ORIGINAL RESEARCH

A Novel Antifibrotic Mechanism of Nintedanib and Pirfenidone
Inhibition of Collagen Fibril Assembly

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Abstract

Idiopathic pulmonary fibrosis (IPF) is characterized by excessive deposition of extracellular matrix, in particular, collagens. Two IPF therapeutics, nintedanib and pirfenidone, decelerate lung function decline, but their underlying mechanisms of action are poorly understood. In this study, we sought to analyze their effects on collagen synthesis and maturation at important regulatory levels. Primary human fibroblasts from patients with IPF and healthy donors were treated with nintedanib (0.01–1.0 μM) or pirfenidone (100-1,000 μM) in the absence or presence of transforming growth factor-β1. Effects on collagen, fibronectin, FKBP10, and HSP47 expression, and collagen I and III secretion, were analyzed by quantitative polymerase chain reaction and Western blot. The appearance of collagen fibrils was monitored by scanning electron microscopy, and the kinetics of collagen fibril assembly was assessed using a light-scattering approach. In IPF fibroblasts, nintedanib reduced the expression of collagen I and V, fibronectin, and FKBP10 and attenuated the secretion of collagen I and III. Pirfenidone also down-regulated collagen V but otherwise showed fewer and less pronounced effects. By and large, the effects were similar in donor fibroblasts. For both drugs, electron microscopy of IPF fibroblast cultures revealed fewer and thinner collagen fibrils compared with untreated controls. Finally, both drugs dose-dependently delayed fibril formation of purified collagen I. In summary, both drugs act on important regulatory levels in collagen synthesis and processing. Nintedanib was more effective in down-regulating profibrotic gene expression and collagen secretion. Importantly, both drugs inhibited collagen I fibril formation and caused a reduction in and an altered appearance of collagen fibril bundles, representing a completely novel mechanism of action for both drugs.

Keywords: idiopathic pulmonary fibrosis; extracellular matrix; nintedanib; pirfenidone

Clinical Relevance

Accumulation of extracellular matrix, mainly collagen, is a main feature of idiopathic pulmonary fibrosis (IPF). Nintedanib and pirfenidone, two recently approved therapeutics for IPF, decelerate disease progression, but their antifibrotic mechanisms of action are incompletely understood. To the best of our knowledge, this study provides the first evidence for the inhibition of collagen fibril formation as a major mechanism of action for nintedanib and pirfenidone and puts forward extracellular collagen self-assembly as a druggable target in IPF.

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Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal interstitial lung disease with a median survival of 3–5 years after diagnosis (1). The underlying pathogenic processes are poorly understood, but the aberrant fibrotic response is likely initiated by repeated microinjuries to the airway and the alveolar epithelium (2). This leads to the secretion of fibrotic mediators, including transforming growth factor β (TGF-β), which results in the accumulation of myofibroblasts in alveolar regions. Multiple progenitor cells may contribute to the myofibroblast population, but the most well-established source is the interstitial fibroblast (3). Myofibroblasts synthesize and deposit excessive amounts of extracellular matrix (ECM) proteins, such as collagen type I, III, and V, and fibronectin (4). The resulting accumulation of ECM in the alveolar region is the ultimate pathological feature of lung fibrosis, leading to progressive lung function decline (5).

A recent study highlights that collagens are the main components of newly synthesized ECM in lung fibrosis (6), but large-scale quantitative proteome approaches have also demonstrated that the ECM composition (the matrisome) is far more complex than previously assumed (7). In addition, the complexity of collagen biosynthesis and maturation is rarely taken into account in mechanistic studies of the evaluation of antifibrotic strategies. Collagen modification and folding in the rough endoplasmic reticulum (rER) requires several enzymes and molecular chaperones essential for post-translational modifications (PTMs) and the processing of procollagen into triple helices, where one of the rate-limiting steps is the *cis-trans* isomerization of proline residues catalyzed by rER resident peptidyl prolyl isomerases (8). Collagen triple helix formation is followed by its secretion, extracellular fibril formation, and fiber assembly (9). Two endoplasmic reticulum proteins participating in this multistep process are the collagen chaperones FK506-binding protein 10 (FKBP10, also called FKBP65) and heat-shock protein 47 (HSP47, also called SerpinH1) (9). Notably, both FKBP10 and HSP47 are up-regulated in bleomycin-induced lung fibrosis and in IPF (10, 11). Secretion of procollagen from HSP47-deficient fibroblasts is reduced compared with control cells (12) and similarly, knockdown of FKBP10 in IPF fibroblasts decreases collagen type I synthesis and secretion (10). In addition, PTMs such as hydroxylation of lysyl or prolyl residues, or glycosylation of hydroxylysines, are essential for proper stability, assembly, and secretion of procollagen, as well as for the final supramolecular structure of these molecules (13). For example, hydroxylation of proline residues on position three (3-Hyp) might play a role in inter-triple-helical interactions and probably assists in the assembly of supramolecular collagen and lateral fibril growth (14, 15).

Nintedanib and pirfenidone were approved recently for IPF therapy, because both drugs have been shown to slow down disease progression as measured by reduced lung function decline. Despite their widespread application in IPF in recent times, their mechanisms of action are poorly understood and remain to be fully elucidated (16, 17). Nintedanib, originally developed as an anticancer drug, is a receptor tyrosine kinase inhibitor of platelet-derived growth factor receptor, fibroblasts growth factor receptor, and vascular endothelial growth factor receptor, all of which play important roles in the pathogenesis of IPF (5). Pirfenidone is an antifibrotic, antiinflammatory, and antioxidant compound with beneficial effects in lung, hepatic, kidney, and cardiac fibrosis, but its direct targets are unknown (18, 19, 20). Several studies have investigated the effects of either nintedanib or pirfenidone on collagen type I expression or secretion in several cell types (10, 21–26). No study to date, however, has directly compared both drugs on the multiple stages of intracellular collagen synthesis and extracellular maturation in the relevant cell type (i.e., the primary human lung fibroblast).

Therefore, the aim of our study was to comprehensively assess and directly compare the effects of nintedanib and pirfenidone on the different steps of collagen synthesis and maturation in primary human lung fibroblasts (phLF) from patients with IPF and healthy donors. We analyzed the expression of various collagens and the collagen chaperones *FKBP10* and *HSP47*, as well as collagen secretion in IPF and healthy phLF. In addition, we examined the effects of both drugs on the levels of selected PTMs of collagen in IPF fibroblasts and on collagen fibril formation.

Materials and Methods

For more details on materials and methods, *see* the online supplement. Statistical analysis was performed in GraphPadPrism 7.02 (GraphPad Software, San Diego, CA).

MTT Cytotoxicity Assay

See the online supplement.

Human Lung Material and Culture of phLF

Primary human lung fibroblasts, isolated from human lung explant material of patients with IPF or healthy donors, were obtained from the BioArchive CPC-M for lung diseases at the Comprehensive Pneumology Center (CPC, Munich, Germany). All participants gave written informed consent, and the study was approved by the local ethics committee of the Ludwig-Maximilians University of Munich, Germany. Isolation of phLF was performed as described previously (10). For more details, *see* the online supplement.

Cotreatment of IPF and Donor phLF with TGF-β1 and Nintedanib or Pirfenidone

Cells were seeded at a density of 20,000–25,000 cells/cm^2, followed by starvation for 24 hours in starvation medium (Dulbecco's modified Eagle medium/F12, 0.5% fetal bovine serum, penicillin/streptomycin, 0.1 mM 2-phospho-L-ascorbic acid). Subsequently, cells were treated with or without TGF-β1 (2 ng/ml) (R&D Systems, Minneapolis, MN) and with nintedanib (0.01 μM, 0.1 μM, 1.0 μM) or pirfenidone (100 μM, 500 μM, 1,000 μM) (both Selleck, Houston, TX) for 48 hours in starvation medium. Nintedanib and pirfenidone were dissolved in dimethyl sulfoxide. The final dimethyl sulfoxide concentration in the medium was always 1%.

