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Pirfenidone, Fluorofenidone and New Antifibrotic Drugs: Biochemistry of Direct and Indirect Effects

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Abstract

As a broad spectrum antifibrotic agent, pirfenidone has been shown to be efficacious in many patients and models of fibrotic diseases including idiopathic pulmonary fibrosis (IPF). Discovery of pirfenidone, along with nintedanib, was a significant progress in fight against IPF, a chronic, progressive disease of uncertain etiology. Pirfenidone has shown antifibrotic and antiinflammatory effects in various models, but its molecular target has not been elucidated. It is plausible that pirfenidone interacts with multiple target molecules. Fluorofenidone and other structurally-related compounds are also gaining researchers' interest for their high potencies in in vitro antifibrotic effects. This review article is aimed to overview recent researches on pirfenidone and some other antifibrotic compounds with a focus on biochemical mechanisms. In particular, potential importance of the antioxidative activities of these drugs in possible linkage to anti-inflammatory and anti-fibrogenic effects are considered. In addition, novel modalities of pirfenidone effects in collagen fibril formation and effects mediated by regulator of G-protein signalling 2 (RGS2) and hedgehog signalling are discussed.

Fibrosis and pirfenidone: overview

Drug development for fibrotic diseases is an important activity in today's medicinal researches. For idiopathic pulmonary fibrosis (IPF), a devastating disease, a variety of drugs have been evaluated based on current paradigm of IPF pathogenesis, that is, recurrent injury to the alveolar epithelium incurs aberrant wound healing processes, which leads to accumulation of excessive amount of extracellular matrix (ECM) components, rather than normal tissue repair [1,2]. For a wide range of fibrotic diseases with various underlying etiologies, common mechanisms have been shown or suggested [2,3]. That is, by some stimulation, fibroblasts are activated and transdifferentiate into myofibroblasts that secrete ECM components. Thus, in addition to loss of alveolar epithelium, activated fibroblasts and myofibroblasts are key pathological features of IPF and their accumulation associate with the progression of fibrosis. In the case of liver fibrosis, hepatic stellate cells (HSCs) exhibits proliferation and production of ECM proteins [4].

In November 2014, the US Food and Drug Administration approved pirfenidone (PFD) and nintedanib as treatments for IPF [5]. Nintedanib can inhibit receptor tyrosine kinase signaling by platelet-derived growth factor (PDGF), fibroblast growth factor, and vascular endothelial growth factor. Given the enhanced activities of these signaling pathways in IPF, understanding molecular mechanisms for antifibrotic effects of nintedanib may be relatively straightforward [6]. However, how PFD works is poorly understood. To our knowledge, as an antifibrotic agent, to date, PFD significantly slowed disease progression in four randomized, placebo-controlled phase III studies, [7-10]. Cell-based and animal model-based studies mainly conducted in 1990s have established that PFD has antiinflammatory, antioxidant, and antifibrotic effects [e.g.,11-13]. PFD has exhibited an antifibrotic effect in several tissues including lung, liver and kidney [14].

Nonetheless, the specific molecular mechanisms by which PFD exerts such effects are still a matter of debate. For example, it is not clear to what extent the PFD-induced suppression of TGF-β and p38 MAPK signaling are crucial [15]. Nor is clear how antioxidative activity is related to the effects on these signaling pathways.

In this review, we will summarize the available literature, with a focus on biochemical aspects of PFD and related compounds. The effects on NADPH oxidases (Nox)/ROS and profibrogenic TGF- β signaling, which can be considered an important link between redox control and fibrogenesis, are discussed in comparison with the effects of long-used antioxidative reagents including N-acetylcysteine (NAC). As novel modalities of PFD, we will also discuss recent findings on pathways mediated by regulator of G-protein signaling 2 (RGS2) and glioma-associated oncogene homolog (GLI) and effects on collagen fibril destabilization. Later we discuss fluorofenidone and other new drugs with some similarities to PFD. We refer readers to a well-balanced review article on animal models and cell-based analyses by Schaefer et al. [14]. Potential benefits PFD to fibrosis of liver heart and kidney have been discussed in a review article by Kreuter [16].

Early studies on pirfenidone

Pirfenidone (PFD, 5-methyl-L-phenyl-2-(1H)-pyridone) is a relatively soluble crystalline powder with a.m.w. of 185.2 [17] (Figure 1A). To our knowledge, antifibrotic activity of PFD as analysed in cell-based studies drew wide attention in 1990s. Unique features of PFD noticed therein include the

finding that a high concentration (over 1 mM) is necessary for the antifibrotic activity, and that both transcriptional and translational controls of mRNAs including those for proinflammatory cytokines become the target.

