

-Supplementary material-

Pyrrolizidine alkaloid-induced transcriptomic changes in rat lungs in a 28-day subacute feeding study

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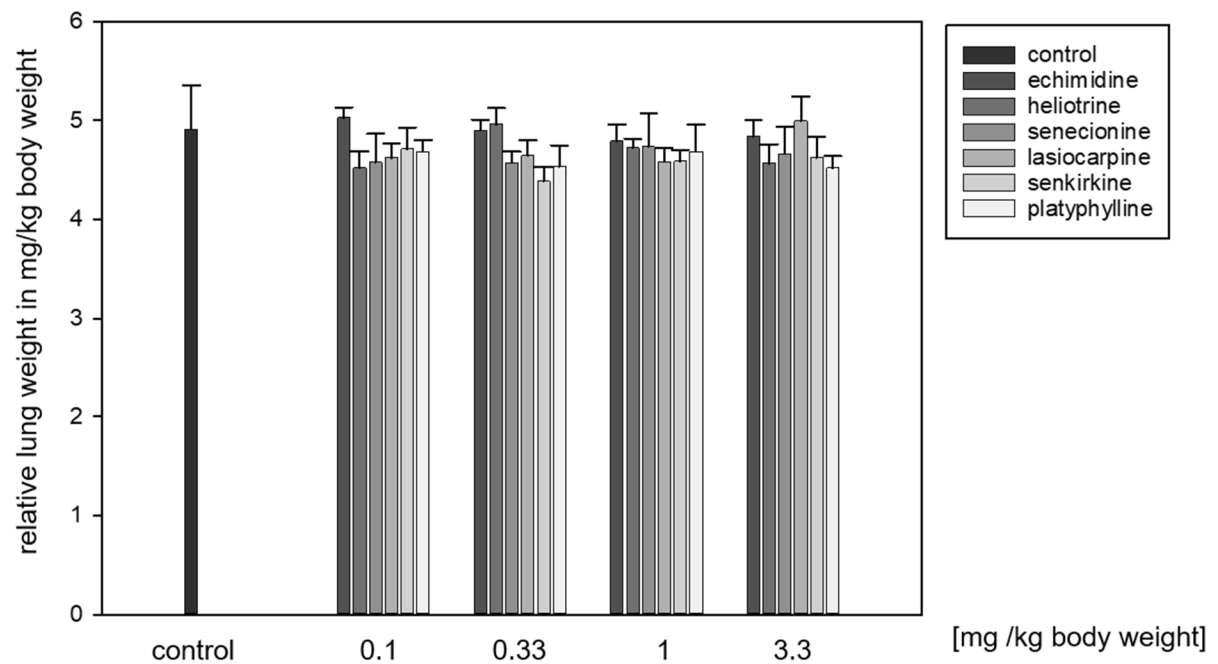


Figure S1: Relative lung weights of rats treated for 28 days with six different PAs. Rats were treated by gavage daily with 3.3 mg/ kg body weight of echimidine, heliotrine, senecionine, lasiocarpine, senkirkine or platyphylline for 28 days. After sacrifice, lungs were extracted and weighed. The bars show the relative organ weights in comparison to the control group.

