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Phenylpyrrolidine structural mimics of pirfenidone lacking antifibrotic activity: A new tool for mechanism of action studies

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ABSTRACT

Pirfenidone recently received FDA approval as one of the first two drugs designed to treat idiopathic pulmonary fibrosis. While the clinical data continues to support the efficacy of pirfenidone, the specific molecular mechanism of action of this drug has not been fully defined. From a chemical perspective the comparatively simple and lipophilic structure of pirfenidone combined with its administration at high doses, both experimentally and clinically, complicates some of the basic tenants of drug action and drug design. Our objective here was to identify a commercially available structural mimic of pirfenidone which retains key aspects of its physical chemical properties but does not display any of its antifibrotic effects. We tested these molecules using lung fibroblasts derived from patients with idiopathic pulmonary fibrosis and found phenylpyrrolidine based analogs of pirfenidone that were non-toxic and lacked antifibrotic activity even when applied at millimolar concentrations. Based on our findings, these molecules represent pharmacological tools for future studies delineating pirfenidone's mechanism of action.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic disease characteristically defined by excessive deposition of extracellular matrix and destruction of the lung's normal architecture. Over time this progressive scarring and alteration in tissue leads to declining lung function and often death. Despite the scope and severity of the disease burden associated with IPF, it has only recently become the target of clinically approved therapeutics, with the approval of nintedanib and pirfenidone. Interest in the latter began with the finding that phenylamine derivative substituted pyridones produce analgesic properties (Scudi et al., 1960), leading to the synthesis of 5-Methyl-1-phenyl-2-(1 H)-pyridone (initially referred to as AMR-69, later referred to as pirfenidone) and description of its analgesic, antipyretic, and anti-inflammatory effects (Gadekar, 1972). Decade's later the antifibrotic properties of pirfenidone began to emerge (Iyer et al., 1995; Kehrer and Margolin, 1997; Schelegle et al., 1997).

The molecular mechanism of action of pirfenidone is still not well defined. In pulmonary fibrosis, activated resident fibroblasts are thought to be the main cellular contributor to matrix deposition and scarring (Barkauskas and Noble, 2014). In an effort to identify the mechanism of action of pirfenidone, its *in vitro* antifibrotic efficacy was established in isolated fibroblasts (Conte et al., 2014; Hewitson et al., 2001; Lehtonen et al., 2016a). A major limitation of pirfenidone is the compound's potency. In order to produce antifibrotic activity *in vitro,* concentrations between 1 and 5 mM are required (Conte et al., 2014; Lehtonen et al., 2016b; Nakayama et al., 2008). Pirfenidone's lack of potency, lipophilic nature, and minimal number of heteroatoms (Fig. 1) has led to debate about the potential for pirfenidone to bind to a selective target or pocket, and has stimulated an alternative hypothesis that pirfenidone simply works as a free radical scavenging antioxidant (Mitani et al., 2008; Salazar-Montes et al., 2008). Some very recent work has suggested pirfenidone is an inhibitor of the p38 mitogen-activated protein kinase (p38 MAPK) pathway (Li et al., 2016; Ma et al., 2014; Neri et al., 2016; Yin et al., 2016). A major barrier to further delineating pirfenidone's mechanism of action is the absence of structurally similar compounds which lack antifibrotic efficacy. Inactive structural analogs are extremely valuable tools when attempting to further define the mechanism of action or molecular target of small molecules (Dancy et al., 2012; Fu et al., 2008; Lim et al., 2004).

We set out to identify a chemical mimic of pirfenidone which retains its low molecular weight and lipophilic nature but does not

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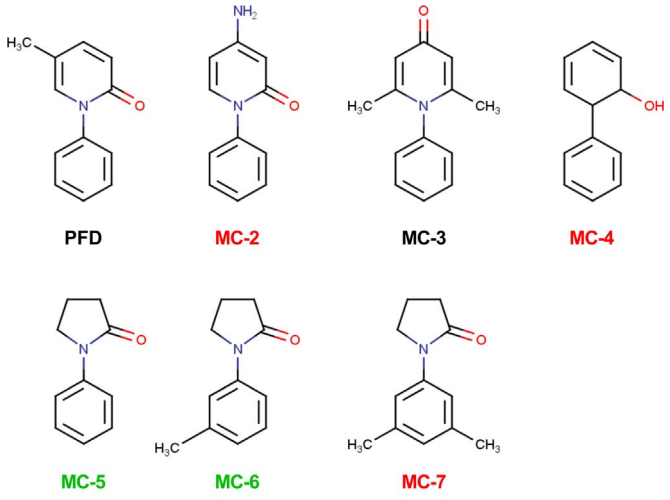

Compound	Mol. Weight	cLogP	TPSA	Heteroatoms
PFD	185	1.82	20.31	N(1) O(1)
MC-2	186	0.49	46.33	N(2) O(1)
MC-3	199	2.69	20.31	N(1) O(1)
MC-4	170	2.97	20.23	O(1)
MC-5	161	1.58	20.31	N(1) O(1)
MC-6	175	1.93	20.31	N(1) O(1)
MC-7	189	2.27	20.31	N(1) O(1)