Iyer et al. reported in hamster lungs that bleomycin induces increases of several lung toxicity markers (superoxide dismutase (SOD) activity, malondialdehyde equivalent levels and the lung propyl hydroxylase activity) and that PFD partially suppressed such increases [11]. Later, Iyer et al. [18] showed that PDF suppresses the increases in procollagen mRNA and TGF-β mRNA in the bleomycin hamster model [18]. Cain et al. showed that PFD>1 mM can downregulate the expressions of proinflammatory cytokines, fibroblast proliferation and collagen matrix synthesis [17]. Both in lipopolysaccharide (LPS)-injected mice serum and in vitro macrophage culture medium, PFD treatment decreased TNF-α production [17].

Interestingly, in addition to the transcriptional level control, control at the translational level also appears the target step of PFD. In Iyer Gurujeyalashmi and Giri and Gurujeyalashmi et al. [18,19], the authors showed in bleomycin-mouse model over-expression of PDGF-A mRNA and that it is down-regulated by IFN- γ at the transcriptional level, ameliorating the lung fibrosis. PDGF-A and PDGF-B transcripts were elevated in BAL cells of bleomycin-treated hamster and the elevations were unchanged with PFD. Nonetheless, both PDGF-A and PDGF-B protein products were downregulated by PFD, suggesting translational (or post-translational) controls of these isoforms by PFD. Nakazato et al. [20] reported the effect of PFD to suppress TNF-α expression at the translational level in LPS-stimulated RAW264.7 macrophage-like cell line at 300 mg/L PFD in medium. This was also translational level as PFD showed no effects on the LPS-induced increase in TNF-α mRNA [20]. Additional analyses using transcriptional arrest by actinomycin D and differential time of administration of PFD supported the importance of translational control [20], with TNF-α mRNA stability or total protein synthesis activity of cells unchanging. In the same RAW264.7 systems, p38 and JNK activation by LPS was not influenced by PFD, arguing against the universal of roles of these signaling molecules as PFD targets. They also showed that LPS/D-gal (D-galactosamine) injection increases IFN-γ and IL-6 in vivo and their mRNAs ex vivo, and that here again PFD did not change the mRNA levels. This is striking because PFD-mediated translational control is likely to occur without changes in the activity of the stress kinases. However, underlying mechanisms for this translational control remain to be studied. Of note, PFD attenuated TGF-β induced increase in α-smooth muscle actin (α-SMA) and procollagen–I mRNA and protein levels, but such effects become evident after a relatively long treatment (48 h treatment was used in Conte et al. [21,22]) that would allow joint effect of multiple pathways, so early events may not be inferred from such studies.

Later, PFD was applied in various animal models and cell-based analyses [14]. In cell-based studies, PFD suppresses LPS-induced expression of TNF-α in a few different settings human mononuclear cell line and mouse macrophage RAW264.7 cell line [20,23] and downregulates the expression of TGF-β in human lung fibroblasts [24], consistent with the findings in clinical trials [25,26]. However, while many studies with animal models and clinical studies have indicated PDF-mediated downregulation of TGF-β, PDGF, basic fibroblast growth factor, TNF-α as well as and matrix metalloproteinase (MMP)-2 and MMP-9 or their transcripts [14], cell-based analyses have been relatively few and detailed molecular mechanisms for PFD-mediated inhibition on expression of these molecules remain unclear.

Antioxidant defence of PFD and N-acetylcysteine

Antioxidative effects of PFD are likely to be tightly linked to its antifibrotic and antiinflammatory effects. To disentangle these linkages, it seems important to take into account the roles for ROS in cellular signaling. It is now known that reactive oxygen species (ROS) are not only the cytotoxic substances but also important second messenger for many cellular processes, and, in particular, TGF-β signaling is linked to the NADPH oxidases (Nox)/ROS system (see the next section). As such, the antioxidative effects should have unexpectedly profound ramifications to both antiinflammatory and anti-fibrogenic roles of PFD.

Analysis using manganese SOD showed that ROS can initiate bleomycin-induced fibrosis [27]. ROS can activate NF-kB that in turn enhances synthesis of TNF-α [28] Despite that interrelationship between Nox system and TGF-β signaling was largely unknown at the time, Giri et al. [12] showed that the reaction rate of PFD was $1.63 \times 10^{10}\ M^{-1}\ s^{-1}$, which is comparable to several well-established antioxidants including ascorbate, glutathione and cysteine. Compared to hydroxyl radical (•OH), the superoxide radical (O_2^{-}•) scavenging was less efficient 42.36 $M^{-1}\ s^{-1}$ with PFD [12]. This was supported by Misra et al. [29] that showed that, while PFD is ineffective as a scavenger of superoxide radical, it is a potent scavenger of hydroxyl radicals, implicating this activity for beneficial effects of PFD [29]. Mei et al. [30] reported that, increase in oxygen radicals (as measured by ESR spectrometer) was observed in an oleic acid (OA)-induced acute lung injury rat model as anticipated from earlier studies on this model [31], and that this increase was most effectively suppressed by PFD at 0.5 h after injection of OA and PFD, which was an earlier time point at which PFD effects on the pathological changes in lung became evident. Salazar-Montes et al. showed that, in hepatic cirrhosis models, PFD showed protective effects based on several metrics including malondialdehyde (MDA) concentration, with PFD yielding better results compared to a Nox inhibitor, diphenyleneiodonium (DPI) [32]. Overall, these findings suggested that direct ROS scavenging accounts for an important part of its effects.