RNA Isolation and Real-Time Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis

See the online supplement.

Protein Isolation and Western Blot Analysis

See the online supplement.

Quantification of Secreted Collagen

Collagen I and III were precipitated from cell culture supernatant of cultured IPF and donor phLF as described previously (10). For more details, *see* the online supplement.

Collagen Precipitation and Analysis of PTM

See the online supplement.

Scanning Electron Microscopy for Assessment of Fibrils in the ECM of phLF

IPF phLF were grown on glass slides, treated with nintedanib (1 μM) or pirfenidone (1,000 μM) in combination with TGF-β1 (2 ng/ml) for 48 hours and fixed with paraformaldehyde and glutaraldehyde, 3% each, in 0.1% sodium cacodylate buffer pH 7.4 (Electron Microscopy Sciences, Munich, Germany). The specimens were dehydrated in gradual ethanol and dried by the critical-point method, using CO_2 as the transitional fluid (Polaron Critical Point Dryer CPC E3000; Quorum Technologies, Ringmer, UK). Specimens were sputter coated with a thin layer of platinum by a sputtering device (Emitech K575; Quorum Technologies) and observed by scanning electron microscopy (JSM 6300F; JEOL, Eching, Germany). Fibril thickness was assessed by measuring the diameter of the smallest unit of fiber forming fibrils, using the length measurement tool of the open source software ImageJ 1.50i (W.S. Rasband, National Institutes of Health, Bethesda, MD).

Collagen I Fibril Formation Assay

This assay was performed essentially as described previously (27). For more details, *see* the online supplement.

Results

Applied Concentrations of Nintedanib and Pirfenidone Were Well Tolerated by IPF phLF

For *in vitro* experiments, we selected a range of nintedanib and pirfenidone concentrations similar to those used in published studies (22, 23, 25). Notably, for pirfenidone, in an effort to adhere to physiologically relevant concentrations, we used 1,000 μM as the highest concentration, although others have used pirfenidone in concentrations of up to 10 mM in similar experiments (21, 26, 28). Initially, we analyzed the effect of increasing doses of nintedanib (0.01–1 μM) and pirfenidone (100-1,000 μM) on the viability of IPF fibroblasts in an MTT assay. The used concentrations of nintedanib and pirfenidone were well tolerated for the treatment period of 48 hours (*see* Figure E1 in the online supplement).

Nintedanib Reduced the Expression and Secretion of ECM Components More Effectively Than Did Pirfenidone in phLF

Next, we assessed the effect of different concentrations of both drugs on the expression and secretion of collagen I (*COL1A1*), III (*COL3A1*), V (*COL5A1*), fibronectin 1 (*FN1*), and plasminogen activator inhibitor 1 (*PAI-1*) in lysates and cell culture supernatants from primary human IPF and donor fibroblasts. Nintedanib consistently down-regulated transcript and protein levels of basal and TGF-β1–induced collagen I in IPF phLF (Figures 1A, 1C, 1E, and 1G). Pirfenidone reduced TGF-β1–induced *COL1A1* transcripts only marginally (Figures 1A and 1C), and collagen I protein remained largely unchanged (Figures 1E and 1G). Similar tendencies were observed in phLF isolated from healthy donor lungs (Figures 1B, 1D, and 1F), with the exception of *COL1A1* transcript (Figure 1B) and levels of basal secreted collagen I (Figure 1H), which both, in contrast to IPF, appeared unaffected by nintedanib in donor fibroblasts.

Similarly, the expression of collagen III was consistently down-regulated by nintedanib in IPF fibroblasts on transcript and protein levels, whereas pirfenidone merely regulated *COL3A1* transcripts, an effect which again did not translate to protein level (Figures 2A, 2C, and 2E). Interestingly, in donor fibroblasts, nintedanib increased and pirfenidone decreased *COL3A1* transcription (Figures 2B and 2D), whereas the amount of secreted collagen III remained largely unchanged or even tended to anticorrelate with transcript levels (Figure 2F).

As for collagen V, TGF-β1–induced collagen V was significantly reduced in IPF fibroblasts by both drugs, an effect that was captured only partly at the transcript level (Figures 3A, 3C, and 3E); similar trends were observed in donor phLF (Figures 3B, 3D, and 3F).

Expression of *FN1* was consistently decreased by nintedanib on transcript and protein levels, in both IPF and donor fibroblasts (Figure E2). In contrast, pirfenidone reduced *FN1* mRNA, but not protein levels, in IPF fibroblasts (Figures E2A, E2C, and E2E) and had no significant effect in donor phLF (Figures E2B, E2D, and E2F). Interestingly, pirfenidone actually showed a trend to increase FN1 protein levels in both IPF and donor phLF (Figures E2E and E2F). Expression of *PAI-1*, a classic TGF-β–induced gene, was reduced significantly by nintedanib in IPF and donor fibroblasts, with a more pronounced effect in the absence of TGF-β1 (Figure E3). Pirfenidone did not affect *PAI-1* expression.

In summary, in comparison with nintedanib, pirfenidone showed fewer effects on collagen expression and secretion and *FN1* and *PAI-1* expression. Notably, exceeding the effective concentration of nintedanib at by least 500 times, a concentration of 500 to 1,000 μM pirfenidone was necessary to achieve significant effects (e.g., on collagen I, III, and V and *FN1* expression) in TGF-β1–treated IPF fibroblasts (Figures 1A, 2A, 3E, and E2A).

Nintedanib, but Not Pirfenidone, Reduces the Expression of the Collagen Chaperone FKBP10 in IPF Fibroblasts

Next, we investigated the effects of nintedanib and pirfenidone on the expression of *FKBP10* and *HSP47*, two collagen I chaperones. Nintedanib moderately, but significantly, down-regulated protein levels of FKBP10 in mock- as well as in TGF-β1–treated IPF fibroblasts (Figure 4E). This effect was already visible on the transcript level for TGF-β–treated samples (Figures 4A and 4C). In contrast, pirfenidone did not influence *FKBP10* expression in IPF fibroblasts (Figures 4A, 4C, and 4E). Interestingly, clearly different results were obtained with donor fibroblasts here, where nintedanib failed to regulate *FKBP10* expression, and pirfenidone down-regulated *FKBP10* transcript but up-regulated FKBP10 protein levels (Figures 4B, 4D, and 4F). Regarding the expression of the major collagen I chaperone HSP47, neither drug reduced HSP47 protein levels in phLF (Figures 5E and 5F), even if both therapeutics decreased basal *HSP47* mRNA (Figures 5A–5D).

