Pharmacometabolic response to pirfenidone treatment in pulmonary fibrosis detected by high resolution MALDI-FTICR-mass spectrometry imaging

Na Sun[1+], Isis E. Fernandez[2+], Mian Wei[1], Michael Witting[3], Michaela Aichler[1], Annette Feuchtinger[1], Gerald Burgstaller[2], Stijn Verleden[4], Philippe Schmitt-Kopplin[3], Oliver Eickelberg[2,5*], Axel Walch[1*]

[1]Research Unit Analytical Pathology, Helmholtz Zentrum München, German Research Centre for Environmental Health (GmbH), Neuherberg, Germany

[2]Comprehensive Pneumology Centre, Helmholtz Zentrum München, Ludwig Maximilian University München. Member of the German Centre for Lung Research (DZL), München, Germany

[3]Research Unit Analytical BioGeoChemistry, Helmholtz ZentrumMünchen, German Research Centre for Environmental Health (GmbH), Neuherberg, Germany

[4]Leuven Lung Transplant Unit, KU Leuven and UZ Gasthuisberg, Leuven, Belgium

[5]Division of Respiratory Sciences and Critical Care Medicine, Department of Medicine, University of Colorado, Denver, CO, USA.

*To whom correspondence should be addressed: Oliver Eickelberg: oliver.eickelberg@ucdenver.edu, Axel Walch: axel.walch@helmholtz-muenchen.de

[+*] These authors contributed equally.

Supplementary information

Bleomycin-induced mouse model of pulmonary fibrosis and pirfenidone treatment

For this study, 8- to 10-week-old female C57BL/6N mice (Charles River Laboratories) were used for the experiments, which were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Pulmonary fibrosis in mice was established as previously described (1). Briefly, bleomycin (3 U/kg) was dissolved in sterile phosphate-buffered saline (PBS) and applied intratracheally as a single dose to C57BL/6N mice. Control mice were given PBS. From day 7 to day 14, experimental animals were administered pirfenidone (Selleckchem, Houston, TX, USA) in carboxymethyl cellulose (CMC) by oral gavage daily. We used a dose of 500 mg/kg body weight pirfenidone, based on literature reports (2, 3). Control mice received CMC only. All animals (control=3, bleomycin=3, pirfenidone treatment=3) were sacrificed on day 14. The lungs were rapidly dissected, fresh-frozen in liquid nitrogen, and stored at -80°C until sectioned.

MADLI-MSI tissue preparation

The frozen tissues were cut into 12-µm thick sections at -20°C using a microtome (Leica CM1950, Leica Microsystems, Germany). The sections were thaw-mounted onto indium tin oxide -coated MALDI target slides (Bruker Daltonics, Bremen, Germany) and stored at -80°C until further analysis.

For endogenous metabolite MALDI-MSI, the matrix solution was prepared by dissolving 9-aminoacridine hydrochloride monohydrate matrix (Sigma-Aldrich, Germany) in 70% methanol to a final concentration of 10 mg/ml. The matrix solution was sprayed by a SunCollect^{TM} automatic sprayer (Sunchrom, Friedrichsdorf, Germany). The flow rate was 10, 20, 30, and 40 µl/min for the first four layers, respectively. The next four layers were sprayed at 40 µl/min. For MALDI-MSI of pirfenidone and the related metabolites 5-hydroxymethyl pirfenidone and 5-carboxy pirfenidone, a 30 mg/ml super-DHB matrix (Sigma-Aldrich) in 50% methanol containing 0.2% trifluoroacetic acid was applied using an automated spray coating machine, ImagePrep (Bruker Daltonics). Following the MALDI imaging experiments, the matrix was removed by washing with 70% ethanol. Then, slides were quickly dipped in distilled water and stained with H&E. Slides were scanned with a MIRAX DESK digital slide-scanning system (Carl Zeiss MicroImaging, Göttingen, Germany).

Data processing and statistical analysis

Data processing

The acquired MSI data underwent spectra processing with FlexImaging v. 4.0 software (Bruker Daltonics). The raw data were normalized against the root mean square of all data points. The average spectra of defined regions of interest were exported as .csv files and subsequently processed by MATLAB R2013a (Mathworks, Natick, MA, USA). A self-implemented MATLAB analysis pipeline was performed as previously described (4), (5), (6) . Basically, baseline correction was performed by interpolating between given equally spaced intervals (spacing 0.02 Da). Resampling was performed using a 0.005-Da step width, and the smoothing operation was carried out using a size 3 Kaiser-Window. Using an adapted version of the LIMPIC algorithm (7), peaks were picked using a minimal peak width of 0.0005, a noise-threshold of 2, and an intensity threshold percentage of 0.01. Isotopes were excluded. To identify statistically significant differences in *m/z* values, the peak lists were analysed using the Student's *t*-test (unpaired, two-tailed) and Benjamini-Hochberg *post hoc* test (alpha=0.05). As a result, a list of significantly different metabolites could be achieved with a corresponding *p*-value of ≤ 0.05 and intensity fold-change of ≥ 1.5.