Later, Mitani et al. elucidated a new ROS scavenging role for PFD; as a complex with Fe^{2+}, PFD can exert superoxide anion-scavenging effects. Of note, ferrous ion

(Fe^{2+}) is long thought to be an important active species that generate oxidants through interaction with O_2. Specifically, Fe^{2+} and H_2O_2 can generate •OH through Fenton reaction:

$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + •OH.$

In Mitani et al. [33], 150 μM Fe^{2+}-PFD complex exhibited the O_2^-• scavenging effect. However, the physiological concentration of Fe^{2+} may be fairly low. In a healthy individual, the plasma concentration of non-transferrin-bound iron typically is considered not to exceed 1 μM [34]. According to the measurement using fluorescent probe calcein. Epsztejn et al., for erythroid and myeloid cells, the labile iron pool (metabolically reactive and accessible to chelators) is at 1-2 μM. [35]. Therefore, it remains to be determined what proportion of PFD is forming a complex with Fe^{2+} and exerting scavenging effect against superoxide anion in clinical settings.

We briefly discuss N-acetylcysteine (NAC) here, as much of knowledge about roles for ROS in inflammation and fibrosis was derived from analyses using NAC. NAC is a precursor of cysteine and glutathione and has widely been used in therapeutic practices [36]. NAC has antioxidative activity through its fast reaction with ROS, such as hydroxyl radicals and H_2O_2, as well as restitution of reduced glutathione, which is an endogenous antioxidant [37].

Cantin et al. showed glutathione deficiency in epithelial lining fluid of the lower respiratory tract of patients with IPF [38], suggesting an oxidant-antioxidant imbalance at the alveolar surface of the patients. Many studies have reported that reduced glutathione (GSH) is significantly decreased in the patients with chronic obstructive pulmonary disease (COPD). NAC is considered to reduce the rate of COPD exacerbations and improve small airway function, a main part of which is ascribed to antioxidative activity of NAC. Oral administration of NAC 600 mg/day for 5 days increased GSH in bronchoalveolar lavage fluid [39]. NAC was shown to be protective in models of lung ischemia–reperfusion injury [40]. Inhibition of TGF-β signaling or its direct modification by NAC has been considered to have therapeutic value in several diseases [36].

Careful reappraisal of studies using NAC seems helpful to better understand mechanisms of PFD effects. It is generally difficult to separate direct effects (that depend on binding to key signaling proteins) from indirect effects (due to ROS scavenging activities). In this regard, comparison with NAC may help distinguish direct from indirect effects of PFD. In human bronchial epithelial cells, TNFα-induced activation of p38 MAPK was attenuated by NAC [41]. More recently, in Nam et al., NAC at 1 mM suppressed the lipoteichoic acid- and peptidoglycan-induced (i.e. TLR2-mediated) production of IL-6 and IL-1β. NAC also suppressed TLR2-mediated increase of NF-κB p65 binding to DNA and activation of JNK, p38-MAPK and Akt in a keratinocyte cell line [42], implying that activation of these signaling pathways may be mediated by ROS.

Such a broad range of target molecules of ROS make it difficult to isolate direct effects from indirect effects of antioxidants. To further complicate matters, many signaling proteins are redox-sensitive. A well-known example is ASK-1 (apoptosis signal-regulating kinase-1) which is a member of the MAPK kinase kinase family and activates both p38 kinase and JNK pathways [43]. ASK-1 is considered to be an important effector of Nox in cellular stress responses [44]. Nox-derived ROS also act to inhibit various protein tyrosine phosphatases, thereby causing enhanced and prolonged phosphorylation of receptor tyrosine kinases, which in turn causes activation of ERK1/2, p38 and JNK pathways. Likely through the latter mechanism, Nox-derived ROS are considered to induce phosphoinositide 3-kinase (PI3K)/Akt activation in a variety of cells [45,46]. Intriguingly, TGF-β signaling activates Smad2/3 and PI3K pathways both of which in turn upregulate Nox4 expression thereby increasing ROS signaling [47]. Overall, in light of ROS-sensitivity of these signaling molecules, careful in vitro analyses using well-defined purified components may become helpful when direct and indirect effects of PFD are to be considered.

