Inhibition of collagen cross-links by novel LOXL2 selective inhibitors in an in-vitro model of fibroblastic foci of IPF

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Background

The lung tissue of patients with idiopathic pulmonary fibrosis (IPF) is characterised by dense collections of myofibroblasts and extracellular matrix (ECM) termed 'fibroblastic foci'. Matrix stiffness is believed to be an important driver of proliferation of fibroblasts and transformation to myofibroblasts, and is increased by cross-linking of collagen molecules by the action of the lysyl-oxidase (LOX) family of enzymes. LOXL2 is an attractive drug target as its expression is increased in IPF (Nat Med 2010;16,9:1009-1018) and increased serum LOXL2 is associated with more rapid disease progression (ERJ 2014;43:1430-1438). Synairgen are collaborating with Pharmaxis to develop selective small molecule inhibitors of LOXL2 for treatment of IPF.

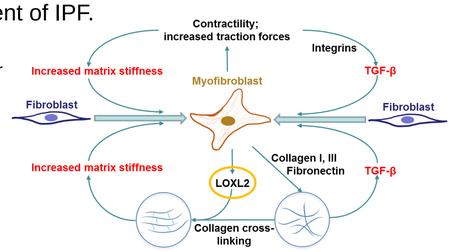

Figure 1: Rationale for targeting LOXL2 in IPF

Summary

Using a novel *in vitro* model of fibroblastic foci (Jones *et al.*, AJRCCM 191;2015:A4912) we have characterised the formation of LOX mediated collagen cross-links and profiled the effects of the non-selective LOX inhibitor β-aminopropionitrile (β-APN) and compound A, a LOXL2-selective inhibitor.

Results

Histochemical analysis showed structural similarity of the *in vitro* model to fibroblastic foci *in vivo.* The number of immature and mature LOX family-mediated collagen cross-links increased over the 6 week duration of the model. Both β-APN and compound A dose-dependently reduced cross-link formation. Second harmonic generation imaging revealed that collagen fibrils were less organised in these cultures compared to controls. Early data indicates reduced tissue stiffness associated with cross-link inhibition.

Overview of Fibroblastic Focus Model

Lung fibroblasts obtained from IPF patients were seeded onto transwell membranes under optimised conditions for mature collagen matrix deposition in the presence of β-APN or LOXL2-selective inhibitors. Following treatment with transforming growth factor β1 (TGF-β1) multicellular foci formed which were histochemically similar in organisation to fibroblastic foci *in vivo*.

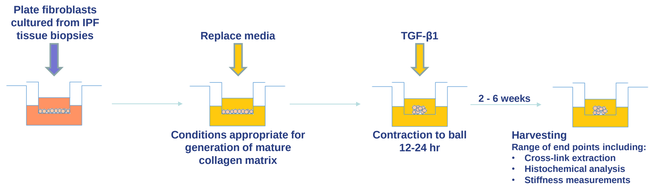

Figure 2: Experimental protocol (Jones et al., AJRCCM 191;2015:A4912)

Characterisation of LOXL2 selective inhibitors

Pharmaxis Ltd are developing first-in-class, mechanism-based small molecules with drug like properties that selectively inhibit Lysyl-Oxidase Like 2 (LOXL-2) for the treatment of fibrotic diseases. Compound A is a tool compound with greater than 300-fold selectivity for LOXL2 over LOX.

LOX enzyme	Cell-free IC_{50} (nM)
LOX	1260
LOXL1	3160
LOXL2	3.98
LOXL3	15.9
LOXL4	251

Table 1: Comparative cell-free IC_{50} values for compound A

Fibroblastic Focus Model Histochemical analysis

Method

IPF lung tissue biopsy portions were formalin fixed and embedded in paraffin. Fibroblastic focus model samples were flash frozen in liquid nitrogen. 7µm sections were stained with Masson's Trichrome stain (Abcam), imaged using the Olympus DotSlide at x40 objective.

Results

Histochemical analysis (Fig. 3) shows similar organisation of collagen matrix in the *in vitro* model and fibroblastic foci *in vivo.*

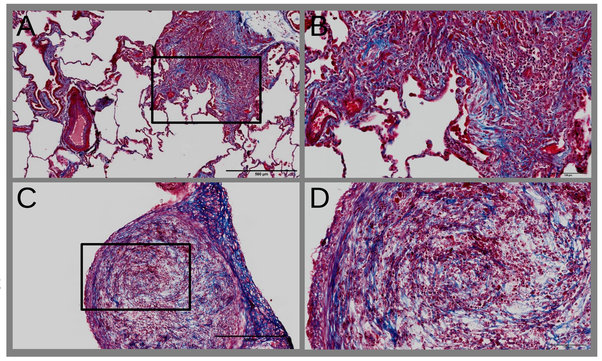

Figure 3: Immunohistochemical characterisation of IPF lung tissue (A, and inset B) and a section of the model (C and inset D). Masson's Trichrome stain: Blue: Collagen; Red: cytoplasm; Black: Nuclei.