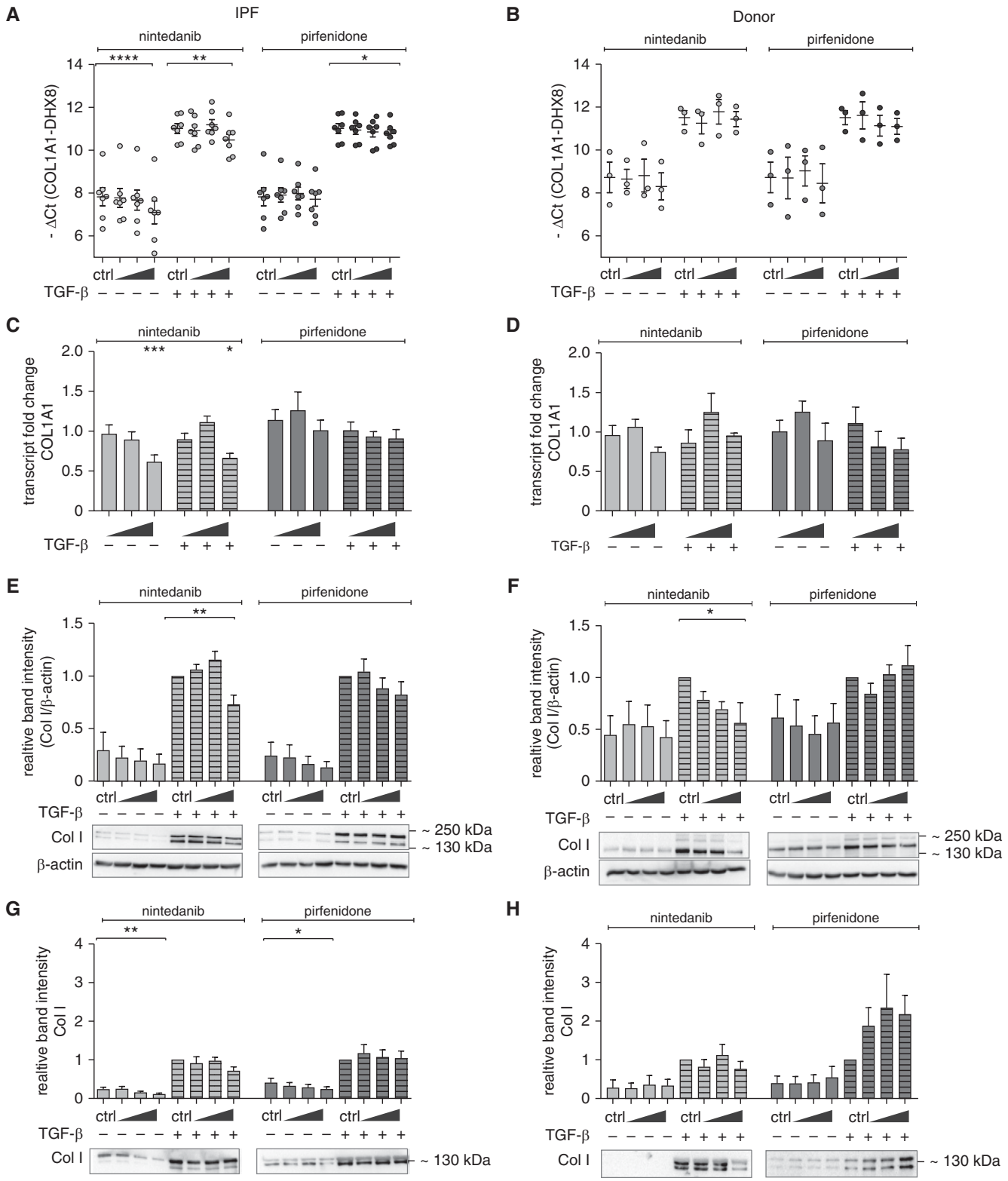

Figure 1. Nintedanib decreases collagen I expression and secretion more potently than does pirfenidone in idiopathic pulmonary fibrosis (IPF) fibroblasts. (*A–D*) Quantitative RT-PCR analysis of primary human lung fibroblasts (phLF) isolated from (*A* and *C*) patients with IPF or (*B* and *D*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of transforming growth factor β (TGF-β) 1 (2 ng/ml). Transcript levels of *COL1A1* are shown as −ΔCt values (*A* and *B*) as well as transcript fold changes (*C* and *D*) to show the effect normalized to control (ctrl). DEAH (Asp-Glu-Ala-His) box polypeptide 8 (*DHX8*) was used as endogenous control. Data are based on

Selected Post-Translational Modifications of Collagen I Are Not Affected by Nintedanib or Pirfenidone

PTMs have a major impact on essential collagen properties such as the three-dimensional structure, thermodynamic stability, and biological functions (13, 14, 15), but, to date, whether antifibrotic drugs affect the PTMs of collagen secreted by IPF fibroblasts has not been assessed. Here, we tested whether nintedanib and pirfenidone affected the levels of selected PTMs of collagen I. More specifically, we compared the levels of prolyl-3-hydroxylation of the A1 site (Pro-986) and the A3 site (Pro-707) of the collagen α1 chain (15), prolyl-3-hydroxylation of the A3 site (Pro-707) of the collagen α2 chain and, finally, the glycosylation site of hydroxylysine (Lys-174) of collagen type I. Neither drug appeared to affect the levels of the assessed PTMs (Figure E4).

Nintedanib and Pirfenidone Affect Collagen Fibril Formation in IPF Fibroblasts

Scanning electron microscopy was used to assess the number, morphology, and thickness of extracellular fibrils formed in cultures of TGF-β1–treated IPF fibroblasts in the absence and presence of nintedanib or pirfenidone. Extracellular collagen fibers were identified as unbranched and dense bundles of thread-like–looking twisted fibrils of variable length, which were oriented randomly, and with a maximal diameter of 1 μm (Figure 6A) (29, 31). Cell cultures that had been treated with 1.0 μM nintedanib or 1.0 mM pirfenidone displayed a markedly reduced number of fibers and changes in overall fiber structure. In the presence of both drugs, fibers were shorter overall and showed a more frayed appearance than in control samples, and the fibril thickness was significantly reduced in nintedanib- and pirfenidone-treated samples compared with control fibrils (Figure 6B).

Nintedanib and Pirfenidone Inhibit Spontaneous Collagen I Fibril Formation

It seemed unlikely that the effects on collagen fibril formation observed by scanning electron microscopy could be accounted for by the effects of both drugs on collagen synthesis and secretion only, in particular in the case of pirfenidone. Therefore, we investigated the direct effect of both drugs on spontaneous collagen I fibril formation in a light-scattering approach. This assay is a well- established method of studying the formation of collagen fibrils in a solution of purified soluble collagen I. It relies on the principle that collagen I, dissolved initially in dilute acid, spontaneously forms fibrils on neutralization in a self-driven process. The resulting fibrils are similar to those formed *in vivo*, and the process can be visualized by dynamic light scattering at 313 nm (30, 31). We found that both therapeutics were able to considerably delay fibril formation of purified collagen I already at micromolar concentrations in a dose-dependent manner (Figure 7 and Table 1).

Discussion

In this study, we demonstrated that nintedanib and pirfenidone affect collagen synthesis and maturation on several regulatory levels, including the inhibition of collagen gene expression, collagen secretion, and, most importantly, fibril formation. In terms of intracellular regulation of the synthesis of ECM components and collagen secretion, nintedanib was clearly more effective, because it (*1*) exerted its effects at substantially lower concentrations (up to 1,000-fold) than did pirfenidone, (*2*) affected the expression and secretion of more ECM and ECM-related genes (i.e., fibronectin, FKBP10, and collagen I), and (*3*) showed more consistent effects on transcript and protein levels. With few exceptions, these effects were mostly similar in IPF and donor fibroblasts. Importantly, both drugs strongly inhibited extracellular fibril formation, and assessment of spontaneous fibril assembly using purified collagen I indicated that both drugs directly inhibited this process with comparable efficiency.

Both nintedanib and pirfenidone inhibited TGF-β–induced transcription of *COL1A1*, *COL3A1*, and *FN1*. In agreement, previous reports by others have shown that nintedanib and pirfenidone counteract TGF-β signaling and down-regulate these TGF-β target genes (23, 24, 32). Although pirfenidone showed these effects only in the presence of exogenously added TGF-β1, nintedanib also affected basal levels of *COL1A1* in IPF fibroblasts and basal levels of another TGF-β target gene, *PAI-1*, in both IPF and donor fibroblasts (Figures 1A and E3). The platelet-derived growth factor receptor (PDGFR) and the fibroblast growth factor receptor are known targets of the receptor tyrosine kinase inhibitor nintedanib, and this might reflect the inhibition of autocrine platelet-derived growth factor or fibroblast growth factor signaling, which has been shown to regulate collagen gene expression (5, 33, 34) via both the ERK and the PI3K/Akt signaling pathways (35). In agreement, phosphorylation of Akt was decreased in

Figure 1. (Continued). seven (IPF) or three (donor) completely independent experiments and are presented as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks* for −ΔCt values and by *asterisks* only for fold changes relative to 1. (*E* and *F*) Western blot analysis of phLF isolated from (*E*) patients with IPF or (*F*) donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Densitometric analysis and representative blots show the effect of nintedanib and pirfenidone on collagen I (Col I) protein expression relative to β-actin as loading control. Data are based on eight (IPF) or three (donor) completely independent experiments and are presented as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks*. (*G* and *H*) Western blot analysis of secreted Col I precipitated from cell culture supernatant of (*G*) IPF or (*H*) donor fibroblasts treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Densitometric analysis and representative blots show the effects of nintedanib and pirfenidone on secreted Col I after 48 hours. Data are based on seven (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks*. Statistical analysis was performed by one-way analysis of variance (post-test, Bonferroni's multiple comparison test: comparison against control). $^{*}P < 0.1$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. The well-known effect of TGF-β1 on these transcripts and proteins was significant, but it is not specified in the interest of clarity.

