Inhalation Study for Cigarette-Smoke-related Lung Tumorigenicity in A/J Mice

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The A/J Mouse as a Lung Tumor Model

• Philip Morris International is committed to the development of Potentially Reduced-Risk Tobacco Products. This requires a state-of-the-art scientific approach to assess the disease risk of new products.

• Animal models with enhanced lung tumor formation after exposure to cigarette smoke are required to substantiate a reduced risk.

• The A/J mouse has been shown to respond to cigarette smoke exposure with enhanced lung tumor formation after a recovery period of several months (Witschi et al., 1997; D’Agostini et al., 2001; Stinn et al., 2005; Curtin et al., 2004).
A/J Mouse History and Strain Description

- A-strain mice inbred by Strong (1921) as a cross between CSH albino and Bagg albino random bred mice, thus most closely related to Balb/c
- Now approx. 270 generations, inbred
- Develop smoke-induced emphysema faster than other mice
- High percentage of mammary adenocarcinomas in multiparous females
- Spontaneous sarcoma development in skeletal musculature
- High spontaneous incidence of lung adenomas (K-ras mutation)
- Lung tumors in response to carcinogens
The A/J Mouse as a Carcinogenicity Model

Used for approx. 30 years as carcinogenicity model (Dragani et al., 1995)

- Application of carcinogens (~70 compounds tested): few i.p. administrations
- Tumors develop 16 to 24 weeks after carcinogen application
- All carcinogens positive in the A/J mouse are genotoxic (Pereira and Stoner, 1985; Maronpot et al., 1986).
## Differences between Humans and Rodents

<table>
<thead>
<tr>
<th>Adult Human Smoker</th>
<th>Smoke-exposed Rodent</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cyclic inhalation of nearly undiluted smoke (bolus)</td>
<td>- Continuous inhalation (hrs) of highly diluted smoke</td>
</tr>
<tr>
<td>- Mouth breather</td>
<td>- Nose breather</td>
</tr>
<tr>
<td>- Long life span</td>
<td>- Short life span</td>
</tr>
<tr>
<td>- Genetically diverse</td>
<td>- Genetically identical copies (inbred)</td>
</tr>
</tbody>
</table>
Study Design

- Male A/J mice
  - bred under SPF conditions, animals from Jackson Laboratories (Maine, U.S.A.)
  - age at start of inhalation: 10 to 14 weeks
  - in total: 1040 mice

- Diluted mainstream smoke (MS) from the Kentucky Standard Reference Cigarette 2R4F

- Total particulate matter (TPM) target concentrations: 150 mg/m³ (low MS) and 300 mg/m³ (high MS)

- Whole-body exposure for 6 hours per day, 5 days per week for up to 18 months
Experimental Conditions

- Cigarettes and smoking conditions
- Cigarette smoke generation and exposure
- Animal housing conditions
- Overview of parameters analyzed in test atmosphere
- Test atmosphere data
Cigarettes and Smoking Conditions

Cigarette type: 2R4F
Source: University of Kentucky
Number: approx. 1,200,000
Storage: temperature 5-10 °C

CONDITIONING
Duration: >7 to <20 days prior to smoking, storage of conditioned cigarettes in airtight containers
Temperature: 22 ± 0.3 °C
Relative humidity: 60 ± 1%

SMOKING CONDITIONS
Basic conformity with ISO standards
Cigarettes smoked on carrousel (30 for high MS and 15 for low MS)
MS diluted with app. 80L filtered fresh air/min
Mean age of smoke in middle of the exposure chamber was ≤6 min
Cigarette Smoke Generation and Mice Exposure
## Housing Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air changes inside the exposure room</td>
<td>&gt;10 times/h</td>
</tr>
<tr>
<td>Room positive pressure</td>
<td>20 to 30 Pa</td>
</tr>
<tr>
<td>Temperature inside the exposure room</td>
<td>22 ± 3 °C</td>
</tr>
<tr>
<td>Rel. humidity inside the exposure room</td>
<td>between 30% and 70%</td>
</tr>
<tr>
<td>Light/dark cycle</td>
<td>12/12 h, light from 06:00 to 18:00</td>
</tr>
<tr>
<td>Animal housing</td>
<td>up to 8 mice/cage, cages changed &gt;2 times/week</td>
</tr>
<tr>
<td>Bedding material</td>
<td>Changed &gt;2 times/week</td>
</tr>
<tr>
<td>Food supply</td>
<td>ad libitum in all groups except during exposure</td>
</tr>
<tr>
<td>Water supply</td>
<td>ad libitum, also during exposure</td>
</tr>
</tbody>
</table>
### Overview of Parameters analyzed in Test Atmosphere

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Frequency</th>
<th>Flow (l/min)</th>
<th>Duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPM</td>
<td>3 filters / exposure day</td>
<td>1.5</td>
<td>60</td>
</tr>
<tr>
<td>Particle size</td>
<td>4 times / study</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>every exposure day, online</td>
<td>1</td>
<td>360</td>
</tr>
<tr>
<td>Nicotine</td>
<td>Once / week during first 4 weeks; thereafter, once / 4 weeks</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Aldehydes</td>
<td>Once / 4 weeks</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Ammonia</td>
<td>Once / 4 weeks</td>
<td>1</td>
<td>30</td>
</tr>
</tbody>
</table>

