dabigatran on the thrombin time (TT) and activated partial thromboplastin time (aPTT) clotting times in mouse plasma in vitro. Data expressed as mean of duplicate determinations. (B) Effect of dabigatran etexilate on level of active thrombin in BALF.

Such prolongation in mouse plasma is consistent with findings in human plasma, where it has also been shown that change in the TT is more sensitive to dabigatran than is the aPTT. Though it is not possible to directly relate plasma levels and/or anticoagulation from mice to humans, it is important to note that the antifibrotic effects observed in our studies were achieved at plasma levels and pharmacological effects consistent with human dosing.

It is also important to note that dose of dabigatran etexilate used in this study significantly reduced but did not completely inhibit thrombin activity in BALF (Fig. 13B). We did not observe any bleeding side-effects during the study suggesting that levels of dabigatran in mouse plasma were not sufficient to completely eliminate thrombin from the normal hemostatic process. However, such doses of dabigatran etexilate ameliorated lung fibrosis even after it had been established, indicating that dabigatran etexilate could safely be administered in chronic forms of lung fibrosis, at least in mice.

## 7. Conclusions

Tissue injury with activation of the coagulation cascade and increased thrombin activity with deposition of fibrin are characteristic features of pulmonary fibrosis – the end result of a heterogeneous group of disorders that includes IPF and SSc-ILD. Characterized by microvascular injury and inflammation, SSc-ILD culminates in excessive deposition of extracellular matrix proteins, often resulting in severe lung dysfunction and death. Although cyclophosphamide treatment may stabilize lung function in some patients, long-term treatment is required and significant toxicity may occur (Berezne et al., 2007; Strange et al., 2008). There is, therefore, a great need for new therapeutic approaches that would be more effective and less toxic than current treatments. Dabigatran etexilate represents the first synthetic oral, reversible, direct inhibitor of thrombin with a very favorable biochemical and pharmacological profile that translates into clinical efficacy and safety in patients with or at risk of thrombotic disorders (Stangier, 2008). The current study provides important preclinical information about the feasibility and efficacy of dabigatran etexilate for the treatment of fibrotic diseases, including IPF and SSc-ILD, in which there is evidence for tissue injury with overexpression of thrombin and downstream fibrogenesis. Any future studies of thrombin inhibition for the treatment of SSc-ILD would need to demonstrate a positive benefit/risk ratio taking into account potential risks such as gastrointestinal tract hemorrhage.

## 8. Acknowledgment

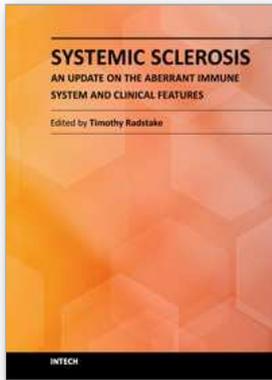
This work was supported in part by a career award from National Institutes of Health K01AR051052 (to GSB), NIH/NCRR Grant RR029882, the Scleroderma Foundation, and Boehringer Ingelheim International GmbH. Ilia Atanelishvili was sponsored by UICC fellowship ICRETT- 10-087.