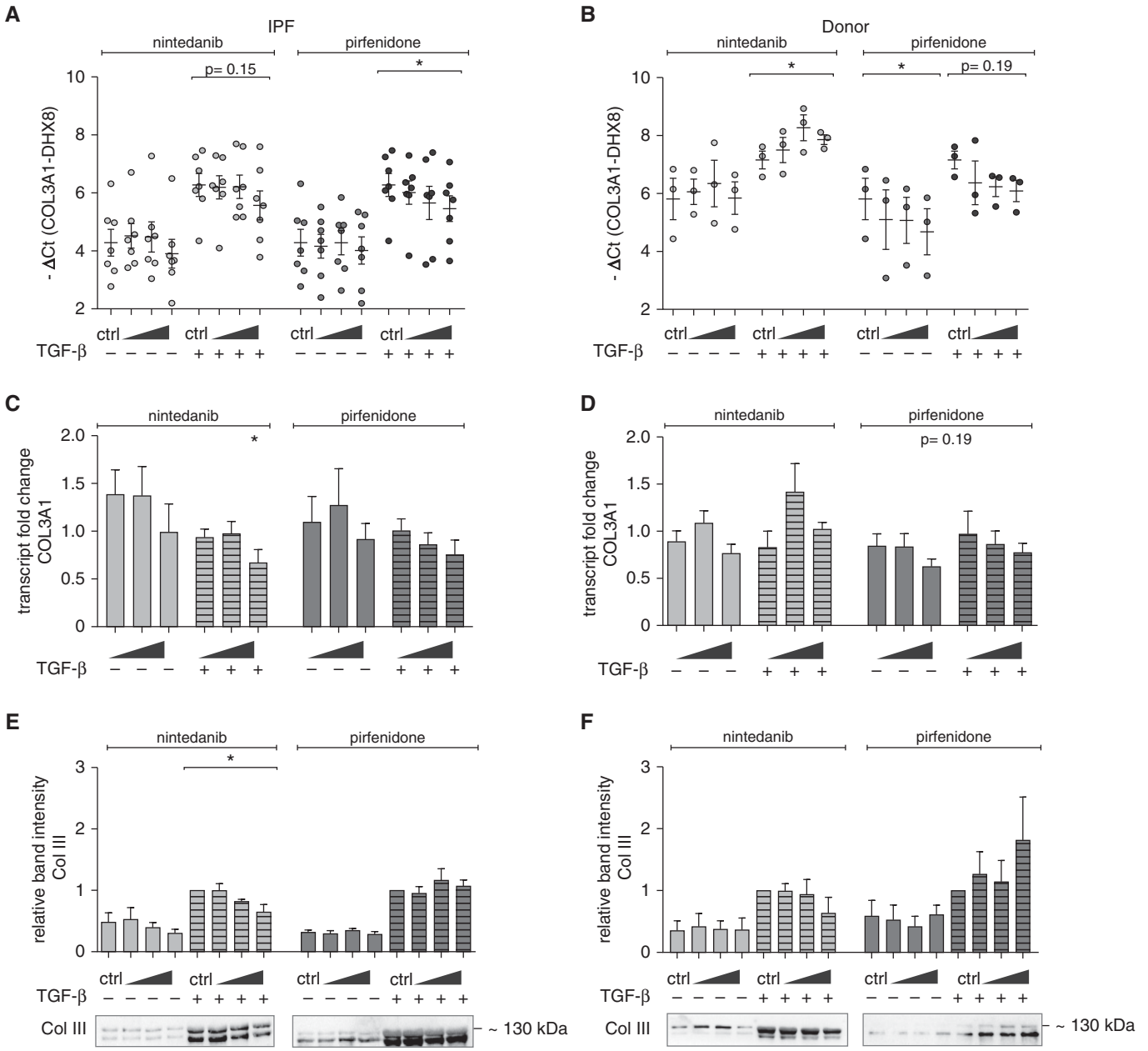

Figure 2. *COL3A1* transcription is decreased by nintedanib in IPF fibroblasts and increased in donor fibroblasts, whereas collagen III (Col III) secretion is decreased in both. (*A–D*) Quantitative RT-PCR analysis of phLF isolated from (*A* and *C*) patients with IPF or (*B* and *D*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Transcript levels of *COL3A1* are shown as −ΔCt values (*A* and *B*) as well as transcript fold changes (*C* and *D*) to show the effect normalized to control. *DHX8* was used as endogenous control. Data are based on seven (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks* for −ΔCt values and by *asterisks* only for fold changes relative to 1. The well-known effect of TGF-β1 on these transcripts was significant, but it is not specified in the interest of clarity. (*E* and *F*) Western blot analysis of secreted Col III precipitated from cell culture supernatant of (*E*) IPF or (*F*) donor fibroblasts after treatment of phLF isolated from patients with IPF treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Densitometric analysis and representative blots show the effects of nintedanib and pirfenidone on secreted Col III after 48 hours. Data are based on seven (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks*. Statistical analysis was performed by one-way analysis of variance (posttest, Bonferroni's multiple comparison test: comparison against control). *$P < 0.1$.