Characterisation of cross-link formation

Method

Cultures were harvested and treated with potassium borohydride (0.4mg/mL, pH7.6) to stabilise the reducible immature cross-links, and hydrolysed in 6N HCl at 100ºC for 16hr. Total collagen content was assessed by hydroxyproline assay (Sigma), and normalised to total protein content measured by Genipin blue amino acid assay (QuickZyme). Immature cross-links were assessed by LC/MS/MS (Pharmaxis), and mature Pyridinoline cross-links by ELISA (Quidel Corp). Cross-link data is expressed as moles of cross-link per mole collagen.

Results

Total collagen content normalized to total protein increased over the six weeks of culture (Fig. 4A). The immature cross-link DHLNL accumulated over the first four weeks of culture up to around 1 mole DHLNL per mole collagen (Fig. 4B), and this was followed by delayed accumulation of mature pyridinoline (Pyd) and deoxy-pyridinoline (DPD) cross-links (Fig. 4C).

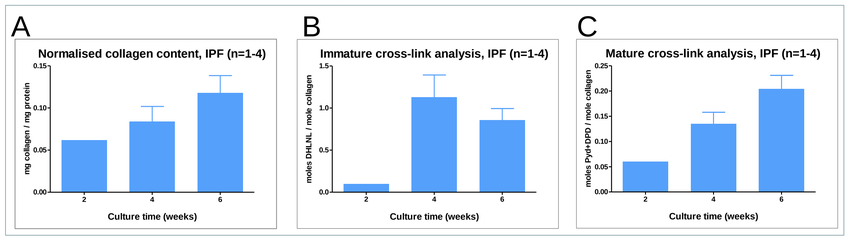

Figure 4: Time course of collagen deposition (A), immature DHLNL (B) and mature Pyd/DPD (C) cross-link formation (mean +SEM)

Inhibition of cross-link formation

Method

After 6 weeks of continuous treatment with TGF-β1 in the presence of β-APN or a dose range of compound A, cultures were harvested and processed as described above.

Results

There was no change in collagen deposition in response to the inhibitors (Fig. 5A). Immature DHLNL cross-links (Fig. 5B) and mature Pyd/DPD cross-links (Fig. 5C) were completely inhibited by 1mM β-APN, and were inhibited in a dose-dependent manner by compound A. Formation of cross-links was significantly reduced at doses below the IC_{50} for LOX, at which LOXL2 is selectively inhibited.

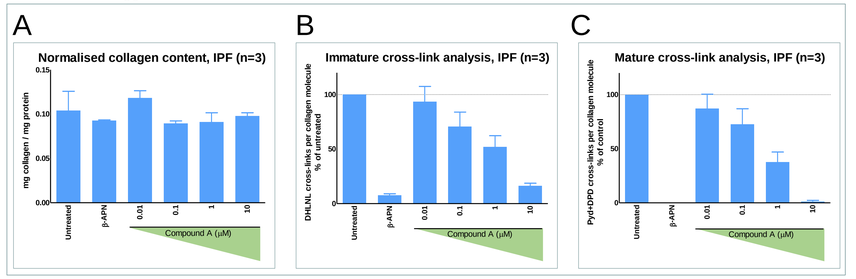

Figure 5: Inhibition of collagen cross-links by 1mM β-APN and compound A. Collagen deposition (A), Immature DHLNL cross-links (B) and mature Pyd/DPD cross-links (C) (mean +SEM)

Second Harmonic Generation imaging

Method

7µm sections of cryopreserved cultures were imaged by second harmonic generation imaging on a laser scanning microscope using a femtosecond Ti/sapphire laser with excitation and emission wavelengths of 800nm and 400nm respectively.

Results

The regular arrangement of collagen fibrils observed in the untreated sample appeared dysregulated at high doses of β-APN and compound A. Further work is required to validate this finding.

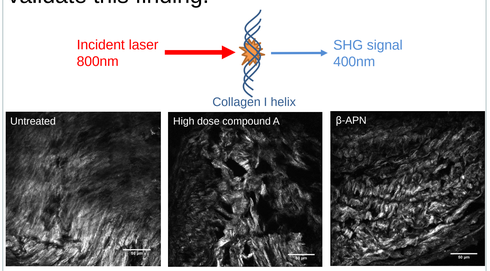

Figure 6: Second Harmonic Generation imaging

Atomic Force Microscopy

Method

50µm sections of cryopreserved cultures were subjected to micro-indentation atomic force microscopy (µi-AFM).

Results

Treatment with 1mM β-APN resulted in a significant reduction in micron-scale tissue stiffness (figure 7). Work is ongoing to determine changes in tissue stiffness in response to LOXL2 selective inhibition.

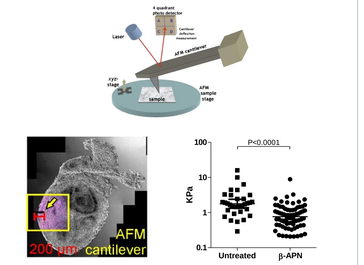

Figure 7: Atomic Force Microscopy

Conclusion: We have shown that LOXL2 selective inhibitors can reduce cross-link formation in a fibroblastic focus model of IPF, demonstrating their potential as a novel treatment for IPF. It is hypothesised that reducing collagen cross-linking will reduce tissue stiffness, tipping the balance in favour of matrix breakdown over deposition, beneficially altering the course of disease.

General conflicts of Interest:
Authors who are Synairgen employees have received share options. Synairgen is in partnership with Pharmaxis for development of LOXL2 selective small molecules as a treatment for IPF.