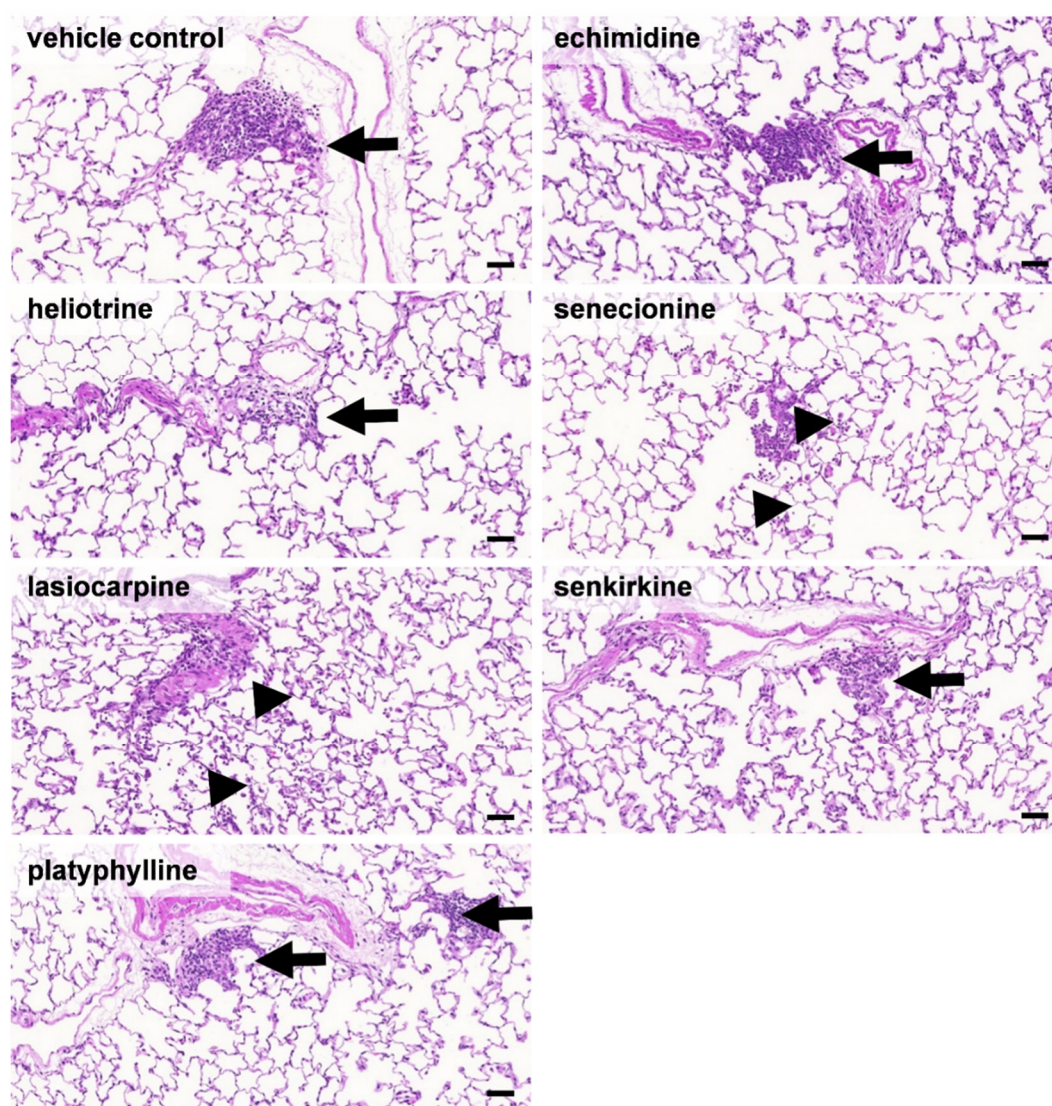


Figure S2: Histological examination of rat lung tissue sections of rats treated daily with 3.3 mg/kg body weight of six different pyrrolizidine alkaloids or vehicle control by oral gavage for 28 days. The lung sections were stained with hematoxylin and eosin. The arrows highlight signs of perivascular mononuclear cell infiltration. The black triangles indicate multifocal mixed inflammatory cells. Bar = 50 μm .

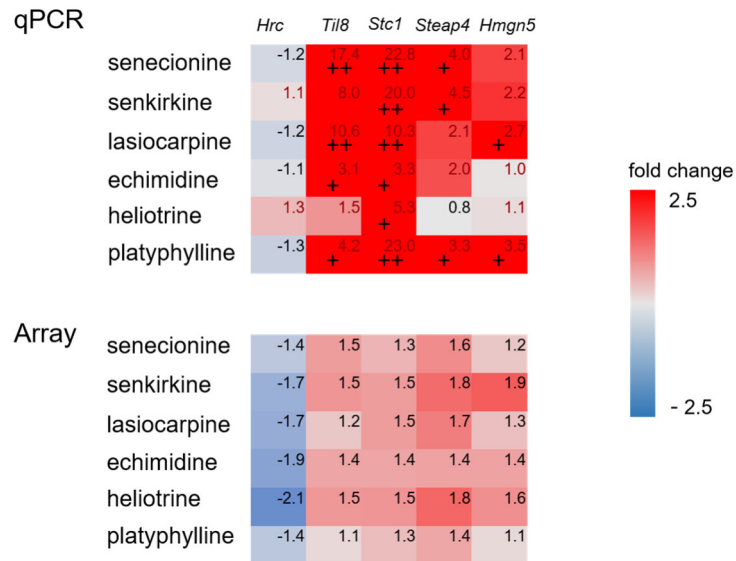


Figure S3: Verification of PA-induced regulation of gene expression. RNA from lungs of rats treated with 3.3 mg PA/kg body weight by gavage daily for 28 days was extracted and the expression of genes was investigated by microarray or qPCR. The qPCR was done by reverse transcribing the RNA into cDNA before adding the appropriate primers and SYBR Green/ROX mix for the qPCR. The results were normalized to housekeeping genes. Fold changes of relative gene expression from four animals are depicted. Fold change values that are out of the color range are additionally marked with + (+ = values up until 10, ++ = values up until 23).