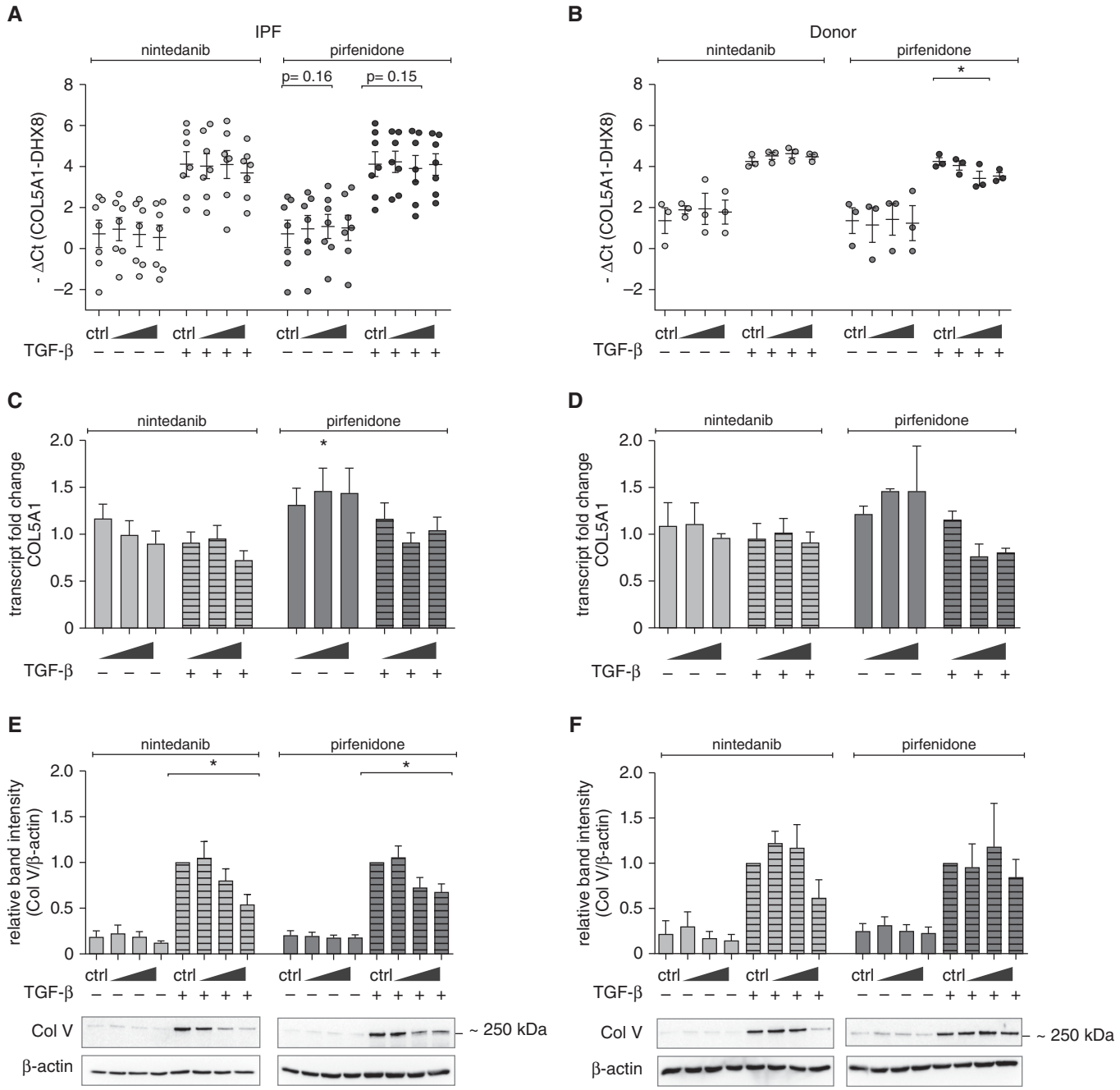

Figure 3. Nintedanib and pirfenidone down-regulate collagen V (Col V). (*A–D*) Quantitative RT-PCR analysis of phLF isolated from (*A* and *C*) patients with IPF or (*B* and *D*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Transcript levels of (*A* and *C*) IPF and (*B* and *D*) donor phLF of *COL5A1* are shown as −ΔCt values (*A* and *B*) as well as transcript fold changes (*C* and *D*) to show the effect normalized to control (ctrl). *DHX8* was used as endogenous control. Data are based on seven (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks* for −ΔCt values and by *asterisks* only for fold changes relative to 1. (*E* and *F*) Western blot analysis of phLF isolated from (*E*) patients with IPF or (*F*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Densitometric analysis and representative blots show the effect of nintedanib and pirfenidone on Col V protein expression relative to β-actin. Data are based on eight (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks*. Statistical analysis was performed by one-way analysis of variance (posttest, Bonferroni's multiple comparison test: comparison against control). *$P < 0.1$. The well-known effect of TGF-β1 on these transcripts and proteins was significant, but it is not specified in the interest of clarity.

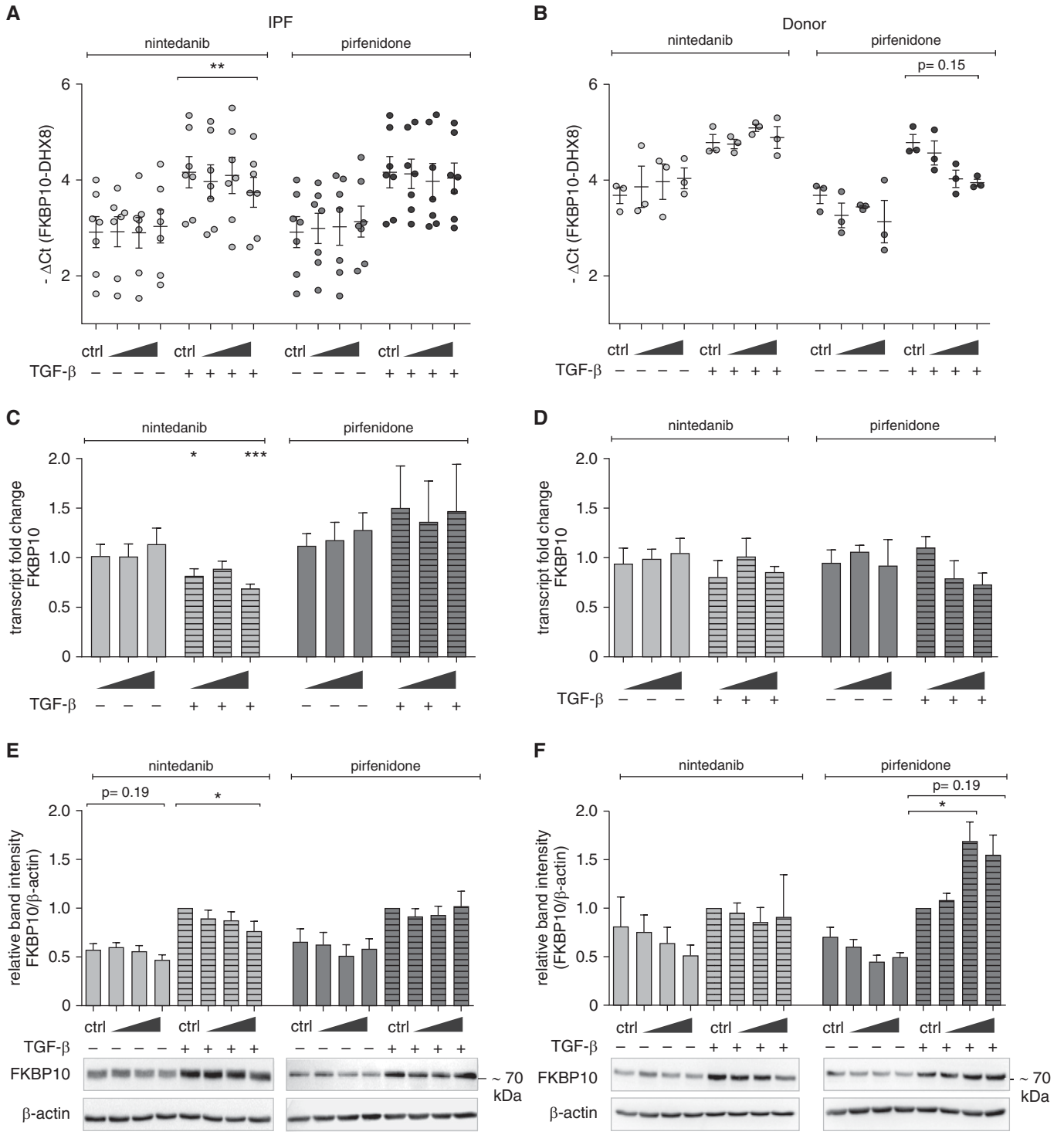

Figure 4. Expression of the collagen chaperone FKBP10 is consistently down-regulated by nintedanib in IPF but regulated more dynamically by pirfenidone in donor fibroblasts. (*A–D*) Quantitative RT-PCR analysis of phLF isolated from (*A* and *C*) patients with IPF or (*B* and *D*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Transcript levels of (*A* and *C*) IPF and (*B* and *D*) donor phLF of *FKBP10* are shown as −ΔCt values (*A* and *B*) as well as transcript fold changes (*C* and *D*) to show the effect normalized to control. *DHX8* was used as endogenous control. Data are based on seven (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks* for −ΔCt values and by *asterisks* only for fold changes relative to 1. (*E* and *F*) Western blot analysis of phLF isolated from (*E*) patients with IPF or (*F*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1

response to nintedanib in all our experiments (Figures E5A and E5B). In contrast, phosphorylation of ERK was not consistently changed, either in IPF or in donor fibroblasts (Figures E5C and E5D). In light of the time point studied (48 h after treatment start), this argues for a stronger and more sustained inhibition of the PI3K/Akt signaling pathway by nintedanib in our studies. Inhibition of PDGFR signaling, however, cannot explain all our *in vitro* results, because a previous study from our laboratory (34) found that small interfering RNA–mediated down-regulation of PDGFR-α actually increased levels of collagen V drastically in primary human lung fibroblasts, which is in contrast to what we observed in the presence of nintedanib.