Position: inside animal cage, center of the chamber
Test Atmosphere Data

- Target concentrations were met
- Particle size indicates that the particles were respirable for the mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>Sham</th>
<th>Low MS</th>
<th>High MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPM (mg/m³)</td>
<td>389</td>
<td>&lt; DL</td>
<td>153 ± 8</td>
<td>298 ± 15</td>
</tr>
<tr>
<td>Particle size (µm)</td>
<td>4</td>
<td>-</td>
<td>0.67</td>
<td>0.72</td>
</tr>
<tr>
<td>CO (ppm)</td>
<td>388</td>
<td>&lt; DL</td>
<td>210 ± 20</td>
<td>375 ± 28</td>
</tr>
<tr>
<td>Nicotine (µg/l)</td>
<td>21</td>
<td>&lt; DL</td>
<td>5.78 ± 0.99</td>
<td>12.78 ± 2.31</td>
</tr>
<tr>
<td>Formaldehyde (µg/l)</td>
<td>26</td>
<td>-</td>
<td>0.19 ± 0.05</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Acetaldehyde (µg/l)</td>
<td>26</td>
<td>-</td>
<td>12.00 ± 1.65</td>
<td>22.34 ± 2.15</td>
</tr>
<tr>
<td>Ammonia (µg/l)</td>
<td>20</td>
<td>&lt; DL</td>
<td>0.30 ± 0.11</td>
<td>0.40 ± 0.14</td>
</tr>
</tbody>
</table>
In-life Observations

• Blood carboxyhemoglobin

• Body weight development

• Mortality
Blood Carboxyhemoglobin

Data represent mean + SE
7 to 8 mice per group and determination
Body Weight Development

18 Months Exposure

Data for BW gain reduction represents mean ± SE.

* Statistically significant difference compared to sham, \( p < 0.05 \)
X Statistically significant difference compared to low MS, \( p < 0.05 \)
Mortality after 18 Months Exposure

![Graph showing mortality over study days for Sham, Low MS, and High MS groups.]

- **Sham**
- **Low MS**
- **High MS**

Study Day:

- 0 60 120 180 240 300 360 420 480 540 600

Mortality (%):

- 0 10 20 30 40 50 60

Key:

- Sham (black line)
- Low MS (yellow line)
- High MS (red line)
Biological Outcome

- Inflammation markers in the lung
- Lung tumor formation
Inflammation Markers in Lungs of Mice

Bronchoalveolar Lavage Fluid (BALF)

↓

Leukocytes

Neutrophiles

Lymphocytes

↓

Inflammatory Mediators

IL1-α

MCP-1

MIP-2

MMP-9
Inflammatory Cells in BALF of Mice

mean ± SE; +++, p <0.001; ++, p <0.01; ANOVA plus Dunnett posthoc test
Inflammatory Mediators in BALF of Mice

median, and variance quartiles; ++++ p <0.001; ++, p <0.01; ANOVA plus Dunnett posthoc test on rank-transformed data

- IL-1α (pg/ml)
- MCP-1 (pg/ml)
- MIP-2 (pg/ml)
- MMP-9 (pg/ml)

Legend:
- Sham
- Low MS
- High MS
Nodules in the A/J Mouse Lung

- Lung nodules
- Hyperplasia
- Bronchioloalveolar adenoma
- Bronchioloalveolar adenocarcinoma
Evaluation of Lung Tumor Response

- Macroscopic counting of nodules after immersion in Telyesniczky’s fixative for 1 day followed by fixation in ethanol
- Histopathological evaluation of serial lung sections cut at a distance of 300 µm
- Lung tumor classification in accordance with WHO guidelines (2001)
Time Course of Lung Tumor Multiplicity

Data represents mean ± SE.
Summary and Conclusions

• Under long-term, carefully controlled conditions, the A/J mouse is a promising model for cigarette-smoke-related lung tumorigenicity

  - Severe inflammation in the bronchoalveolar lavage of smoke-exposed mice

  - Significant and concentration-dependent enhancement of lung tumors, i.e., adenomas and adenocarcinoma was observed

• The relevance of the A/J mouse model for cigarette-smoke-induced lung tumors in humans requires further validation
Acknowledgements

Co-authors

• Ansgar Büttner (Philip Morris Research Laboratories, Cologne, Germany)
• Baerbel Friedrichs (Philip Morris Research Laboratories, Cologne, Germany)
• Walter Stinn (Philip Morris Research Laboratories, Cologne, Germany)
• Frans van Overveld (Philip Morris Research Laboratories, Leuven, Belgium)

Special thanks also to all colleagues at Philip Morris Research Laboratories (Leuven, Belgium, and Cologne, Germany) for providing excellent support.

This work was supported in part by Philip Morris USA, Inc. prior to the spin-off of Philip Morris International, Inc. by Altria Group, Inc. on March 28, 2008.