Relative quantification of endogenous metabolites

The comparison of relative abundance of endogenous metabolites between different groups was performed using One-Way analysis of variance, with followed by Bonferroni test. Data is presented as bars or whiskers vertical graphs with mean ± standard deviation.

Unsupervised hierarchical clustering

Hierarchical clustering was performed using the built-in FlexImaging v. 4.0 (Bruker Daltonics, Germany). Similar spectra are grouped using multivariate statistical analysis (8), (9). The created segmentation maps were used to identify areas in which similar spectra occur across the tissue sample. List of m/z species with respective intensities for selected regions were uploaded to MetaboAnalyst, processed with a mass tolerance of m/z 0.0001 and no data filtering. Heatmap-based clustering was created with MetaboAnalyst (http://www.metaboanalyst.ca) (10).

Metabolite identification by LC-MS

The targeted metabolite identification by LC-MS was performed as described previously (5). Briefly, 30 mg of lung tissue was lysed with 1 ml cold 80% MeOH/20% water in NucleoSpinBeadTube (Macherey Nagel, Düren, Germany) for 3 min at a frequency of 30 Hz using a TissueLyser (Qiagen, Hilden, Germany). The supernatant was collected by centrifugation at 17,900 x *g* at 4°C for 15 min. Then, 10 µl of tissue sample and 10 µg/ml of single metabolite standards were injected into an XBridge Amide column (100 mm x 4.6 mm ID, 3.5 µm; Waters, Eschborn, Germany) via full-loop injection. LC-MS was performed on a Waters Acquity UPLC (Waters) coupled to maXis UHR-TOF-MS (Bruker Daltonics) using an established protocol (11). For confirmation of metabolite identity, the resulting mass spectra were compared against reference spectra from the measured standards.

References

1. Fernandez IE, Amarie OV, Mutze K, Konigshoff M, Yildirim AO, Eickelberg O. Systematic phenotyping and correlation of biomarkers with lung function and histology in lung fibrosis. American journal of physiology Lung cellular and molecular physiology 2016; 310: L919-927.
2. Schaefer CJ, Ruhrmund DW, Pan L, Seiwert SD, Kossen K. Antifibrotic activities of pirfenidone in animal models. European respiratory review : an official journal of the European Respiratory Society 2011; 20: 85-97.
3. Sun N, Fernandez IE, Wei M, Wu Y, Aichler M, Eickelberg O, A. W. Pharmacokinetic and pharmacometabolomic study of pirfenidone in normal mice tissues using high mass resolution MALDI-FTICR-mass spectrometry imaging. Histochemistry and cell biology 2015.
4. Aichler M, Borgmann D, Krumsiek J, Buck A, MacDonald PE, Fox JEM, Lyon J, Light PE, Keipert S, Jastroch M, Feuchtinger A, Mueller NS, Sun N, Palmer A, Alexandrov T, Hrabe de Angelis M, Neschen S, Tschop MH, Walch A. N-acyl Taurines and Acylcarnitines Cause an Imbalance in Insulin Synthesis and Secretion Provoking beta Cell Dysfunction in Type 2 Diabetes. Cell metabolism 2017; 25: 1334-1347 e1334.
5. Sun N, Ly A, Meding S, Witting M, Hauck SM, Ueffing M, Schmitt-Kopplin P, Aichler M, Walch A. High-resolution metabolite imaging of light and dark treated retina using MALDI-FTICR mass spectrometry. Proteomics 2014; 14: 913-923.
6. Buck A, Ly A, Balluff B, Sun N, Gorzolka K, Feuchtinger A, Janssen KP, Kuppen PJ, van de Velde CJ, Weirich G, Erlmeier F, Langer R, Aubele M, Zitzelsberger H, Aichler M, Walch A. High-resolution MALDI-FT-ICR MS Imaging for the analysis of metabolites from formalin-fixed paraffin-embedded clinical tissue samples. The Journal of pathology 2015.
7. Mantini D, Petrucci F, Pieragostino D, Del Boccio P, Di Nicola M, Di Ilio C, Federici G, Sacchetta P, Comani S, Urbani A. LIMPIC: a computational method for the separation of protein MALDI-TOF-MS signals from noise. BMC bioinformatics 2007; 8: 101.
8. Alexandrov T, Becker M, Deininger SO, Ernst G, Wehder L, Grasmair M, von Eggeling F, Thiele H, Maass P. Spatial segmentation of imaging mass spectrometry data with edge-preserving image denoising and clustering. Journal of proteome research 2010; 9: 6535-6546.
9. Alexandrov T, Becker M, Guntinas-Lichius O, Ernst G, von Eggeling F. MALDI-imaging segmentation is a powerful tool for spatial functional proteomic analysis of human larynx carcinoma. Journal of cancer research and clinical oncology 2013; 139: 85-95.
10. Xia J, Wishart DS. MetPA: a web-based metabolomics tool for pathway analysis and visualization. Bioinformatics 2010; 26: 2342-2344.
11. Yuan M, Breitkopf SB, Yang X, Asara JM. A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. Nature protocols 2012; 7: 872-881.