## 9. References

- [1] Ando S, Otani H, Yagi Y, Kawai K, Araki H, Nakamura T, Fukuhara S, Inagaki C. Protease-activated receptor 4-mediated Ca<sup>2+</sup> signaling in mouse lung alveolar epithelial cells. *Life Sci.* 2007;81(10):794-802.
- [2] Armstrong MT, Fenton JW 2nd, Andersen TT, Armstrong PB. Thrombin stimulation of matrix fibronectin. *J Cell Physiol* 1996;166:112-20.
- [3] Ashcroft T., Simpson J. M., Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J. Clin. Pathol.*, 41: 467-470, 1988
- [4] Bachhuber BG, Sarembock IJ, Gimble LW, and Owens GK. alpha-Thrombin induces transforming growth factor-beta1 mRNA and protein in cultured vascular smooth muscle cells via a proteolytically activated receptor. *J Vasc Res* 1997; 34:41-48.
- [5] Bérezné A, Valeyre D, Ranque B, Guillevin L, Mouthon L. Interstitial lung disease associated with systemic sclerosis: what is the evidence for efficacy of cyclophosphamide? *Ann N Y Acad Sci.* 2007;1110:271-84.
- [6] Bogatkevich GS, Gustilo E, Oates J, Feghali-Bostwick C, Harley RA, Silver RM, Ludwicka-Bradley A: Distinct PKC isoforms mediate cell survival and DNA synthesis in thrombin-induced myofibroblasts. *Am J Physiol: Lung Cell Mol Physiol* 2005, 288, L190-L201.
- [7] Bogatkevich GS, Ludwicka-Bradley A, Paul J. Nietert, Silver RM: Scleroderma Lung Fibroblasts: Contractility and Connective Tissue Growth Factor. In *Myofibroblasts* (Gabbiani G, Chaponnier C, Desmouliere A, eds), Landes Bioscience, Georgetown, TX, 2006, 25-31.
- [8] Bogatkevich GS, Ludwicka-Bradley A, Silver RM. Dabigatran, a direct thrombin inhibitor, demonstrates antifibrotic effects on lung fibroblasts. *Arthritis Rheum.* 2009; 60(11):3455-64.
- [9] Bogatkevich GS, Tourkina E, Abrams CS, Harley RA, Silver RM, Ludwicka-Bradley A. Contractile activity and smooth muscle alpha-actin organization in thrombin-induced human lung myofibroblasts. *Am J Physiol Lung Cell Mol Physiol.* 2003;285(2):L334-43.
- [10] Bogatkevich GS, Tourkina E, Silver RM and Ludwicka-Bradley A: Thrombin differentiates normal lung fibroblast to a myofibroblast phenotype via proteolytically activated receptor-1 and protein kinase C-dependent pathway. *J Biol Chem* 2001; 276, 45184-92.
- [11] Chambers RC, Dabbagh K, McAnulty RJ, Gray AJ, Blanc-Brude OP, Laurent GJ. Thrombin stimulates fibroblast procollagen production via proteolytic activation of protease-activated receptor 1. *Biochem J* 1998;333:121-7.
- [12] Chambers RC, Leoni P, Blanc-Brude OP, Wembridge DE, Laurent GJ. Thrombin is a potent inducer of connective tissue growth factor production via proteolytic activation of protease-activated receptor-1. *J Biol Chem* 2000;275:35584-91.
- [13] Chambers RC. Procoagulant signalling mechanisms in lung inflammation and fibrosis: novel opportunities for pharmacological intervention? *Br J Pharmacol.* 2008;153 Suppl 1:S367-78.
- [14] Chujo S, Shirasaki F, Kawara S, Inagaki Y, Kinbara T, Inaoki M, Takigawa M, Takehara K. Connective tissue growth factor causes persistent pro  $\alpha$ 2(I) collagen gene expression