TGF-β and Nox interplay

Exaggerated TGF-β signaling has strongly been implicated in numerous fibrotic diseases including those causing liver heart and lung fibrosis [48,49]. Understandably from general role of TGF-β in fibrosis, the antifibrotic effect of PFD has been shown to be through blockade of TGF-β signaling [50]. TGF-β activates a unique signal transduction pathway that acts via the Smad family of proteins, and in addition, via Smad-independent pathway [51]

Here we briefly review Nox-dependent redox signaling and its implications in TGF-β biology. We would like to refer readers to excellent review articles by Jiang et al. [52]. Among the five Nox isoforms of the Nox catalytic subunits (i.e., Nox1, Nox2, Nox3, Nox4 and Nox5), it is important to note that Nox4 plays pivotal roles in fibroblasts from different organs. Gene expression analysis, as well as Nox4 specific gene suppression using siRNA, and dominant negative Nox4 expression and Nox4 knockout mouse analysis all pointed to the view that Nox4 is the main Nox isoform that mediates profibrotic actions of TGF-β in a variety of cells including pulmonary fibroblasts, lung mesenchymal cells and kidney fibroblasts and liver stellate cells. It is important to note that Nox and ROS not only mediate profibrotic effects of TGF-β, ROS has been shown to potentiate the TGF/Smad signaling, although mechanisms for this is largely unclear as yet. For example, ROS elicit conversion of the latent form of TGF-β to its active form. Further, treatment with either various antioxidants or Nox4 gene silencing suppresses the TGF-β – induced Smad2/3 phosphorylation.

It should be noted that in addition to the ROS-scavengers, drugs specifically target the Nox components are currently drawing much attention for potential benefits in fibrotic disease [53]. Notably, Genkyotex (Page and coworkers) performed systematic structure-activity relationship analysis starting from a pyrazolopyridine dione core structure and discovered Nox4/Nox1 inhibitors including GKT136901 and GKT137831 [54,55]. Some of their

compounds exhibited inhibition of collagen deposition with higher efficacies in bleomycin-induced pulmonary fibrosis model compared to PFD. Hecker et al. showed that [56] established fibrosis in lungs of aged mice can be partially reversed by administration of GKT137831.

MAPK as a target of PFD

TGF-β is generally considered to be the most important cytokine for fibrogenesis [57]. As the downstream signaling pathway downstream to TGF-β1, besides Smad2/3 pathway, MAP kinases (MAPK) that are ERK1/2, p38 and JNK have been implicated [58]. It is understandable that PFD has suppressive effects on MAPK activation induced by profibrotic factors. However, molecular mechanisms for these suppressive effects remain unclear. In particular, it is not clear whether the antioxidative effects indirectly abrogate the TGF-β effects or whether some more direct modulation may be taking in place.

Conte et al. [21] showed that 24 h treatment with PFD 0.3 mg/ml significantly inhibited the TGF-β induced increase in phosphorylation of p38 MAPK and Akt in human lung fibroblasts. Analyses using different cells also showed similar PFD inhibited p38 phosphorylation induced by various stimulations in various cell types [59-61].

In Li et al. [62], in both unilateral ureteral obstruction (UUO)-rats, which is an experimental renal fibrosis model, and in vitro analysis with a human renal proximal tubular epithelial cell line, PFD inhibited the phosphorylation of ERK, p38, and JNK, without changing the expression levels of these proteins. In Li et al [62], PFD at 0.5 mg/ml inhibited E-cadherin and α-SMA protein expression, type III collagen production. By and large, the extent of the suppressions on protein expression of α-SMA and type III collagen and phosphorylation of MAPK are largely similar to the case with a mixture of SB203580 (p38 inhibitor), U0216 (ERK1/2 inhibitor) and SP600125 (JNK inhibitor). However, as Li et al. performed cell-based analyses, not purified proteins, it is difficult to discuss direct and indirect effects.

Ma et al. [63] hypothesized that p38 may be a direct target for PFD, based on the understanding that a member of the stress-activated protein family. Their molecular docking study showed that binding energy is as large as -33.60 kcal/mol, which is much greater unsigned energy than that for TNF-α TGF-β. Compared with PFD that required a high concentration for p38 inhibition (IC_{50} : 165.4 μM) all tested derivatives showed superior inhibitory activity, with compounds named 7d and 8d showing 4-5.2 μM IC_{50}. These suggested some structurally-related compounds might have increased inhibitory activity against p38. Ma et al. was informative especially because of their use of inhibition assay using purified p38, which made interpretation unarguable [63]. Of note, the IC_{50} of PFD (165.4 μM) is not as high as 1-5 mM required for PFD effect against cells, but it could be that a higher concentration may become necessary for PFD for other effects involving antioxidative activities, which may indirectly suppress activation of signaling molecules involving p38.

These studies suggested the p38 MAPK pathway as a target important in operating in inhibitory mechanism of PFD. However, it is possible that simultaneous inhibition of other targets is a requirement of PFD action. First, Nakazato et al. showed that in RAW264.7 systems, p38 and JNK phosphorylation induced by LPS was not influenced by PFD [20]. Further, in Haak et al. [64], one of the compounds named MC-6 significantly inhibited increase of phosphorylated p38 (based on Western blotting) in TGF-β-stimulated IPF lung fibroblasts, but strangely, it showed no antifibrotic activity. The latter finding may reflect that simultaneous suppression of multiple targets is important for the PFD-mediated antifibrotic action.