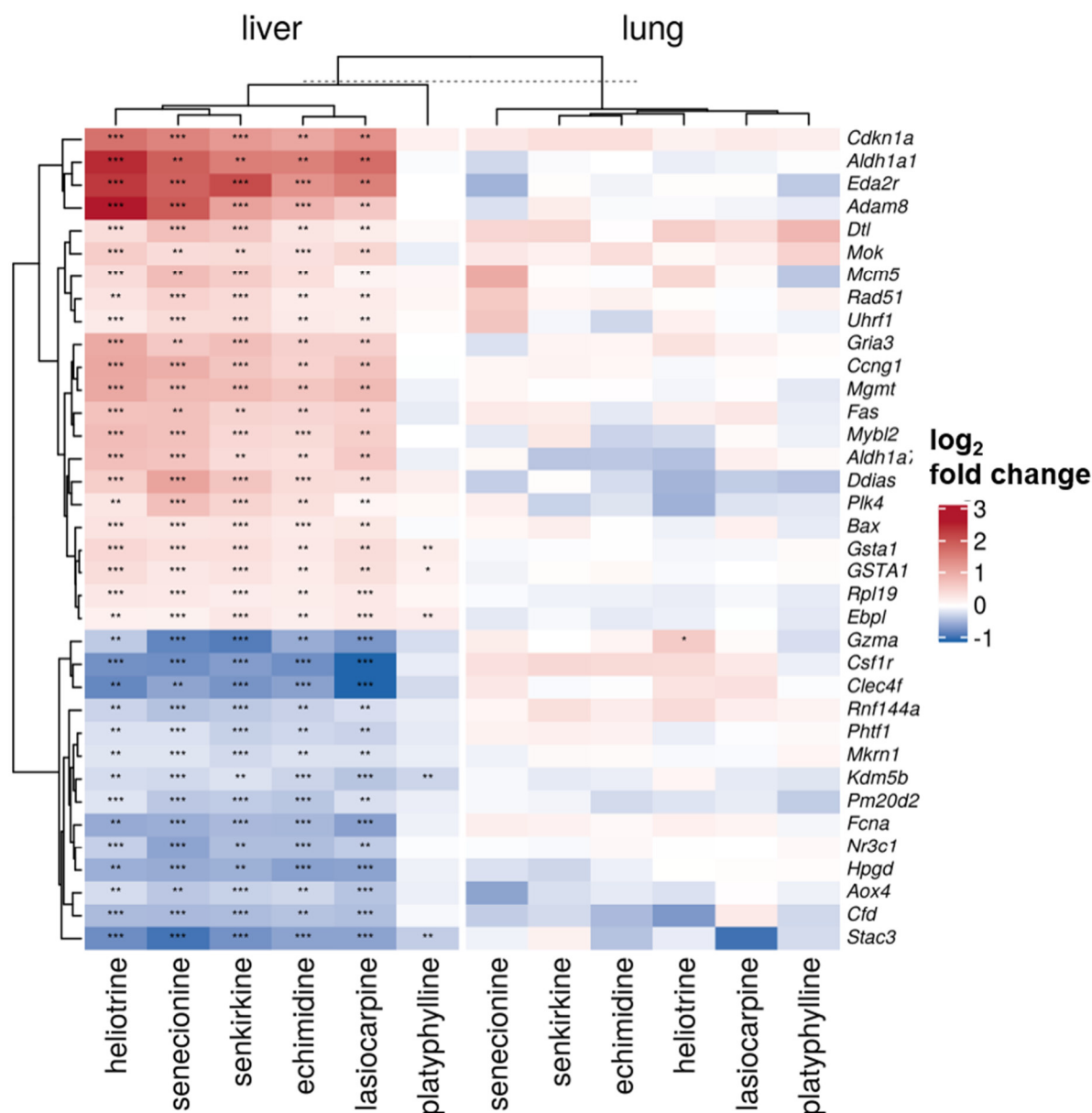


Figure S4: Comparison of gene regulation in liver and lung. Rats were treated by gavage for 28 days daily with 3.3 mg PA/kg body weight. The RNA of lungs was extracted and whole genome microarray analysis was performed. The data were compared to data previously published by Ebmeyer et al. (2020). They found 36 genes commonly significant regulated in all treatment groups in the liver (except for platyphylline). These genes were used for the comparison between the two organs. The heatmap shows the log₂ fold change of this gene expression in both organs. Significance of gene expression alteration in the lung is indicated (*) with the definitions of *p<0.05, ** p<0.01, ***p<0.001.