It is striking that many of the observed effects translated to the protein level only in the presence of nintedanib, but not pirfenidone. For instance, both drugs inhibit TGF-β–induced *COL1A1*, *COL3A1*, and *FN1* transcription, whereas the levels of collagen I protein, secreted collagen III, and fibronectin protein were reduced only by nintedanib in IPF fibroblasts (Figures 1, 2, and E2). These results suggest that post-transcriptional regulation mechanisms are affected differently by the drugs and highlight the importance of analysis at the protein level in this context. Nevertheless, other studies have reported effects of pirfenidone on collagen I and/or fibronectin protein levels in normal phLF (26), alveolar epithelial cells (21), and nasal polyp fibroblasts (28). These discrepancies, however, may be the result of the use of substantially higher concentrations of pirfenidone in those studies (1.6–10 mM). Notably, during standard treatment with pirfenidone of patients with IPF (three daily doses of 801 mg pirfenidone), serum levels of pirfenidone do not exceed 100 μM (36), a concentration at which we did not observe any effect on phLF gene expression.

Both nintedanib and pirfenidone significantly down-regulated collagen V in IPF fibroblasts, and a similar trend was observed in donor fibroblasts (Figures 3E and 3F). To our knowledge, down-regulation of collagen V levels in response to nintedanib or pirfenidone has not been reported before. Type V collagen is a minor component of collagen type I fibrils that plays an important role in fibrogenesis and regulation of fiber size (37–39). In IPF lungs, collagen V is heavily overexpressed compared with that in normal lungs (38). Importantly, in the context of the observed effects on fibril thickness (Figure 6B), collagen V has been shown to be crucial for the initiation of collagen fibril assembly (39). Therefore, down-regulation of collagen V by both drugs likely contributes to the phenomenon of fewer and thinner fibrils in the extracellular space of primary human IPF fibroblasts described here (Figure 6). Interestingly, Hostettler and colleagues (25) found that matrix metalloprotease 2, an ECM metalloprotease that cleaves collagen V, is up-regulated, and its inhibitor, metalloproteinase inhibitor 2 (TIMP2), is down-regulated in response to nintedanib. This provides indirect evidence for increased extracellular degradation of collagen V, and, collectively, this suggests that nintedanib may decrease collagen V via both an intracellular and an extracellular mechanism.

To date, few studies have assessed the effects of nintedanib and pirfenidone on collagen secretion. Previously, using Sirius Red–based quantification of total collagen in cell culture supernatant, we showed that nintedanib, but not pirfenidone, dose dependently inhibited collagen secretion in IPF fibroblasts (10). Similarly, Hostettler and colleagues reported a reduction of total secreted collagens in IPF and control fibroblasts on nintedanib treatment (25). Because collagen I and III are the most abundant fibrillar collagens in the lung interstitium and both are known to be increased in IPF (40), we further characterized the inhibition of collagen secretion by both drugs, looking at these two specific collagen subtypes in this study. Clearly, nintedanib was more effective in inhibiting basal collagen I and TGF-β–induced collagen III secretion. Pirfenidone showed only weak inhibitory effects on basal collagen I secretion (Figure 1G), and, notably, no significant effects on collagen III secretion (Figure 2E). Given the observed inhibitory effect of nintedanib on total collagen secretion in our previous study (10), this suggests that nintedanib-induced down-regulation of collagen III secretion contributes more strongly to the decrease of total secreted collagen than does collagen I in IPF fibroblasts.

FKBP10 and HSP47 are rER-resident chaperones, critical for the proper folding of triple-helical procollagen (9, 12). Deficiency of both proteins leads to changes in the extracellular appearance of collagen fibrils as, for example, reduced collagen crosslinking (41, 42) or aberrant fibril formation (12). Both collagen chaperones are increased in animal models of bleomycin-induced lung fibrosis and in patients with IPF (7, 10, 11), and we showed recently that small interfering RNA–mediated down-regulation of FKBP10 attenuates the expression and secretion of collagen in pHLF (10). Interestingly, it had been reported in two independent studies that pirfenidone down-regulated the expression of HSP47 in A549 cells and human lung fibroblasts, which suggested that pirfenidone exerted its antifibrotic effects in part via the inhibition of intracellular collagen folding (21, 26). Therefore, we also assessed the regulation of HSP47 and FKBP10 by nintedanib and pirfenidone in IPF and donor fibroblasts. Notably, nintedanib marginally, but significantly, down-regulated TGF-β–induced FKBP10 expression on transcript and protein levels in IPF fibroblasts, whereas pirfenidone had no effect (Figures 4A, 4C, and 4E). Strikingly, a different pattern was observed here for donor fibroblasts: FKBP10 expression remained unaffected by nintedanib, but decreased at the transcript and increased at the protein level by pirfenidone (Figures 4B, 4D, and 4F). Regarding HSP47 expression, neither drug had an effect on

Figure 4. (Continued). (2 ng/ml). Densitometric analysis and representative blots show the effect of nintedanib and pirfenidone on FKBP10 protein expression relative to β-actin. Data are based on eight (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks*. Statistical analysis was performed by one-way analysis of variance (posttest, Bonferroni's multiple comparison test: comparison against control). $^{*}P < 0.1$, $^{**}P < 0.01$, $^{***}P < 0.001$. The well-known effect of TGF-β1 on these transcripts and proteins was significant, but it is not specified in the interest of clarity.