Blockade of Smad2/3 and other pathways

Smad3 is the major contributor in regulation of myofibroblast differentiation from fibroblasts [65]. It is well known that TGF-β1 promotes Smad3 phosphorylation, which accelerates the translocation of the Smad2/3-Smad4 heterotrimer complex to the nucleus and facilitates the transcription of target genes. Smad2/3s are activated by phosphorylation, and are released and translocate onto the nucleus, activating genes expression including α-SMA [51]. Yang et al. [66] showed that the Smad-dependent pathway is involved in antifibrotic effect of PFD.

Recent supports of this view are seen in Shin et al. [67] that showed that in TGF-β1–induced NPDF (nasal polyp-derived fibroblasts) 0.5 mg/ml PFD blocks phosphorylation of Smad2/3. In Ji et al., PFD attenuated phosphorylated Smad (pSmad) level without changing Smad level in kidneys of Dahl salt-sensitive rats fed a high-salt diet [68]. Conte et al. [22] showed 0.3 mg/ml PFD impaired TGF-β induced phosphorylation of Smad3 in human lung fibroblasts. However, to our knowledge, lack of data from in vitro analysis with purified components, molecular mechanisms of PFD effect on Smad pathways remain unclear. In lung fibroblasts proliferation and differentiation into myofibroblasts, PI3K pathway plays a major role [21].

Novel mechanisms - collagen fibril bundle destabilization

Interestingly, Knüppel et al. showed that both PFD and nintedanib can inhibit collagen I fibril formation, reducing the size and number of collagen fibril bundles [69,70]. Although neither of nintedanib and PFD treatment lead to differential degrees of post-translational modifications of collagen in IPF fibroblasts compared to fibroblasts from healthy donors, both drugs shortened the fibre size and fibril thickness based on electron microscopy analysis. This is an interesting finding as these drugs can directly interact collagen molecules. Another notable feature was that, while nintedanib showed inhibitory effects superior to PFD against fibrotic gene expression and fibril formation, clinical data have not shown superiority of

nintedanib relative to PFD. It would be interesting to envisage that there are still unknown targets that collectively cover a wide variety of molecules.

RGS2

Recent findings have highlighted the importance of regulator of G-protein signaling 2 (RGS2) as protective factor in fibrosis and as a central mediator of PFD function. Xie et al. reported that deletion of RGS2 significantly enhanced IL-13-induced airway remodelling, including increased peribronchial fibrosis and smooth muscle in mice [71]. RGS2 can inhibit the progression of kidney fibrosis following UUO in mice [72]. In an analysis by Xie et al. using mRNA expression profiling with GeneChip microarray, RGS2 was among the top six genes upregulated at 2 hour treatment with 10 mM PFD in HFL1, a fetal lung fibroblast cell line. In several human lung fibroblast cell lines, RGS2 mRNA increased to 6-fold at 2 h after treatment. Further, overexpression of RSG2 attenuated the thrombin-stimulated increase of connective tissue growth factor (CTGF) mRNA expression by more than 50% and inhibited the profibrotic effects of thrombin in HFL1 cells. Analysis using knockout mice also showed that the upregulation of RGS2 by PFD treatment is crucial for PFD protection of mice against bleomycin-induced pulmonary fibrosis [71].

How does RGS2 control fibrosis? Although RGS2 is known as a GTPase-activating protein (GAP) for Gq, GTP is essential molecule for initiation of protein synthesis (translation). Strikingly, Nguyen et al. showed that RGS2 can control initiation of translation [73]. Based on yeast two-hybrid screening, Nguyen et al. [73] showed that eIF2Bε (eukaryotic initiation factor 2B ε subunit) is a binding partner of RGS2. eIF2B serves as a promoting factor in guanine nucleotide exchange on the eIF2γ subunit, thereby promoting eIF2-GTP-tRNA ternary complex formation required for initiation of translation. In an in vitro translation system, 4 μM RGS2 addition suppressed translational activity to 30%.

Further, the ability of eIF2B to promote dissociation of GDP from eIF2 was observed to be greatly inhibited by RGS2. Transfection analysis also showed that total protein synthesis is reduced by RGS2. Although it remains to be studied whether such a translational control of gene expression takes place to mediate the effects of PFD, currently available data about RGS2 localization and functions jointly point to a view of multifaceted role of this molecule, not solely limited to the role as a GAP. Interestingly, RGS2 may have regulatory activity distinct from its known RGS domain function [73].

For example, RGS2, through its peptide fragments located outside of the RGS domain, interacts with the TRPV6 channel and inhibits both Na and Ca currents [74]. Other non-RGS segments are involved to enhancement by RGS2 of microtubule polymerization. Of note, one of well-studied functions of RGS2 is that as a negative regulator against α-1 adrenergic receptor–stimulated cardiomyocyte hypertrophy [75]. However, the finding in Nguyen et al. argues the possibility that stress-induced up-regulation of RGS2 expression may impede the development of cardiac hypertrophy by inhibiting global protein synthesis [73].