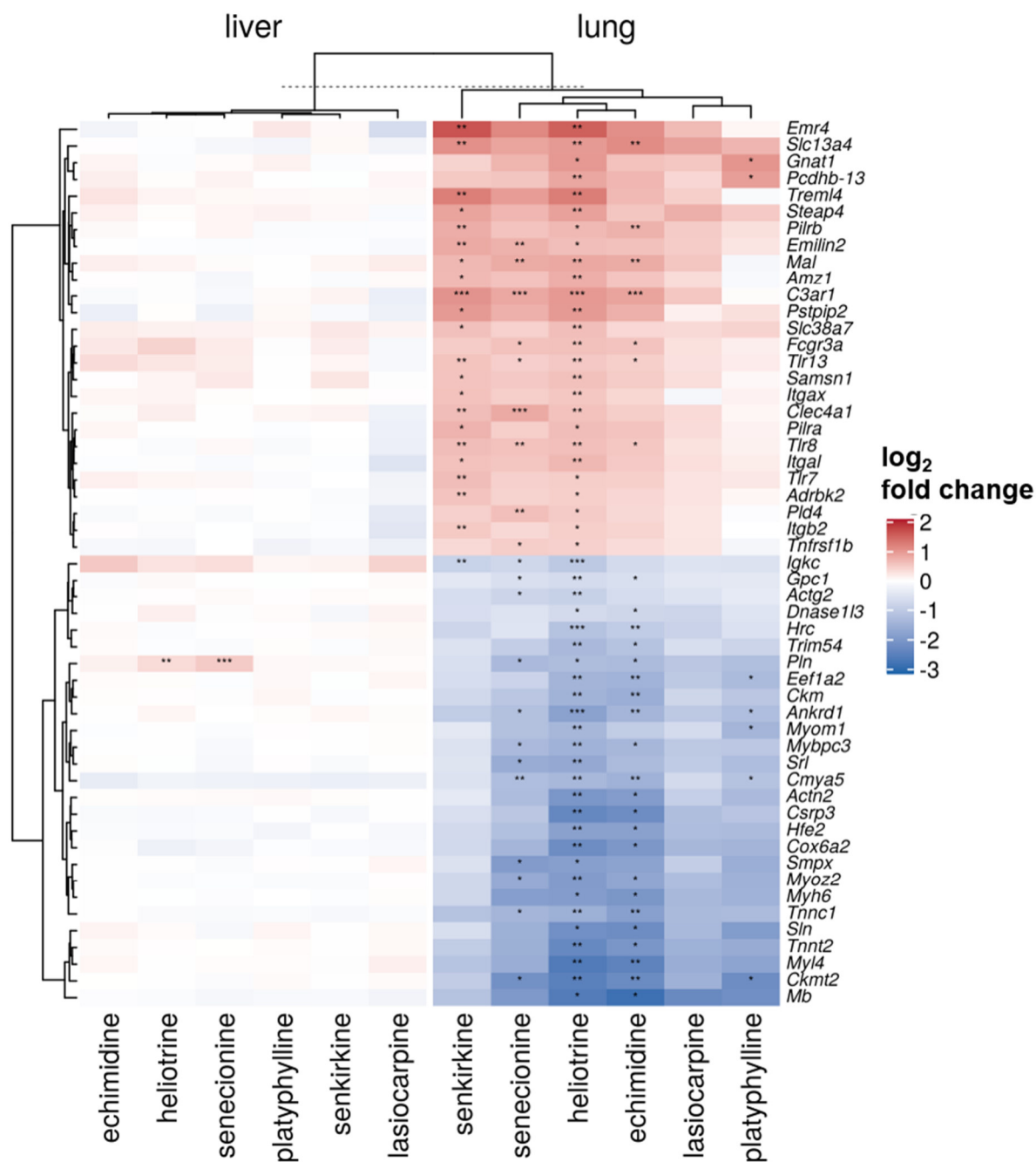


Figure S5: Comparison of gene regulation in lung and liver. Rats were treated by gavage for 28 days daily with 3.3 mg PA/kg body weight. The RNA of lungs was extracted before a whole genome microarray analysis was performed. The data were compared to data previously published by Ebmeyer et al. (2020). Thereby, genes showing significant regulation in two treatment groups in the lung were chosen. The heatmap shows the log₂ fold change of the gene expression in the lung and liver. Significance of gene expression changes is indicated as follows: p_{adj} < 0.1, ** p_{adj} < 0.05, *** p_{adj} < 0.01.