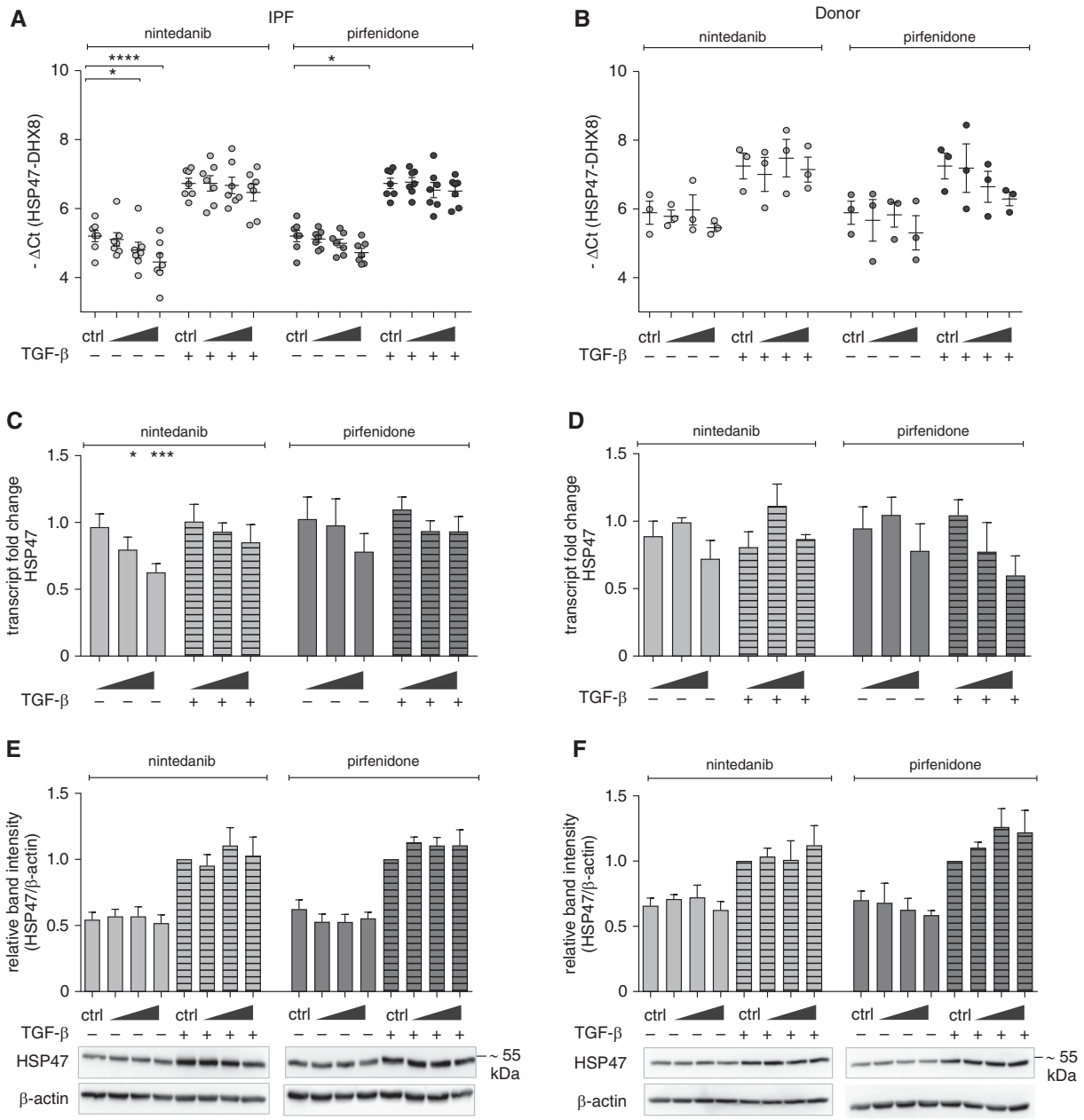

Figure 5. Expression of the major collagen I chaperone HSP47 is reduced only on the transcript level by both drugs in IPF fibroblasts. (*A–D*) Quantitative RT-PCR analysis of phLF isolated from (*A* and *C*) patients with IPF or (*B* and *D*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) in the absence or presence of TGF-β1 (2 ng/ml). Transcript levels of (*A* and *C*) IPF and (*B* and *D*) donor phLF of *HSP47* are shown as −ΔCt values (*A* and *B*) as well as transcript fold changes (*C* and *D*) to show the effect normalized to control. *DHX8* was used as endogenous control. Data are based on seven (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks* for −ΔCt values and by *asterisks* only for fold changes relative to 1. (*E* and *F*) Western blot analysis of phLF isolated from (*E*) patients with IPF or (*F*) healthy donors treated for 48 hours with increasing concentrations of nintedanib (0.01, 0.1, and 1 μM) or pirfenidone (100, 500, and 1,000 μM) cotreated with or without TGF-β1 (2 ng/ml). Densitometric analysis and representative blots show the effect of nintedanib and pirfenidone on FKBP10 protein expression relative to β-actin. Data are based on eight (IPF) or three (donor) completely independent experiments and are given as mean ± SEM. Statistical significance between control and different concentrations of nintedanib or pirfenidone is indicated by *horizontal brackets* and *asterisks*. Statistical analysis was performed by one-way analysis of variance (posttest, Bonferroni's multiple comparison test: comparison against control). $^{*}P < 0.1$, $^{***}P < 0.001$, $^{****}P < 0.0001$. The well-known effect of TGF-β1 on these transcripts and proteins was significant, but it is not specified in the interest of clarity.

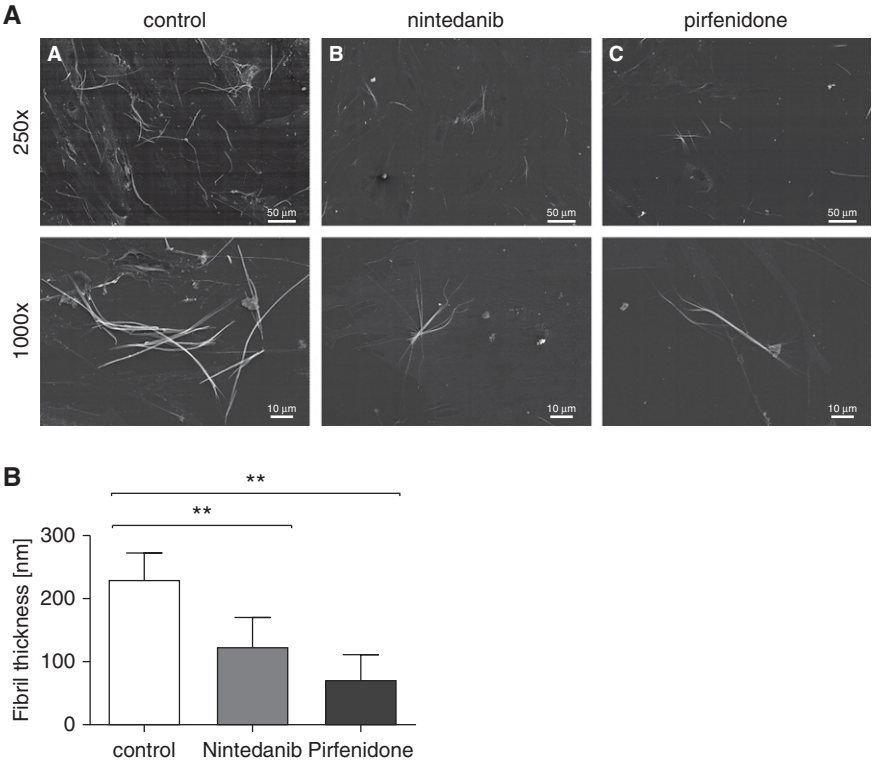

Figure 6. Nintedanib and pirfenidone decrease the number and thickness of collagen fibrils and alter their appearance. (*A*) Scanning electron microscopy of extracellular matrix fibrils deposited by phLF treated for 48 hours with nintedanib (1 μM, *middle panel*) or pirfenidone (1,000 μM, *right panel*) and TGF-β (2 ng/ml) showed fewer, thinner, and more frayed fiber bundles when compared with control (*left panel*). Magnification is indicated on the *left side* (*upper panel*: 250 × ; *lower panel*: 1000 ×). Results shown are representative images of three independent experiments with similar results. (*B*) Thickness of single fibrils was measured in scanning electron microscopy pictures in three experiments using independently derived IPF fibroblasts. Statistical analysis was performed by a paired *t* test. *Scale bars*, 50 μm in top panels of *A–C*, and 10 μm in bottom panels of *A–C*. $^{**}P < 0.01$.

protein levels (Figures 5E and 5F), even if both drugs showed effects on the transcript level (Figures 5A and 5C). Again, this is in contrast to previously reported results, but may be due to higher pirfenidone concentrations used in those studies (21, 26).

Importantly, we found much fewer, thinner, and aberrantly structured collagen fibrils in the extracellular space of IPF fibroblasts treated with nintedanib or pirfenidone (Figure 6). This was particularly unexpected for pirfenidone, because we had observed only minor effects on collagen synthesis and secretion. As pointed out earlier in the text, a partial explanation for this result may be the observed down-regulation of type V collagen, which, even if it represents a minor constituent of collagen fibrils, appears to be crucial for collagen fibrogenesis (39). Because deficiency of 3-Hyp has been shown to have major effects on lateral fibril growth (15), we also assessed hydroxylation of three 3-Hyp sites in collagen I, a comparatively rare collagen PTM, next to a lysyl glycosylation site in collagen I. We did not observe any effect of nintedanib or pirfenidone on these PTMs of collagen I (Figure E4). However, there are clearly many more collagen PTMs to consider and, in light of the recently reported profibrotic properties of ECM of patients with IPF (43), a broader PTM fingerprinting of collagens in the context of fibrotic disease would undoubtedly be warranted.

Extracellular collagen fibril formation is mainly an entropy-driven self-assembly process (31). The so-called collagen D-stagger is formed by specific interactions of the residues along the triple-helical molecules with regularly staggered ends. After cleavage of the propeptides, the collagen molecules become competent for fibril formation. We took advantage of the fact that this process can be studied in a straightforward manner using purified pepsin-digested collagen (27) and found that low micromolar concentrations of both drugs inhibited collagen I fibril formation with comparable efficiencies in a dose-dependent manner (Figure 7). Even if the exact molecular mechanisms remain obscure, it can be speculated that pirfenidone and nintedanib directly bind to collagen triple helices and mask or alter interaction sites caused by changes in hydrophobicity or charges on the surface of the triple helix. Moreover, considering that higher concentrations of pirfenidone than of nintedanib were necessary to achieve the same amplitude of effect, nintedanib likely displays a stronger affinity to collagen than does pirfenidone. Finally, because pepsin-digested solubilized collagen is used in our cell-free system, it can be concluded that the direct drug–collagen interaction takes place in the collagenous region of collagen and not in the telopeptides and propeptides, which are typically removed by pepsin digestion.

