Quantitation of hydroxyproline in a mouse model of bleomycin-induced lung fibrosis: comparison with histological analysis

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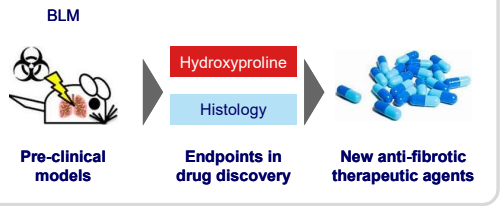
AIM



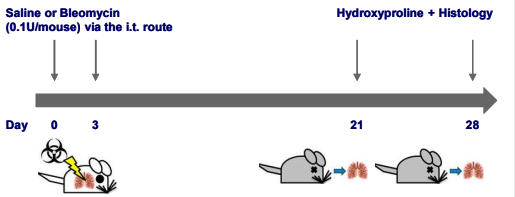
Biochemical evaluation of lung collagen content is considered one of the gold standard endpoints in the preclinical assessment of novel therapeutic agents for IPF.

For the evaluation of collagen levels in lung tissues, several methodologies have been described, including histological analysis, gene expression, HPLC and colorimetric assays. However, some of these techniques may not always reflect the lung collagen accumulation and fibrosis in *in vivo* models. For instance, some assays allow only the detection newly formed collagen, not taking into account the insoluble collagen of the scar tissue fraction[1]. We aimed to validate a robust and reproducible hydroxyproline technique that allows accurate assessment of collagen deposition.

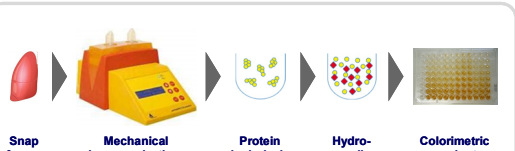
For this purpose, we analysed sample stability, intra-assay precision and linearity of samples used in the hydroxyproline assay.

Finally, we correlated the validated biochemical readout with histological analysis.

METHODS



***In vivo* Bleomycin model.** Lung samples were obtained from an *in vivo* model in which bleomycin is administered twice to male C57BL/6J mice. On days 21 and 28, animals were euthanized and the right lobe was snap-frozen for hydroxyproline assay, while the left lobe was fixed and embedded in paraffin for histological analysis.



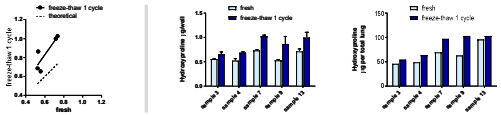
Hydroxyproline assay procedure. For hydroxypyproline assessment, lung samples were homogenised in ddH2O using gentleMACS Dissociator (Miltenyi Biotech). 12M HCl was added 1:1 to lung homogenate and samples were incubated at 120°C for 3h on a heating platform. Evaporation was prevented by the use of appropriate glass vials and screw caps, limiting sample loss to <10%.
Hydroxyproline assay was then carried out according to kit manufacturer instructions (Sigma Aldrich).

1 RESULTS – Hydroxyproline assay validation

A. Sample stability of lung homogenates

Lung homogenates

Sample #	3	4	7	9	13
Mean (fresh)	0.556	0.525	0.735	0.531	0.717
CV (%)	0.4	7.1	1.3	0.9	6.3
Mean (frozen)	0.655	0.687	1.026	0.864	1.000
CV (%)	7.7	7.7	2.6	17.9	10.3
Δ / fresh (%)	17.822	30.887	39.524	62.771	39.400

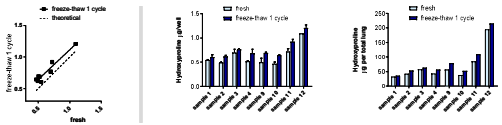


Sample stability during freeze-thaw in lung homogenates. Sample stability assessment was performed on lung homogenate samples analysed fresh and after 1 freeze-thaw cycle. Hydroxyproline levels were significantly increased after one freeze-thaw cycle when compared to fresh material (Table A).

B. Sample stability of hydrolysed samples

Hydrolyzed samples

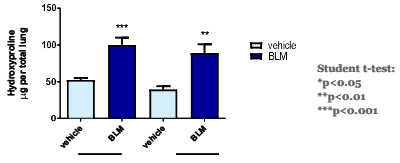
Sample #	1	2	3	4	9	10	11	12
Mean (fresh)	0.546	0.493	0.698	0.523	0.502	0.469	0.720	1.094
Mean (frozen)	0.604	0.618	0.768	0.686	0.695	0.644	0.920	1.207
Δ / fresh (%)	10.5	25.2	9.9	31.1	38.5	37.2	27.8	10.3



Sample stability during freeze-thaw in hydrolysed samples. Sample stability assessment was performed on hydrolysed samples analysed fresh and after 1 freeze-thaw cycle. Hydroxyproline levels were significantly increased after one freeze-thaw cycle when compared to fresh material (Table B).

3 RESULTS – Hydroxyproline assay in the Bleomycin model

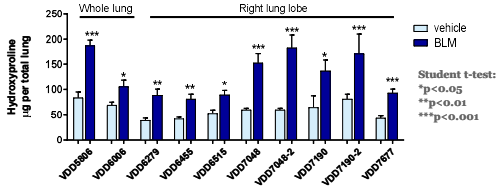
Hydroxyproline assay time-course study



Student t-test:
*p<0.05
**p<0.01
***p<0.001

Bar graph showing hydroxyproline levels per total lung in an *in vivo* time-course study at day 21 and day 28. BLM-treatment induced a significant increase in hydroxyproline levels compared to controls at both time points analysed.