- induced by transforming growth factor- $\beta$  in a mouse fibrosis model. *J Cell Physiol* 2005;203:447-56.
- [15] Coughlin SR. How the protease thrombin talks to cells. *Proc Natl Acad Sci USA* 1999; 96:11023-11027.
- [16] DeMaio L, Tseng W, Balverde Z, Alvarez JR, Kim KJ, Kelley DG, Senior RM, Crandall ED, Borok Z. Characterization of mouse alveolar epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol*. 2009 Jun;296(6):L1051-8.
- [17] Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003; 200:500-03.
- [18] Gore-Hyer E, Shegogue D, Markiewicz M, Lo S, Hasen-Martin D, Greene E, Grotendorst G, Trojanowska M. TGF and CTGF have overlapping and distinct fibrogenic effects on human renal cells. *Am J Physiol Renal Physiol* 2002;283:F707-F716.
- [19] Gray AJ, Bishop JE, Reeves JT, Laurent GJ. A alpha and B beta chains of fibrinogen stimulate proliferation of human fibroblasts. *J Cell Sci* 1993;104:409-13.
- [20] Grinnell F. Signal transduction pathways activated during fibroblast contraction of collagen matrices. *Curr Top Pathol* 1999; 93:61-73.
- [21] Grotendorst GR, Rahmanie H, Duncan MR. Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. *FASEB J*. 2004; 18: 469-479.
- [22] Hernández-Rodríguez NA, Cambrey AD, Harrison NK, Chambers RC, Gray AJ, Southcott AM, duBois RM, Black CM, Scully MF, McAnulty RJ, et al. Role of thrombin in pulmonary fibrosis. *Lancet*. 1995;346:1071-3.
- [23] Hinz B, Gabbiani G, Chaponnier C. The NH2-terminal peptide of alpha-smooth muscle actin inhibits force generation by the myofibroblast in vitro and in vivo. *J Cell Biol* 2002;157:657-63.
- [24] Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. *Am J Pathol* 2007;170:1807-16.
- [25] Howell DC, Goldsack NR, Marshall RP, McAnulty RJ, Starke R, Purdy G, Laurent GJ, Chambers RC. Direct thrombin inhibition reduces lung collagen, accumulation, and CTGF mRNA levels in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 2001;159:1383-95.
- [26] Howell DC, Johns RH, Lasky JA, Shan B, Scotton CJ, Laurent GJ, Chambers RC. Absence of proteinase-activated receptor-1 signaling affords protection from bleomycin-induced lung inflammation and fibrosis. *Am J Pathol* 2005;166:1353-65.
- [27] Howell DC, Laurent GJ, Chambers RC. Role of thrombin and its major cellular receptor, protease-activated receptor-1, in pulmonary fibrosis. *Biochem Soc Trans*. 2002;30:211-6.
- [28] Köhler C, Orrenius S, Zhivotovsky B. Evaluation of caspase activity in apoptotic cells. *J Immunol Methods*. 2002;265(1-2):97-110.
- [29] Ludwicka A, Trojanowska M, Smith EA, Baumann M, Strange C, Korn JH, Smith T, Leroy EC, Silver RM. Growth and characterization of fibroblasts obtained from bronchoalveolar lavage of patients with scleroderma. *J Rheumatol*. 1992;19:1716-23.
- [30] Luo GH, Lu YP, Song J, Yang L, Shi YJ, Li YP. Inhibition of connective tissue growth factor by small interfering RNA prevents renal fibrosis in rats undergoing chronic allograft nephropathy. *Transplant Proc* 2008;40:2365-9.
- [31] Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. *Pharmacol Rev*. 2001;53:245-82.
- [32] Mercer PF, Deng X, Chambers RC. Signaling pathways involved in proteinase-activated receptor1-induced proinflammatory and profibrotic mediator release following lung injury. *Ann N Y Acad Sci* 2007;1096:86-8.

- [33] Moeller A, Ask K, Warburton D, Gaudie J, Kolb M. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol*. 2008;40(3):362-82.
- [34] Moore BB, Hogaboam CM. Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2008;294(2):L152-60.
- [35] Ohba T, McDonald JK, Silver RM, Strange C, LeRoy EC and Ludwicka A. Scleroderma BAL fluid contains thrombin, a mediator of human lung fibroblast proliferation via induction of the PDGF- $\alpha$  receptor. *Am J Resp Cell Mol Biol* 1994; 10:405-12.
- [36] Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, Leask A, Abraham DJ. Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/ phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol Biol Cell* 2004;15:2707-19.
- [37] Shrivastava S, McVey JH, Dorling A. The interface between coagulation and immunity. *Am J Transplant*. 2007;7:499-506.
- [38] Sorbera LA, Bozzo J, Castaner J. Dabigatran/ Dabigatran etexilate. *Drugs Fut* 2005; 30(9):877.
- [39] Stangier J. Clinical pharmacokinetics and pharmacodynamics of the oral direct thrombin inhibitor dabigatran etexilate. *Clin Pharmacokinet* 2008;47:285-95.
- [40] Strange C, Bolster MB, Roth MD, Silver RM, Theodore A, Goldin J, Clements P, Chung J, Elashoff RM, Suh R, Smith EA, Furst DE, Tashkin DP; Scleroderma Lung Study Research Group. Bronchoalveolar lavage and response to cyclophosphamide in scleroderma interstitial lung disease. *Am J Respir Crit Care Med*. 2008;177(1):91-8.
- [41] Suzuki T, Moraes TJ, Vachon E, Ginzberg HH, Huang TT, Matthay MA, Hollenberg MD, Marshall J, McCulloch CA, Abreu MT, Chow CW, Downey GP. Proteinase-activated receptor-1 mediates elastase-induced apoptosis of human lung epithelial cells. *Am J Respir Cell Mol Biol*. 2005;33(3):231-47.
- [42] Tomasek JJ, Gabbiani G, Hinz B et al. Myofibroblasts and mechano-regulation of connective tissue remodeling. *Nature Rev Mol Cell Biol* 2002; 3:349-363.
- [43] Tourkina E, Hoffman S, Fenton JW 2nd, Lipsitz S, Silver RM, Ludwicka-Bradley A. Depletion of protein kinase C $\epsilon$  in normal and scleroderma lung fibroblasts has opposite effects on tenascin expression. *Arthritis Rheum* 2001;44:1370-81.
- [44] van Ryn J, Stangier J, Haertter S, Liesenfeld K-H, Wienen W, Feuring M, Clemens A. Dabigatran etexilate - a novel, reversible, oral direct thrombin inhibitor: Interpretation of coagulation assays and reversal of anticoagulant activity. *Thromb Haemost* 2010; 103(6):1116-27.
- [45] Vyalov SL, Gabbiani G, Kapanci Y: Rat alveolar myofibroblasts acquire -smooth muscle actin expression during bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1993; 143:1754-1765.
- [46] Walker GA, Guerrero IA, Leinwand LA. Myofibroblasts: molecular crossdressers. *Curr Top Dev Biol* 2001; 51:91-107.
- [47] Wienen W, Stassen JM, Pripke H, Ries UJ, Huel N. In-vitro profile and ex-vivo anticoagulant activity of the direct thrombin inhibitor dabigatran and its orally active prodrug, dabigatran etexilate. *Thromb Haemost* 2007;98:155-62.
- [48] Zhang H, Gharraee-Kermani M, Zhang K, Karmioli S, Phan S: Lung fibroblast  $\alpha$ -smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 1996; 148:527-37.
- [49] Zhang K, Rekhter MD, Gordon D et al. Myofibroblasts and their role in lung collagen gene expression during pulmonary fibrosis: A combined immunohistochemical and in situ hybridization. *Am J Pathol* 1994; 145:114-25.