In the context of the PFD effects, however, it still remains to be analysed how the suppression of total protein synthesis observed by Nguyen et al. [73] can be related to translational (or post-translational) inhibition of TNF-α and other cytokines/growth factors that we have discussed above [19,20]. At present, it may be speculated that the changes in short-lived protein such as the cytokines may be easily detected in the 1990s' studies due to technical difficulty observing degradation of stable proteins. Or alternatively, it is possible that some specific molecules that target the translation of TNF-α mRNA are playing roles.

To our knowledge molecular mechanisms for PFD effect on RGS2 are unknown as yet, but there are ongoing studies on transcriptional control of RGS2 dependent on profibrotic stimulations [76,77], which may eventually help further analysis.

Hedgehog–Glioma-associated oncogene homolog (GLI) pathway as a new target of PFD

The hedgehog pathway plays a role in lung development, likely through epithelial-mesenchymal interactions [78]. Recent studies uncovered a new modality of PFD antifibrotic effects, which is through inhibition of glioma-associated oncogene homolog (GLI) transcription factors [79]. Didiasova et al. demonstrated that PFD selectively destabilizes the glioma-associated oncogene homolog 2 (GLI-2) protein, the primary activator of hedgehog-mediated gene transcription [79].

Although we can cite only a limited number of papers, basics related to this finding is as follows. Hedgehog signaling plays critical roles in embryonic development, tissue patterning and organogenesis. Hedgehog signaling responses are mediated by several receptors including Smoothened. Sonic hedgehog, one of ligands, is considered important for branching morphogenesis of the lung [80]. Sonic hedgehog produced by distal epithelium diffuses and activate nearby mesenchyme via Smoothened and the receptors and GLIs, a family of transcriptional activators. In a simplified description of canonical pathway, binding of the hedgehog ligand including Sonic hedgehog to the receptor PTCH-1 releases tonic inhibition against (and induces structural change in) Smoothened and then the latter allows GLI to dissociate from Suppressor of fused (Sufu) and to translocate into the nucleus and transcriptional regulation.

Sonic hedgehog expression is upregulated in lung fibrosis. [81] and it has been shown that TGF-β dependent differentiation of myofibroblast in human is mediated by hedgehog system machinery [82] Bolanos et al. showed that the main components of Sonic signaling pathways, Sonic hedgehog, PTCH-1, Smoothened, GLI-1 and GLI-2, are overexpressed in IPF lungs [83]. Sonic hedgehog was observed mainly in the alveolar and bronchiolar epithelium, whereas PTCH-1 observed in mesenchymal cells and in

interstitial inflammatory cells [83]. Sonic hedgehog is likely to increase the proliferation, migration, ECM production and survival of fibroblasts, and, in addition, may be a potent chemoattractant for lymphocytes and monocytes. In a bleomycin-induced lung model, Moshai et al. [83] observed an increased nuclear localization of GLI-1 and GLI-2 at day 14 after bleomycin treatment of mice and that inhibition of GLI activity with GANT61 (an inhibitor of GLI transcription factors in the nucleus) decreased lung fibrosis and lung collagen accumulation [84]. The presented findings also suggest that GLI inhibition has more relevance compared to Smoothened inhibition. It may be that GLI can be activated by a hedgehog-independent pathways such as TGF-β signaling,

MA-35, another TNF-α and TGF-β1 signaling inhibitor

While the success of PFD was an important achievement, the human therapeutic dose of PFD is high, ranging from 1400 to 2800 mg per day. It is quite possible that drugs with longer half-life and higher antifibrotic activity than PFD will be discovered in future. This and following sections try to cover a few compounds that have structural and/or functional similarity to PFD. From such comparisons among these compounds, insights into the molecular mechanisms of PFD may also be gained.

A novel indole compound named MA-35 (5-(3,5-dimethoxybenzyloxy)-3-indoleacetic acid) resembles PFD in its ability of dual inhibition against TNF-α and TGF-β1 signaling (Figure 1E). In a quest for renal fibrosis treatment, Shima et al. [85] screened their library of indole derivatives and observed that MA-35 shows antifibrotic effects in liver-derived Hep3B and L929 fibrosarcoma cells. IκB kinase (IKK) phosphorylation induced by TNF-α was suppressed by MA-35. (But MA-35 unchanged the TNF-α induced phosphorylation of JNK, p38 and ERK, here again demonstrating the non-universal feature of effects on p38 and JNK in antifibrotic phenomenon). MA-35 is likely to suppress the positive feedback pathway of TNF-α and IKK. TGF-β-induced Smad3 phosphorylation was significantly inhibited by MA-35 in both LX-2 (hepatic stellate cell line) and NRK-49F (rat kidney interstitial fibroblast) cells, thereby reducing the expression of fibrotic genes.