Interestingly, inhibition of collagen I self-assembly has been proposed as a strategy for antifibrotic therapy, but this concept has received little attention in the field of lung fibrosis thus far (44). Instead, efforts have been undertaken to evaluate the inhibition of collagen crosslinking by the enzyme lysyl oxidase-like 2, notably a step subsequent to spontaneous fibril formation that stabilizes existing fibrils (31, 45). Recently, however, a phase II study with a monoclonal anti–lysyl oxidase-like 2 antibody was terminated because of a lack of efficacy (46). Here we show, to our knowledge for the first time, that nintedanib and pirfenidone down-regulate collagen V, a minor collagen important for the initiation of extracellular fibrillogenesis, and directly inhibit collagen fibril formation. This suggests that both therapeutics exert their antifibrotic actions at least in part via the inhibition of collagen fibril formation, which provides additional support for the concept of inhibition of collagen self-assembly as a promising antifibrotic strategy. This is a particularly interesting finding for pirfenidone, in which the well-known antifibrotic effects *in vivo* to date stay in

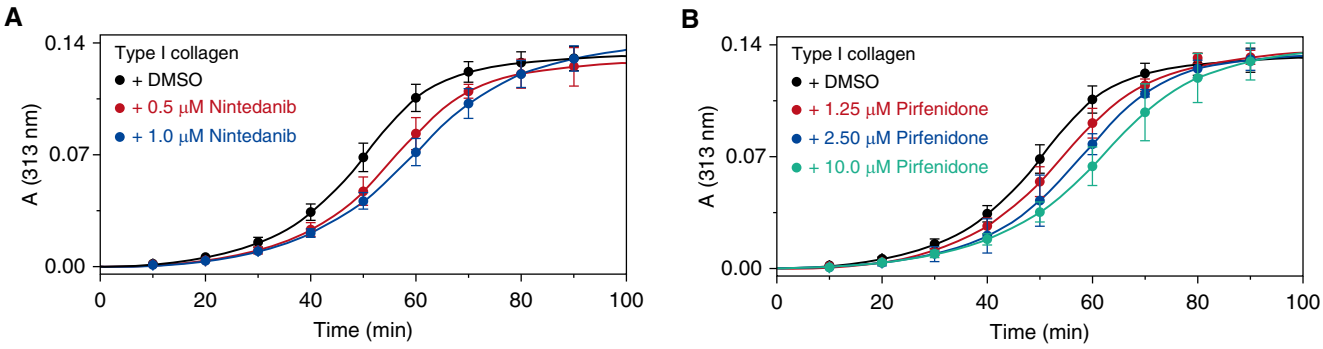

Figure 7. Spontaneous collagen fibril formation is inhibited by both drugs in a dose-dependent manner. A collagen type I stock solution in 50 mM acetic acid was diluted to a final concentration of 0.1 μM into a 0.1 M $NaHCO_3$ buffer (pH 7.8) containing 0.15 M NaCl and 1 mM $CaCl_2$ and heated up to 34°C, followed by monitoring of absorbance (light scattering) at 313 nm. (*A*) Nintedanib (*red*, 0.5 μM [$n = 2$]; *blue*, 1.0 μM [$n = 5$]) and (*B*) pirfenidone (*red*, 1.25 μM [$n = 3$]; *blue*, 2.50 μM [$n = 2$]; *green*, 10.0 μM [$n = 3$]) on collagen type I fibril formation in comparison to dimethyl sulfoxide control (*black* [$n = 4$]) are shown. The resulting half-time values for fibril formation are given in Table 1.

sharp contrast to concentrations in the millimolar range required to observe effects on fibrotic marker expression (19–21, 26, 36). Notably, we believe our study is the first to offer evidence for an antifibrotic effect of pirfenidone *in vitro*, which occurs at a micromolar concentration. For nintedanib, which affects the intracellular collagen pathway more strongly and in physiologically relevant concentrations, the reduced fibril formation is probably a combined result of intracellular and extracellular events. Our findings furthermore emphasize that collagen V plays a hitherto underestimated role in fibrogenesis.

We observed only few differences between IPF and donor fibroblasts regarding the effects of nintedanib and pirfenidone on the expression of the studied targets. The most striking difference was observed in the regulation of the collagen chaperone FKBP10, the expression of which was consistently down-regulated by nintedanib in IPF fibroblasts without evidence for down-regulation in donor fibroblasts. Even more surprisingly, pirfenidone, which did not affect FKBP10 expression in IPF fibroblasts, decreased FKBP10 transcript and increased FKBP10 protein levels in donor fibroblasts. Another difference was that nintedanib had a stronger negative effect on *COL1A1*, *COL3A1*, and *HSP47* transcription in IPF fibroblasts than in donor fibroblasts; at the same time, however, protein levels were unchanged or regulated similarly. Finally, both drugs down-regulated basal collagen I secretion only in IPF fibroblasts and not in donor fibroblasts. Apart from these differences, by and large, the tendencies of the effects of nintedanib and pirfenidone between IPF and donor fibroblasts on fibrotic markers, collagen chaperones, and collagen secretion were similar. Notably, in a previous study, we also observed that the effects on collagen synthesis and secretion were similar in IPF and normal control fibroblasts, and we ultimately pooled those results for data presentation (10). This is also in agreement with the findings of two previously published studies (25, 47). Lehtonen and colleagues (47) examined the effect of nintedanib and pirfenidone on fibroblast and myofibroblast properties and also observed only marginal differences between cells from control and IPF lungs. Hostettler and colleagues (25) studied collagen secretion by nintedanib in phLF and IPF fibroblasts and found that collagen secretion was down-regulated to the same extent in both cell types.

In conclusion, our findings provide an overview and a direct comparison of the effects of the Food and Drug Administration/European Medicines Agency-approved IPF drugs nintedanib and pirfenidone on different stages of expression and maturation of collagen in primary human lung fibroblast derived from patients with IPF as well as from healthy donors. Nintedanib clearly was more efficient than pirfenidone in inhibiting profibrotic gene expression and collagen secretion, both in terms of the required effective concentration as well as in the number, consistency, and

Table 1. Half-Time Values for Fibril Formation (Fibril $Formation_{50}$)

Concentration of Indicated Drug (*μM*)	Fibril $Formation_{50}$ (*min*)	*P* Value*
0 (DMSO control)	49.6 ± 2.1	
Nintedanib		
0.5	55.6 ± 2.1	n.d.
1.0	59.1 ± 2.0	0.000218†
Pirfenidone		
1.25	54.2 ± 4.7	0.133
2.5	57.3 ± 4.6	n.d.
10	61.6 ± 3.1	0.00165‡

Definition of abbreviation: n.d., not determined ($n = 2$).
Nintedanib and pirfenidone increase half-time values for fibril formation (fibril $formation_{50}$) dose dependently. Half-time values for fibril formation (fibril $formation_{50}$) are defined as the time at which the absorbance reaches half the value of the total absorbance change. Data are derived from graphs shown in Figure 7 and are presented as mean ± SD.
*Statistical analysis was performed on half-time values relative to DMSO control using one-way analysis of variance.
†$P < 0.001$.
‡$P < 0.01$.

magnitude of its effects in independently derived IPF fibroblast lines. Finally, nintedanib and pirfenidone inhibited collagen fibril self-assembly, which represents a novel antifibrotic mechanism of action for both drugs. We suggest two independent potential mechanisms for this observation, namely, down-regulation of collagen V and inhibition of extracellular fibril formation by direct interaction of the drugs with triple-helical collagen. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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