Hydroxyproline assay at 28 days



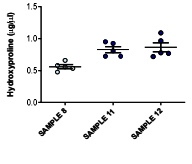
Student t-test:
*p<0.05
**p<0.01
***p<0.001

Manhattan plot showing hydroxyproline levels per total lung at 28 days in different studies

2 RESULTS – Hydroxyproline assay validation

A. Intra-assay precision

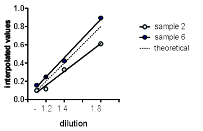
	Mean result	St.Dev.	CV%	Mean result	St.Dev.	CV%
Sample 6 (vehicle)	0.535	0.014	2.6	0.54	0.04	8.2
	0.558	0.021	3.7			
	0.579	0.024	4.2			
Sample 11 (BLM)	0.477	0.075	15.7	0.85	0.11	12.4
	0.725	0.036	4.9			
	0.927	0.163	17.6			
	0.809	0.001	0.1			
	0.952	0.133	13.9			
Sample 12 (BLM)	0.968	0.215	22.2	0.81	0.11	13.5
	0.77	0.037	4.8			
	0.72	0.008	1.1			
	0.778	0.094	12.1			



Intra-assay. Intra-assay validation shows the reproducibility between different hydrolysed samples within an assay plate. Intra-assay variation was evaluated by testing 4 replicates each for three samples in the same assay (Table A). The resulting low CV% for each sample indicates good reproducibility within the assay.

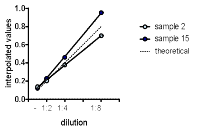
B. Linearity on lung homogenates

Dilution factor (Theoretical)	Sample 2 Mean	Sample 2 Dilution factor (measured)	Sample 6 Mean	Sample 6 Dilution factor (measured)
Undiluted	0.611	–	0.8945	–
2	0.327	1.9	0.424	2.1
4	0.118	5.2	0.249	3.6
8	0.100	6.1	0.158	5.7



C. Linearity on hydrolysed samples

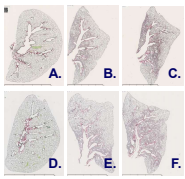
Dilution factor (Theoretical)	Sample 2 Mean	Sample 2 Dilution factor (measured)	Sample 15 Mean	Sample 15 Dilution factor (measured)
Undiluted	0.699	–	0.952	–
2	0.378	1.85	0.461	2.07
4	0.204	3.4	0.228	4.1
8	0.136	5.1	0.121	7.8



Linearity. Lung homogenates (Table B) and hydrolysed samples (Table C) were diluted in water across the dynamic range of the assay. The measured interpolated values versus expected values show good assay linearity up to 1 in 4 dilution and a significant interference from sample matrices on samples diluted 1 in 8.

4 RESULTS – Histological Analysis in the Bleomycin model

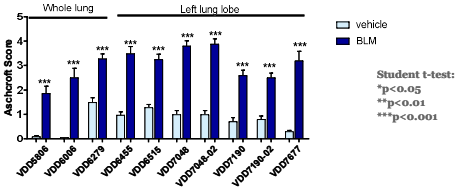
Histological analysis at 21 days and 28 days



Ashcroft scoring in representative images at day 21 and day 28. Sections were stained with picrosirius red and fast green and imaged through a Hamamatsu NanoZoomer digital slide scanner. Parenchymal fibrosis was evaluated according to the modified Ashcroft method[2].

A. 21 days, saline, mean score 0
B. 21 days, BLM, mean score 3.9
C. 21 days, BLM, mean score 4.4
D. 28 days, saline, mean score 0.2
E. 28 days, BLM, mean score 3.8
F. 28 days, BLM, mean score 4.7

Histological analysis at 28 days



Student t-test:
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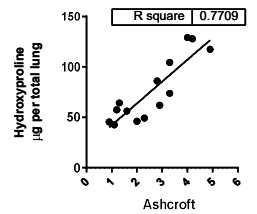
Manhattan plot showing Ashcroft scoring at 28 days in different studies

5 RESULTS – Correlation between hydroxyproline levels and Ashcroft scoring

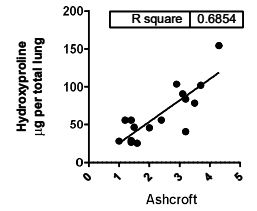
DAY 21

A



DAY 28

B



Linear regression of hydroxyproline levels measured as µg per total lung versus Ashcrosft scoring of vehicles and bleomycin treated mice at **A.** day 21 (R^2=0.77) and **B.** day 28 (R^2=0.68).

CONCLUSIONS

- Extensive validation of the hydroxyproline assay has been performed, identifying the best working conditions for the assessment of hydroxyproline levels in lung homogenates
- Stability studies showed signal alteration of hydroxyproline levels after one thawing cycle of frozen lung homogenate and in hydrolysed samples when compared to fresh samples. The use of fresh material is thus highly recommended
- Intra-assay precision studies showed good reproducibility of data within the same assay and in different studies
- Linearity studies suggests that lung homogenates or hydrolysed samples should be used undiluted or diluted up to 1 in 4
- BLM-treatment induced a reproducible ~2-fold increase in hydroxyproline compared to controls
- Hydroxyproline and Ashcroft scoring endpoints showed a good degree of correlation in the same animals (R^2: 0.77 and 0.68 at 21 and 28 days respectively)
- **The hydroxyproline assay can be used as a reliable method of quantification of fibrosis, correlating with the modified Ashcroft score, in preclinical studies investigating the effects of current and potential new therapeutic agents**

REFERENCES

- [1] Jenkins R.G. et al. 2017, Am J Respir Cell Mol Biol Vol 56, 5:667–679.
- [2] Hübner et al. 2008, BioTechniques 44:507-517.

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