In addition to the evidence for that MA-35 ameliorates renal fibrosis in vivo, they further showed that MA-35 inhibits the TGF-β-induced binding of H3K4me1 (methylated histone H3K4) binding to the promotor of the collagen I gene and that of the gene for plasminogen activator inhibitor-1 (PAI-1). This is important as SET7/9 that methylates H3K4 increases in a renal fibrosis mice model, [86]. It would be interesting to examine whether MA-35 has antioxidative activity and whether these dual inhibition of IKK and Smad pathways can be attributed to antioxidative activities. Prior to this study, the authors had noticed that some indole derivatives show interesting biological activities including prolonged life span of a mitochondrial disease model [85]. Although many of steps of drug design are unpredictable, it is plausible that many more compounds will be discovered in future that have a good balance of activities and exhibit clinical efficacies.

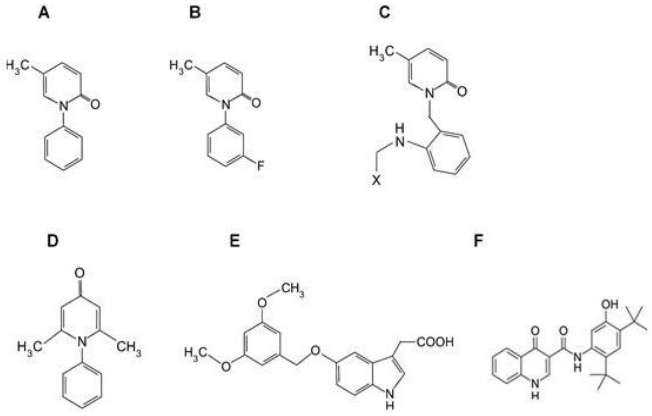

Figure 1: Structure of pirfenidone (PFD) and other antifibrotic compounds. (A) PFD; (B) Fluorofenidone [87]; (C) Carbohydrate-modified 1-(substituted aryl)-5-trifluoromethyl-2(1H) pyridine [91]. X: carbohydrate; (D) MC-3 [64]; (E) MA-35 [85]; (F) Ivacaftor [94].

Pirfenidone-related compounds - fluorofenidone (AKF-PD), 5-carboxypifenidone and MC-3

Recent studies have shown several compounds that have structurally similarity to PFD and exhibit antifibrotic activities. A well-studied example is fluorofenidone [87] (Figure 1B). Fluorofenidone (1-(3-fluorophenyl)-5-methyl-2-(1H)-pyridone) is structurally similar to PFD but the hydro- at the meta-position of the benzene ring of PFD is replaced by fluoro- in fluorofenidone. Fluorine has greater electronegativity compared to hydrogen and this change can be significantly change affinities toward some target molecules. Nonetheless, it showed efficacy similar to PFD.

In renal tubulointerstitial fibrosis, angiotensin II (AngII) is the key inducer of ROS, mediated by Nox system [87]. Using NRK-52E, a rat proximal tubular epithelial cell line, Peng et al. [87] compared DPI, PFD and fluorofenidone. PFD and fluorofenidone (both at 8 mM) showed similar efficacy in inhibition of TGF-β mRNA expression and collagen I expression in AngII-stimulated cells. Interestingly, DPI was more effective than PFD and fluorofenidone in suppressing the increase of the Nox activity, but less effective than PFD and fluorofenidone in suppressing fibrotic proteins expression. This result supports the view that, unlike NAC that is a solely antioxidative molecule, PFD and fluorofenidone have multiple targets besides Nox/ROS system, modulating many signaling pathways and machineries for transcription and translation.

Fluorofenidone exhibited efficacy similar to PFD (or slightly more efficacious) in inhibiting platelet-derived growth factor (PDGF)-BB-induced increase in α-smooth muscle actin and tissue inhibitor of metalloproteinases-1 (TIMP-1) expression, which are the marker of hepatic stellate cells

(HSCs) activation, in two activated HSCs lines [61]. Both fluorofenidone and PFD showed similar effects to inhibit phosphorylation of ERK, p38 and JNK in these cells. In dimethylnitrosamine (DMN)-induced liver fibrosis rat model, fluorofenidone and PFD treatment led to similar levels of histological improvement. The similar effect of fluorofenidone with PFD corroborates with the view that instead of binding a selective target or pocket, an action as a free radical-scavenging antioxidant may be the most important mechanism for PFD effects. Qin et al. also showed that flurofenidone inhibits AngII-induced Akt phosphorylation in NRK-52E cells. [88], reinforcing the notion that Nox-derived ROS induces phosphoinositide 3-kinase (PI3K)/Akt activation in a variety of cells [45,46].