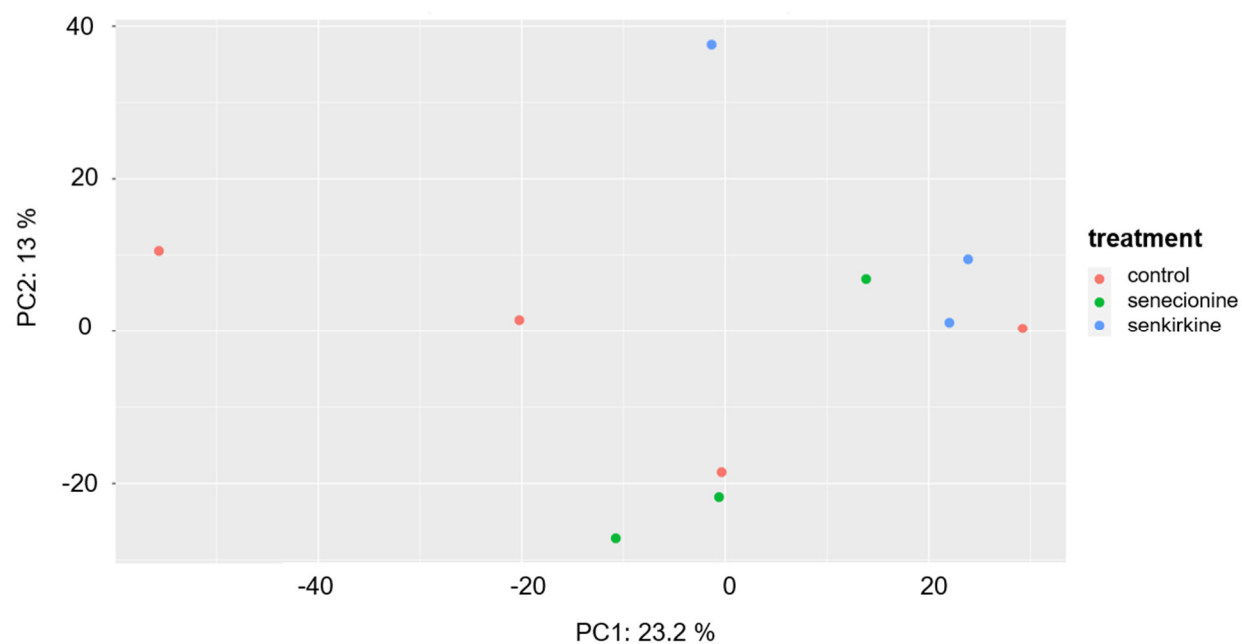


Figure S6: PCA scores plot of genes in kidneys from rats treated orally with 3.3 mg/kg body weight of senecionine or senkirkine for 28 days. The RNA was extracted from the kidneys before performing a whole genome microarrays analysis as described in material and methods section.

Table S1: Gene expression data of genes involved in inflammatory responses in mouse lungs.
Mice were treated with different doses of senecionine for 36 h or with 60 mg/kg body weight for different time intervals as described in more detail in Hessel-Pras et al., 2020.

	[mg/kg body weight]					[h]						fold change
	control	15	30	60		control	12	24	36	48	72	
<i>Cxcl12</i>	1.0	1.8	-1.1	-1.4		1.0	-1.2	-1.4	-1.9	-2.3	-2.0	
<i>Cxcl13</i>	1.0	-1.2	-1.2	2.7		1.1	2.0	-2.8	-1.2	-3.0	-1.2	
<i>Lcn2</i>	1.0	-4.0	-1.0	-2.9		1.1	-1.1	-2.4	-4.7	-2.4	-1.6	
<i>Saa3</i>	1.0	1.8	-1.2	-2.4		1.1	-1.6	-1.4	-2.4	-1.2	-1.2	

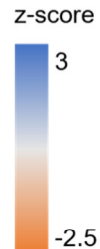
Table S2: Relative expression data for genes that are known to play a role in the progression of PAH. Rats were treated daily with 3.3 mg PA/kg body weight for 28 days. The RNA of the lungs was extracted and the gene expression was analyzed with microarray analysis as described in material and methods section.

