Correlative Histopathology and Systems Biology Approach in Product Risk Assessment

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Background and Objective

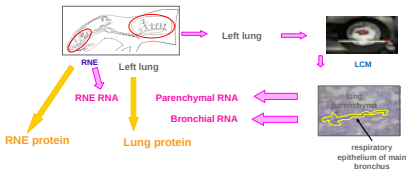
The OECD 28-day rat inhalation study is used in hazard identification and thus forms a part of our risk assessment process. Following a 28-day inhalation with cigarette smoke, rats develop mainly stress-related adaptive changes in the upper airways and signs of inflammation in the lung, a common symptom related to the pathogenesis of COPD and Lung Cancer.
With a view to developing a Systems Biology-based product risk assessment approach, classical toxicology was combined with analysis of gene expression and protein abundance to investigate disease-relevant molecular perturbations at the sites of histopathological changes in a 28-d rat inhalation study, using state-of-the-art technologies for sample preparation and analysis of gene expression arrays and Reverse Protein Arrays (RPA) in addition to the classical toxicology end points.

Materials and Methods

Male Sprague-Dawley rats (15 per group) were exposed for 28 days to filtered air (sham) or to a low, medium, or high concentration of mainstream smoke from the Kentucky Reference Cigarette 3R4F. Sections of the respiratory tract were taken from 10 rats per group at defined sites for histopathology. Care and use of the animals was in accordance with the American Association for Laboratory Animal Science Policy (1996). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC).

Exposure (28 days)	Sham	3R4F_Low	3R4F_Medium	3R4F_High
nicotine concentration (µg/l)	0	7	15	23
number of rats for histopathology	10	10	10	10
number of rats for Systems Biology	5	5	5	5

For Systems Biology, RNA and protein samples from specific areas in the respiratory tract were obtained from an additional five rats per exposure group.

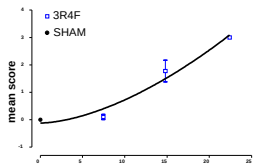


Respiratory nasal epithelium (RNE) was isolated bilaterally and divided for RNA and protein samples. From the lung, 20-µm cryosections were prepared and used for protein samples. For RNA isolation, respiratory epithelium of main bronchus and lung parenchyma was separated by Laser Capture Microdissection (LCM).

Results and Discussion

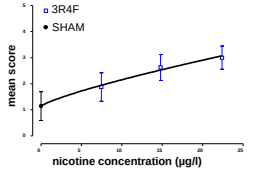
Histopathological Findings

Respiratory Epithelium, Amount of Cornification



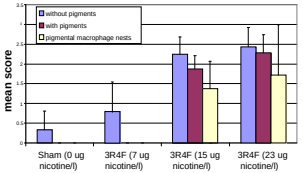
Squamous metaplasia, nose (maxilloturbinate)

Main Bronchus (Left Lung), Goblet Cell Hyperplasia



Goblet cell hyperplasia, bronchial epithelium

Lung parenchyma, alveolar macrophages



The usual dose-dependent adaptive and inflammatory responses for exposure to 3R4F were observed in the nose and in the lung , e.g., squamous metaplasia with cornification of the RNE, goblet cell hyperplasia in the bronchus, and increased number of macrophages and macrophage nests in the lung alveolar parenchyma.

Gene Expression Analysis from RNE and LCM samples from main bronchus and lung parenchyma

RNA from lung parenchyma, bronchus, and RNE was prepared, further processed, and analyzed on whole genome Affymetrix microarrays (GeneChip® Rat Genome 230 2.0 Array).

Affymetrix GeneChip®

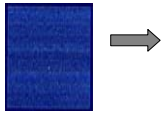
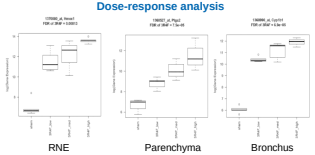
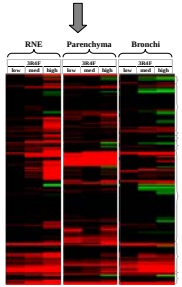


Image of a scanned gene array containing over 500,000 single probes

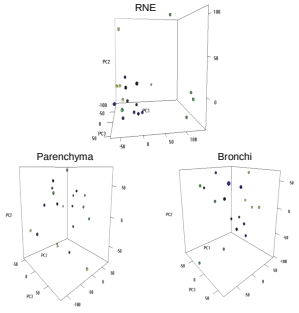
Dose-response analysis





Heat map of representative differentially expressed genes with an absolute fold change larger than two and a p-value < 0.15.
= up-regulated genes
= down-regulated genes

Principal component analysis (PCA)

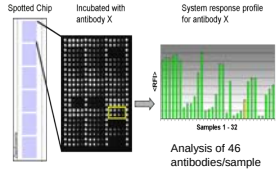


Green: 3R4F_HIGH; Black: 3R4F_MEDIUM; Blue: 3R4F_LOW; Yellow: sham.

The number of significantly up- or down-regulated genes in the three tissues was dose-dependent for all treatment conditions. The differentially regulated genes were mainly related to inflammatory processes, oxidative and ER stress, heat shock, cell growth, and xenobiotic metabolism, thus being consistent with previous findings for cigarette smoke effects in the respiratory tract. Interestingly, the stress-related changes were more pronounced in the RNE, while the inflammatory changes were more pronounced in the lung parenchyma.

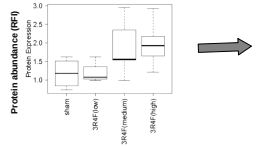
Reverse Protein Array (RPA) from RNE and whole lung tissue

Total protein extracts from whole lung tissue and from RNE of smoke-exposed rats were analyzed with specific antibodies for protein expression/modification on ZeptoMARK reverse protein arrays to generate data to describe the 'protein system response profiles'.
40 tissue samples were analyzed (20 lung, 20 RNE; 5 samples per tissue and exposure group).
System response profiles with antibodies representing different cellular signaling pathways were successfully generated: 47 for lung and 46 for RNE.

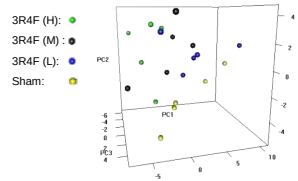


RPA provides analysis of dynamic changes in protein abundance and modification (currently approximately 250 validated antibodies available) *in vitro* and *in vivo*. Here, RPA was used to investigate proteins and pathways which play a key role in response to cigarette smoke exposure. In a novel computational pipeline for RPA data analysis, Significance Analysis of Microarrays (SAM) was found to be suitable to identify sets of differential protein levels in smoke- treated versus untreated animals; the empirical null distribution was generated by 150 random permutations, whereby delta was set to 0.01 (Xiang, Y. et al., German Conference on Bioinformatics, Technische Universität Braunschweig, Germany, September 2010)

p38.MAPK in RNE



Each box represents the relative fluorescence intensity (RFI) distribution over all biological replicates.



Principal component analysis (PCA) shows dose-related separation of 3R4F_HIGH (green points), 3R4F-MEDIUM (black points), and 3R4F_LOW (blue points) from sham (yellow points) in RNE.

Conclusion

The correlative evaluation of classical histopathology with molecular patterns (gene expression and RPA) is feasible and may facilitate a Systems Biology-based product risk assessment approach.

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Society of Toxicology
50th Annual Meeting & ToxExpo™
Washington DC, USA
6-10 March 2011

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