In support of this "loose recognition and weak binding" concept, a compound MC-3, which retains phenylpyridine core of PFD but has two CH_3 and para-O instead of meta-CH_3, exhibited inhibitory effect on collagen I production in Haak et al. [64] (Figure 1D). It may be reasonable to consider that these molecules have many targets with generally low and diverse degrees of affinity, modulating functions of many molecules simultaneously. Nonetheless there seems to be some specificity given that MC-2 and MC-4 showed distinct effects.

Another support to loose recognition and weak binding of PFD has come from studies on its metabolites. It has been shown that PFD is rapidly metabolized to 5-hydroxypirfenidone (PFD-OH) and 5-carboxypifenidone (PFD-COOH), the latter being the major metabolite and is excreted in the urine [89,90]. Interestingly, both PFD-OH and PFD-COOH exhibited an inhibitory effect on collagen synthesis in vitro WI-38 cells, a human lung fibroblast cell line. The effects of these metabolites were not as strong as PFD, raising a pharmacokinetical concern in PFD treatment. On the other hand, this provides biochemical insights because, despite the distinct structures (5-hydroxy *vs.* 5-carboxy), these were similarly efficacious in WI-38 cells.

Drug development among structurally-related compounds is a hot area and in the near future, it is likely that some highly potent and safe antifibrotic drugs are discovered. For more PFD-derivatives that showed high cell-based antifibrotic activities, the following studies are good examples. Lou et al. examined carbohydrate-modified 1-(substituted aryl)-5-trifluoromethyl-2(1H) pyridones and found a compound that shows high cell-based inhibitory activity against NIH 3T3 (IC_{50}=0.17 mM) (Figure 1C) [91]. Chen et al. examined (5-substituent)-2(1H)-pyridone derivatives and showed a compound that exhibited a high potent of anti-fibrosis with a IC_{50} of 0.08 mM, about 34 times of fluorofenidone. [92]. Wu et al. examined N1-substituted phenylhydroquinolinone derivatives and found that 5-hydroxy-1-(4'-bromophenyl)-5,6,7,8-tetrahydroquinolin-2(1H)-one (6j) displayed 13 times higher potency (IC_{50} = 0.3 mM) than that of fluorofenidone [93].

Derivatives of Ivacaftor (4-oxoquinoline-3-carboxamide)

Zhu et al. [94] focused on derivatives of Ivacaftor (Figure 1F), a drug whose benefit has been shown for cystic fibrosis and also shows inhibitory effects on TGF-β–induced collagen production in rat fibroblasts cells [95]. Some derivatives exhibited increased inhibitory potency relative to Ivacaftor in TGF-β1-induced collagen accumulation in NRK-49F rat fibroblast cell as well as a higher inhibitory efficacy against TNF-α production by LPS challenge of RAW 264.7 cells than Ivacaftor. Derivative named '10l' significantly reduced the number of inflammatory cells in BALF in bleomycin-induced lung fibrosis model. Based on hydroxyproline levels, '10l' exhibited significant antifibrotic effects, while Ivacaftor showed little effects at the same dose (20 mg/kg/day) (Table 1).

1. Are the following mechanisms common to many cell types and tissues? If they are not, what is behind the between-cell differences?
- inhibition of p38 and JNK phosphorylation
- increased expression of *RGS2*
- GLI-2 destabilization-mediated mechanism
2. Structural details and effects of conditions (ionic strength, pH, etc) remain unclear in;
- how PFD inhibits p38 MAPK activity
- how PFD destabilizes collagen fibril
3. Mechanisms for translational control of TNF-α and PDGF shown in early studies are not clear. Is RGS2-mediated suppression of translation related to these observations ?
4. Do antifibrotic efficacies of PFD-related compounds correlate with their antioxidative activities?

Table 1: Remaining questions about PFD biochemistry.

Conclusion and perspectives

As we have seen above, there seem to be many target molecules for PFD that are collectively modulated by PFD and cause a change of 'tone' of intracellular signaling in favor of suppression of fibrogenic signaling. It is not straightforward to dissect out the molecular mechanisms of PFD effects because many signaling pathways involved in

fibrogenesis are interconnected in a complicated manner, especially through ROS. So, besides in vitro cell-based analyses, it would be informative to test PFD effects against biochemically-defined systems made up of purified candidate target molecules. For example, PFD destabilizes GLI-2, thereby downregulating hedgehog signaling [79], but it is not clear as yet to what extent this is a direct effect of PFD on GLI-2. In addition to the use of purified molecules, comparison of various cells and tissues in PFD effect on GLI-2 stability may become informative. Comparison of several PFD-related compounds may also provide insights.

From a perspective of drug development, it is encouraging that compounds that have similar but subtly differing combinations of targets relative to PFD have been found. This will increase the chance to find better drugs, when differences in pharmacodynamics and tolerability are also considered. Of note, interest is growing in the potential utility of small molecules in the search of antifibrotic drugs [3]. It is interesting to envisage that PFD and related compounds is just the tip of iceberg in antifibrotic drug development. There seems to be currently unknown intricacy between 'necessity and probability', in the area of small molecule-mediated antifibrotic effects.

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