Fig. 1. Pirfenidone (PFD) and structural mimics: physical chemical properties. Molecular weight (Mol. Weight), calculated logarithm of the partition coefficient (cLogP), topological polar surface area (tPSA), and the non-carbon heteroatoms are compared. Based on the data presented here the compounds are color-coded to summarize whether they were antifibrotic (black), acutely toxic (red) or inactive structural mimics of pirfenidone (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

display any antifibrotic activity against pulmonary fibroblasts. Our criteria for an inactive mimic thus included commercial availability and non-toxicity at the very high doses (millimolar) at which pirfenidone shows antifibrotic activity. For our initial testing we identified a diverse pilot set of compounds with physical chemical properties consistent with pirfenidone (To ease communication the compounds are abbreviated MC-2 through MC-7) (Fig. 1). We tested these compounds in primary human pulmonary fibroblasts obtained from patients with IPF. As a proof of concept demonstrating the utility of these compounds, we used the inactive analogs identified here to address the role of p38 MAPK in mediating antifibrotic effects of pirfenidone in cultured lung fibroblasts.

2. Materials and methods

2.1. Compounds

Pirfenidone (PFD) (PubChem CID:40632) was purchased from Sigma Aldrich, St. Louis, MO. 4-amino-1-phenyl-1,2-dihydropyridin-2-one (MC-2) (PubChem CID:664174); 4,6-dimethyl-1-phenyl-1,2-dihydropyridin-2-one (MC-3) (PubChem CID:N/A); 2-phenylphenol (MC-4) (PubChem CID: 24938931); 1-phenylpyrrolidin-2-one (MC-5) (PubChem CID: 78375); 1-(3-methylphenyl)pyrrolidin-2-one (MC-6) (PubChem CID: 314213); and 1-(3,5-dimethylphenyl)pyrrolidin-2-one (MC-7) (PubChem CID: 847897) were purchased from MolPort, Riga, Latvia. All compounds were dissolved in DMSO for a stock concentration of 600 mM. TPSA and cLogP were calculated using the OSIRIS Property Explorer available through the Organic Chemistry Portal: http://www.organic-chemistry.org/prog/peo/.

2.2. Cell culture

Primary human lung fibroblasts (generously provided by Peter Bitterman and Craig Henke at the University of Minnesota) were isolated by explant culture from the lungs of subjects diagnosed with IPF who underwent lung transplantation, under a protocol approved by the University of Minnesota Institutional Review Board. Primary fibroblasts were maintained in EMEM (ATCC, Manassas, VA) containing 10% FBS, unless otherwise noted. All primary cell culture experiments were performed with cells at passage six or less.

2.3. Viability assay

Lung fibroblasts were plated into 96-well plates in EMEM containing 10% FBS and allowed to attach for 6 h. Media was then exchanged with EMEM containing 0.1% FBS + the indicated compound concentration. All wells were treated with a final concentration of 0.5% DMSO (We have previously found these cells tolerate up to 1% DMSO without any effects on viability). After 24 h the cellular viability/toxicity was analyzed using the WST-1 reagent (Sigma Aldrich) following the manufacturer's protocol. Results are expressed as the absorbance at 450 nm relative to DMSO control.

2.4. Immuno-ECM assay

Adapting from previously published methods (Jones et al., 2010; Vogel et al., 2014), lung fibroblasts were plated to confluence in clear-bottom 96-well plates in EMEM containing 10% FBS and allowed to attach for 6 h. Media was then exchanged with EMEM containing 0.1% FBS plus the indicated compound concentration. All wells were treated with a final concentration of 0.5% DMSO. After 72 h cells were fixed with 4% formalin, then treated with 0.25% Triton X-100 and blocked with Li-Cor Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) before overnight incubation in a polyclonal rabbit antibody for collagen I (Novus NB600-408) diluted 1:150 in blocking buffer. Wells where then incubated with IR-dye-conjugated secondary antibody (Li-Cor #926–32211) diluted 1:500. Plates were imaged via a Li-Cor OdysseyXL system with quantification performed via densitometry. Results are represented as collagen I signal intensity relative to DMSO control.

2.5. qPCR analysis

Cells were plated and treated as indicated prior to RNA isolation using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNA (250 ng) was then used to synthesize cDNA using SuperScript VILO (Invitrogen, Carlsbad, CA). Quantitative PCR was performed using FastStart Essential DNA Green Master (Roche Applied Science, Penzberg, Germany) and analyzed using a LightCycler 96 (Roche Applied Science). Results are expressed as a fold change by ΔΔCt relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers: GAPDH; F: GGAAG GGCTCATGACCACAG, R: ACAGTCTTCTGGGTGGCAGTG. ACTA2; F: GTGAAGAAGAGGACAGCACTG, R: CCCATTCCCACCATC ACC. COL 1A1; F: AAGGGACACAGAGGTTTCAGTGG, R: CAGCACCAG TAGCACCATCATTTC. COL1A2; F: CTTGCAGTAACCTT ATGCCTAGCA, R: CCCATCTAACCTCTCTACCCAGTCT.

2.6. Western blot analysis

Cells were plated in EMEM containing 10% FBS and allowed to attach for 6 h. Cells were then starved for 24 h in EMEM containing 0.1% FBS prior to stimulation with 2 ng/mL TGFβ with or without the indicated concentration of compound for 30 min. Total protein was isolated using RIPA buffer (Thermo Scientific, Rockford, IL) and quantified with a BCA protein assay kit (Thermo Scientific, Rockford,