gene	senecionine	senkirkine	platyphylline	lasiocarpine	echimidine	heliotrine
<i>Kcnk3</i>	0.6	1.0	1.0	0.9	0.9	1.0
<i>Notch1</i>	0.9	0.9	1.1	1.0	1.0	1.1
<i>Smad9</i>	0.9	1.1	1.0	1.1	1.0	1.1
<i>Nos3</i>	1.1	1.2	1.0	1.3	1.1	1.1
<i>Sirt3</i>	0.8	1.0	1.0	0.9	1.0	0.8
<i>Agtr1a</i>	0.9	0.9	1.0	1.0	1.1	1.1
<i>Serpine1</i>	1.6	1.2	1.1	1.1	1.1	1.1
<i>Edn1</i>	1.6	1.2	1.1	1.3	1.1	1.2
<i>Tbx4</i>	1.0	1.1	1.0	1.2	1.2	1.2
<i>Trpc6</i>	1.1	1.1	1.2	1.1	1.1	1.1
<i>Thbs1</i>	1.1	1.1	1.1	1.1	1.0	1.0

fold change

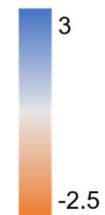
Table S3: Complete list of predicted diseases and biofunctions from IPA analysis. Rats were treated with 3.3 mg PAs per kg body weight for 28 days. RNA of lungs was extracted and used for micro array analysis. The significantly regulated genes were uploaded in IPA for the prediction of affected diseases and biofunctions. Indicated are the specific activation z-scores for each treatment.

diseases and biofunctions	senecionine	senkirkine	platyphylline	lasiocarpine	echimidine	heliotrine
Activation of leukocytes	2.0	1.8				1.9
Immune response of leukocytes	1.7	2.2			-0.3	2.4
Immune response of cells	1.9	2.4				2.6
Contractility of ventricular myocardium					-2.0	
Cardiac contractility	0.3				-1.7	-1.2
Blood pressure					-2.0	-1.4
Contractility of muscle	-0.8				-2.2	-2.0
Function of muscle					-2.0	-1.8
Contractility of cardiac muscle					-2.0	-1.7
Quantity of neutrophils		-1.6				-1.0
Quantity of phagocytes		-0.9				-1.7
Quantity of cytokine		-2.1				-2.4
Quantity of myeloid cells		-1.6				-1.6
Infection of mammalia		-1.7				-2.0
Quantity of protein in blood		-1.6				-2.0
Cell movement of leukocytes		1.1	-0.4			-0.4
Activation of cells	0.9	2.2				1.5
Inflammatory response	0.9	2.4				0.9
Response of myeloid cells		2.0			-0.2	2.0
Adhesion of immune cells		2.0				1.7
Activation of lymphocytes		1.3				2.0
Phagocytosis of cells		1.7				2.1
Activation of lymphoid cells		1.5				2.2
Activation of T lymphocytes		1.5				2.0
Aggregation of cells		2.2				
Cell movement		2.1				
Cell movement of neutrophils		2.1				
Recruitment of cells		2.0				
Aggregation of blood cells		2.0				
Cell movement of macrophages		2.0				
Activation of phagocytes		1.9				0.7



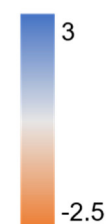
Cell movement of phagocytes		1.6			0.8
Cell movement of myeloid cells		1.6			1.0
Migration of cells		1.5			
Phosphorylation of protein		1.2			
Transport of molecule	0.3	1.3			
Cellular infiltration by myeloid cells		1.3			0.5
Cellular infiltration by phagocytes		1.3			0.5
Inflammation of body cavity		1.7			0.3
Inflammation of absolute anatomical region		1.6			0.5
Inflammation of organ		1.5			0.3
Synthesis of reactive oxygen species		1.1		2.0	2.1
Recruitment of phagocytes				1.2	2.2
Recruitment of myeloid cells				1.2	2.2
Failure of heart				2.0	2.2
Necrosis of muscle				1.6	1.9
Failure of heart				1.6	1.9
Necrosis of muscle				-0.7	1.7
Accumulation of leukocytes		0.0			0.9
Migration of phagocytes					0.9
Binding of mononuclear leukocytes					1.2
Antimicrobial response					1.2
Flux of Ca ²⁺					1.1
Accumulation of myeloid cells					1.1
Accumulation of phagocytes					1.1
Ion homeostasis of cells					1.4
Adhesion of blood cells					1.4
Interaction of blood cells					1.4
Activation of blood cells					1.5
Systemic autoimmune syndrome					1.7
Cytotoxicity of natural killer cells					1.7
Apoptosis of muscle cells					1.6
Recruitment of leukocytes					1.7
Interaction of leukocytes					1.7
Cell death of immune cells					2.6
Cell death of lymphocytes					2.5
Cell death of fibroblasts					2.4
Apoptosis of leukocytes				-0.4	2.2

