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# Murine lung tumor response after exposure to cigarette mainstream smoke or its particulate and gas/vapor phase fractions

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ABSTRACT

Knowledge on mechanisms of smoking-induced tumorigenesis and on active smoke constituents may improve the development and evaluation of chemopreventive and therapeutic interventions, early diagnostic markers, and new and potentially reduced-risk tobacco products. A suitable laboratory animal disease model of mainstream cigarette smoke inhalation is needed for this purpose. In order to develop such a model, A/J and Swiss SWR/J mouse strains, with a genetic susceptibility to developing lung adenocarcinoma, were whole-body exposed to diluted cigarette mainstream smoke at 0, 120, and 240 mg total particulate matter per $m^3$ for 6 h per day, 5 days per week. Mainstream smoke is the smoke actively inhaled by the smoker. For etiological reasons, parallel exposures to whole smoke fractions (enriched for particulate or gas/vapor phase) were performed at the higher concentration level. After 5 months of smoke inhalation and an additional 4-month post-inhalation period, both mouse strains responded similarly: no increase in lung tumor multiplicity was seen at the end of the inhalation period; however, there was a concentration-dependent tumorigenic response at the end of the post-inhalation period (up to 2-fold beyond control) in mice exposed to the whole smoke or the particulate phase. Tumors were characterized mainly as pulmonary adenomas. At the end of the inhalation period, epithelial hyperplasia, atrophy, and metaplasia were found in the nasal passages and larynx, and cellular and molecular markers of inflammation were found in the bronchoalveolar lavage fluid. These inflammatory effects were mostly resolved by the end of the post-inhalation period. In summary, these mouse strains responded to mainstream smoke inhalation with enhanced pulmonary adenoma formation. The major tumorigenic potency resided in the particulate phase, which is contrary to the findings published for environmental tobacco smoke surrogate inhalation in these mouse models.



## 1. Introduction

Cigarette smoking is the cause of morbidity and mortality from several diseases including lung cancer, chronic obstructive pulmonary disease (COPD), and cardiovascular disease (CVD) (US Department of Health and Human Services, 2004). The most effective way of avoiding these risks is not to smoke, but in the foreseeable future there will be a significant part of the population who will continue to smoke (Morgan et al., 2007; World Health Organization, 2004). Apart from smoking cessation, several approaches are being followed in order to contain the risk of developing smoking-related diseases. For lung cancer, this includes the development and evaluation of technologies for early detection (Brambilla et al., 2003), for therapies based on translational medicine (Sato et al., 2007), for chemopreventive interventions (Witschi, 2005b), and for novel potentially reduced-risk tobacco products with the aim of reducing the harm from smoking (US Institute of Medicine, 2001).

Improved mechanistic understanding of lung cancer and the availability of surrogate models with endpoints validated against the disease are needed (Haussmann, 2007; Kim et al., 2005). In particular, laboratory animal studies for lung cancer induced by cigarette mainstream smoke inhalation would be the most appropriate model for smoking-induced lung cancer, but these studies have not been successfully established in the past (Coggins, 2007; Schleef et al., 2006). Two recently published standardized life-time mainstream smoke inhalation studies with F344 rats and B6C3F1 mice showed statistically significantly increased lung tumor incidences in females. However, these studies used 30 months of smoke

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inhalation and have so far not been reproduced (Hutt et al., 2005; Mauderly et al., 2004).

A/J mice may serve as a model for human adenocarcinoma (Malkinson, 2001; Witschi, 2005b), which is a major histological type of human smoking-related lung cancer (Devesa et al., 2005). The A/J mouse model has reproducibly been shown to develop enhanced lung tumor multiplicities after exposure to an environmental tobacco smoke surrogate (ETSS), a mixture mainly consisting of sidestream smoke with some mainstream smoke. This tumorigenic response became only apparent at the end of a 4-month post-inhalation period which followed the 5-month ETSS inhalation period (Witschi, 2005b). However, the active smoker is mainly exposed to mainstream smoke, which differs in its quantitative composition from sidestream smoke (International Agency for Research on Cancer, 2004). The A/J mouse model has already been used in some mainstream smoke inhalation studies of variable design and with a mixture of positive and negative responses (Curtin et al., 2004; D'Agostini et al., 2001; Essenberg, 1952, 1957; Finch et al., 1996; Gordon and Bosland, 2009; Hamm et al., 2007).

The pronounced lung tumor susceptibility of the A/J mouse has been associated with the presence of pulmonary adenoma susceptibility (PAS) loci in the A/J genome, in particular PAS1 (Malkinson, 1989; Manenti and Dragani, 2005). This locus might contain the proto-oncogene Kras (Liu et al., 2006; Wang et al., 2003), which upon mutation, and as a function of expression levels of wild-type and mutant alleles, leads to the development of lung adenoma and adenocarcinoma in mice (Fisher et al., 2001; To et al., 2006). Mutational activation of Kras has also been associated with a high proportion of smoking-associated lung adenocarcinoma in humans (Gazdar et al., 2004). Hierarchical clustering of various inbred laboratory mouse strains by PAS1 locus polymorphisms identified other mouse strains, which demonstrate similar susceptibility to both spontaneous and chemically induced lung tumorigenesis (Manenti and Dragani, 2005), such as SWR/J mice. Inhalation exposure of 'Swiss' and 'SWR' mice (De Flora et al., 2003; Witschi et al., 2002) to ETSS followed by a post-inhalation period was indeed found to enhance lung tumorigenesis, even with a higher dynamic response than in A/J mice.