## **Systemic Sclerosis - An Update on the Aberrant Immune System and Clinical Features**

Edited by Dr Timothy Radstake

ISBN 978-953-307-869-4

Hard cover, 206 pages

**Publisher** InTech

**Published online** 03, February, 2012

**Published in print edition** February, 2012

Systemic sclerosis (SSc), or often referred to as Scleroderma (tight skin), is characterized by an exaggerated formation of collagen fibers in the skin, which leads to fibrosis. Accumulating evidence now points toward three pathological hallmarks that are implicated in Ssc, the order of which has yet to be determined: endothelial dysfunction, autoantibody formation, and activation of fibroblasts. This current book provides up-to-date information on the pathogenesis and clinical features of this severe syndrome. It is our hope that this book will aid both clinicians and researchers in dealing with patients with this clinical syndrome. In addition, we hope to shed more light on this rare and severely disabling syndrome, ultimately leading to better research and successful therapeutic targeting.

### **How to reference**

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Galina S. Bogatkevich, Kristin B. Highland, Tanjina Akter, Paul J. Nietert, Ilia Atanelishvili, Joanne van Ryn and Richard M. Silver (2012). Inhibition of Thrombin as a Novel Strategy in the Treatment of Scleroderma-Associated Interstitial Lung Disease, Systemic Sclerosis - An Update on the Aberrant Immune System and Clinical Features, Dr Timothy Radstake (Ed.), ISBN: 978-953-307-869-4, InTech, Available from: <http://www.intechopen.com/books/systemic-sclerosis-an-update-on-the-aberrant-immune-system-and-clinical-features/inhibition-of-thrombin-as-a-novel-strategy-in-the-treatment-of-scleroderma-associated-interstitial-l>

**INTECH**  
open science | open minds

### **InTech Europe**

University Campus STeP Ri  
Slavka Krautzeka 83/A  
51000 Rijeka, Croatia  
Phone: +385 (51) 770 447  
Fax: +385 (51) 686 166  
[www.intechopen.com](http://www.intechopen.com)

### **InTech China**

Unit 405, Office Block, Hotel Equatorial Shanghai  
No.65, Yan An Road (West), Shanghai, 200040, China  
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

© 2012 The Author(s). Licensee IntechOpen. This is an open access article distributed under the terms of the [Creative Commons Attribution 3.0 License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.