z-score

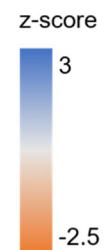


Apoptosis of lymphocytes					0.0	2.3
Endotoxycosis						2.2
Cytotoxicity of lymphocytes						2.2
Apoptosis of hematopoietic progenitor cells						2.2
Apoptosis of T lymphocytes						2.1
Cytolysis						2.0
Apoptosis of thymocytes						1.9
Binding of neutrophils						1.9
Fibrogenesis						1.9
Vascularization of absolute anatomical region						1.9
Migration of myeloid cells						2.0
Congestive heart failure						2.0
Cell-mediated response						2.0
Migration of neutrophils						2.0
Recruitment of neutrophils						2.0
Anemia						
Cell death of cancer cells						
Quantity of heavy metal						
Transport of metal						
Cell death of osteosarcoma cells						
Transport of heavy metal						
Fibrosis of heart					1.9	0.9
Fibrosis					1.9	0.7
Production of reactive oxygen species					2.0	
Necrosis					1.0	
Organismal death					1.4	
Morbidity or mortality					1.3	-0.1
Quantity of cells		-0.8				
Quantity of leukocytes		-1.5				
Apoptosis			-0.6		0.6	
Chemotaxis of granulocytes						
Leukocyte migration					0.2	0.1
Quantity of blood cells						
Differentiation of hematopoietic progenitor cells						
Cell death of pancreatic cancer cell lines						
Leukopoiesis			-0.2			
Homing of mononuclear leukocytes						
Cell proliferation of vascular endothelial cells						

z-score

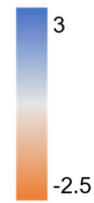


Endothelial cell development						
Proliferation of endothelial cells						
Relaxation of heart	-1.1					-1.1
Quantity of granulocytes						-2.0
Carditis						-1.5
Quantity of interleukin						-1.7
Cell movement of mononuclear leukocytes		0.4				-1.2
Quantity of TNF in blood						-1.2
Migration of mononuclear leukocytes						-1.0
Quantity of macrophages						-1.0
Cell spreading of blood cells						-1.0
Quantity of eosinophils						-1.0
Quantity of mononuclear leukocytes		-1.4				
Quantity of lymphocytes		-1.1				
Proliferation of lymphocytes		0.5	-1.1			
Activation of DNA endogenous promoter			1.2			
Contraction of heart	0.1				-0.6	-0.8
Cellular infiltration by leukocytes					-1.0	-0.5
Survival of organism					-1.1	-0.4
Engulfment of myeloid cells					-1.1	
Engulfment of leukocytes					-1.1	
Engulfment of phagocytes					-0.7	
Phagocytosis of myeloid cells					-0.7	
Phagocytosis of leukocytes					-0.7	
Infiltration by neutrophils		0.7				
Proliferation of immune cells		0.8				
Cell spreading		0.8				
Growth of tumor	0.7					
T cell development			-0.6			
Quantity of Ca ²⁺					0.4	
Quantity of metal					0.7	
Transcription of RNA			0.5			
Liver Damage						0.5



Mobilization of Ca ²⁺		0.0			-0.6
Quantity of antigen presenting cells					-0.4
Size of body					
Endocytosis by eukaryotic cells				-0.3	
Quantity of IgG		0.3			
Quantity of B lymphocytes		-0.2			
Secretion of molecule	-0.2				
Production of antibody		0.0			0.1
Cell movement of blood cells					-0.1
Activation of B lymphocytes					-0.2
Quantity of IL-6 in blood					-0.2
Transcription			0.1		
Expression of RNA			0.2		

z-score



Material and Methods:

Animal study

Briefly, 128 adult male Fischer rats (strain-344/DuCrI) were purchased from Charles River Deutschland (Sulzfeld, Germany) and divided randomly into eight groups (control: n = 8, others: n = 5). They were treated daily by oral gavage with the PAs echimidine, heliotrine, lasiocarpine, senecionine, senkirkine, platyphylline or vehicle (0.15 M NaCl). The rats were maintained under conventional laboratory conditions.

For this study, gene expression analysis in lungs and kidneys of five randomly chosen animals of the control group and four animals of each treatment group dosed with 3.3 mg PA/kg body weight was investigated. After 28 days the animals were killed, organ weights were determined and tissue samples for transcriptomics analysis and histopathological examination were prepared. Shock-frozen samples of lungs, livers and kidneys were used for transcriptomics analysis. For histopathological pulmonary examination the left lung lobe was fixed in formaline (10 %), sliced in pieces with maximum 7 mm diameter and stained with hematoxylin and eosin.

Additionally, mice were treated with senecionine at the Leibniz Research Centre for Working Environment and Human Factors. Mice were treated once with 60 mg per kg body weight and killed after 12 h, 24 h, 36 h, 48 h and 72 h or with doses of 15, 30 and 60 mg per kg bodyweight and killed after 36 h. Lungs were extracted after the different time points.

Gene expression analysis

Gene expression analysis with RT-qPCR

The results of the microarray were verified by analyzing five randomly chosen genes (*Hrc*, *Rnase6*, *Tlr8*, *Stc1*, *Steap4*, *Hmgn5*) using RT-qPCR. These genes were identified to be significantly regulated in the whole genome microarray analysis of the lung samples.

2.4.1 cDNA synthesis and real-time PCR

In total, 1000 ng RNA from rat lungs was reverse transcribed into cDNA using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA) with random primers and MultiScribe™ Reverse Transcriptase. Thermal cycling conditions included 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C. The resulting cDNA was diluted with 40 µl nuclease- free water.

A volume of 1 µl cDNA and 300 nM of each primer was used in a total volume of 10 µl with Maxima SYBR Green/ ROX qPCR Master Mix (Biozym, Oldendorf, Germany) for amplification in the Agilent technologies Aria DX Real-Time PCR System (Agilent, Santa Clara, California, USA). Primer sequences are listed in Table 1. The primers were designed with Primer 3 (<https://bioinfo.ut.ee/primer3-0.4.0/>) and quality was checked with the help of netprimer (<http://www.premierbiosoft.com/netprimer/>). The specific sequences for primer design were gained from the NCBI gene bank (<https://www.ncbi.nlm.nih.gov/gene/>).

Thermal cycling was initiated by a denaturation step at 95 °C, followed by 40 denaturation-primer annealing cycles for 0.5 min at 95 °C and 1 min at 60 °C, respectively. As last step, the template was elongated for 15 min at 60 °C before dissociation curves were recorded for verification of the effective amplification. The relative expression was calculated with the $2^{-\Delta\Delta C_t}$ -method (Livak and Schmittgen 2001). Thereby the C_t values were normalized to the mean of the housekeeping genes *Gapdh* and *Actb*.

Statistics were performed with SigmaPlot 14 by using one way ANOVA followed by Dunnett's test and the definitions *p<0.05, ** p<0.01, ***p<0.001.

Table S4: Primer sequences for the verification of gene expression analysis data with RT-qPCR.

gene	forward primer (5' → 3')	reverse primer (5' → 3')
<i>Actb</i>	TGTGTTGTCCCTGTATGCCT	AGCGCGTAACCCTCATAGAT
<i>Actc</i>	CACGGCATTATCACCAACTG	AACAATGCCTGTGGTTCTCC
<i>C3ar1</i>	ATCTGCTCGTTGTGGATGATTAC	AGGAGGGTTGATTTCTGGTAGAT
<i>Gapdh</i>	GGCCGAGGGCCCACTA	TGTTGAAGTCACAGGAGACAACCT
<i>Hmgn5</i>	TACGGAGAGCTGCAACAATG	CACGACATTTGTGGTCTTCG
<i>Hrc</i>	CTTTCACCATTATCCCAAACCCA	TAGTTCTCATACTCCTGGGCATC
<i>Lilrb3</i>	CCGGGAAGAATATGCTGAATTGA	CTCATTTTCTGACATGGCTGAGT
<i>Rnase6</i>	GGTTTGAAATTCAGCACATATGGG	GTATCGGGTATAGTTGTTGACACC
<i>Steap4</i>	GCTTGACCATTGGATGCTTT	GCCAGGCAGTAGAGGAACTG
<i>Stc1</i>	GCTCATTGCCTTCCATCAAT	TCCAGCTGCCAGGACTACTT
<i>Tlr8</i>	CTCACCTACCTGCTGGCTTC	TGTCACACGTGCAGTCAAAA

Table S5: Results of differential gene expression analysis in lung for all treatments versus control conditions (See Excel sheet).

Bibliography

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402-8
doi:10.1006/meth.2001.1262