For a number of applications, such as the development of potentially reduced-risk tobacco products or for chemopreventive interference with the metabolic activation of smoke carcinogens, it would be helpful to understand the etiology of smoking-induced lung cancer. In previous chronic A/J mouse inhalation studies on ETSS, the tumorigenic potential was associated with the gas/vapor phase-enriched fraction (GVP) (Witschi et al., 1997a; Witschi, 2005a). On the other hand, in sub-chronic mainstream smoke inhalation studies in rats, most of the histopathological changes in the lower respiratory tract and the pulmonary accumulation of inflammatory cells were associated with the particulate phase-enriched smoke fraction (PP) (Coggins et al., 1980; Friedrichs et al., 2006).

Therefore, a series of studies was initiated aimed at establishing and validating a mouse inhalation model for lung tumorigenesis with cigarette mainstream smoke. The objective of the current study was to investigate and compare lung tumor incidence and multiplicity in a concentration-dependent manner in two genetically susceptible mouse strains, A/J and SWR/J, using cigarette mainstream smoke with the same chronic inhalation/post-inhalation study design that was shown to enhance lung tumorigenesis in previous ETSS inhalation studies (Stinn et al., 2005b; Witschi, 2005b). To further unravel the etiology of smoking-induced tumorigenesis in this model, exposure to two mainstream smoke fractions enriched in either the PP or GVP were included in parallel to the exposure to mainstream whole smoke (WS). To improve comparisons of the principal difference in the tumorigenic potential of PP and GVP observed in the current study to that observed in previous ETSS inhalation studies (Witschi, 2005a), chemical characterizations of the aerosol types were conducted. Because inflammatory reactions have been associated with tumorigenesis (Bauer and Rondini, 2009; Engels, 2008; Walser et al., 2008), inflammatory mediators and cells in bronchoalveolar lavage (BAL) fluid were also investigated.

## 2. Materials and methods

### 2.1. General design

This mainstream smoke inhalation study was designed in analogy to previous ETSS inhalation studies (Stinn et al., 2005b; Witschi, 2005b). Male mice were whole-body exposed to WS from the Reference Cigarette 2R4F (University of Kentucky) for 6 h per day, 5 days per week, for 5 months, at 120 or 240 mg total particulate matter (TPM) per $m^3$ (Table 1), followed by a 4-month post-inhalation period. The high WS concentration was selected aiming at a similar effect on body weight development as a sign of systemic toxicity as in previous smoke inhalation studies (Curtin et al., 2004; Hamm et al., 2007; Stinn et al., 2005b; Witschi et al., 2002; Witschi, 2005b). Assuming a respiratory minute volume of 60 ml (Hodge-Bell et al., 2007) and an approximate average body weight of 24 g (A/J mice) during the inhalation period, the upper concentration translates to a daily dose of up to 200 mg TPM/kg body weight. This would translate to approximately 5 packs of cigarettes per day for a human smoker using body surface for interspecies scaling.

This study was conducted under good laboratory practice conditions (Organization for Economic Co-operation and Development Environment Directorate, 1998). Care and use of the laboratory mice was in conformity with national and international regulations and recommendations (Association for the Assessment and Accreditation of Laboratory Animal Care International, 1991). All laboratory animal procedures were approved by the TNO Commission of Animal Welfare.

### 2.2. Cigarettes and smoke generation

The Reference Cigarette 2R4F is a research cigarette representative of the filtered American-blend cigarettes that are currently marketed in many parts of the world (characterized by Chen and Moldoveanu, 2003). The cigarettes were obtained from the University of Kentucky (Lexington, KY) and conditioned and smoked according to standard conditions (International Organization for Standardization, 1991; International Organization for Standardization, 2000) using automated 30-port smoking machines as previously described (Vanscheeuwijck et al., 2002). The low and high concentrations were achieved by smoking 15 and 30 cigarettes at a time, respectively. The PP fraction was generated by filtering the WS through activated charcoal (granular, 2.5 mm, extra pure: Merck, Darmstadt, Germany) to filter out the GVP constituents. The charcoal was replaced every exposure day. The GVP fraction was obtained by passing the WS through an electrostatic filter to trap particulate matter (air cleaner Vortronic 35 RF: Heinisch, Vienna, Austria). The filter was cleaned every day.

### 2.3. Experimental animals

Male A/J and Swiss SWR/J mice, bred under specified pathogen-free conditions, were obtained from Jackson Laboratories (Bar Harbor, ME) at an age of 5–8 weeks, checked for their health status, and individually marked. After a 5-week acclimatization period, the mice were randomly allocated to the groups (Table 1). The mean body weight of all mice at the start of the inhalation was 25 g and did not vary more than 20%. The mice were fed an irradiated rodent pellet diet (2014 Teklad Global 14% Protein Rodent Maintenance diet Harlan Teklad, Blackthorn, Bicester, UK). Food and drinking water were provided ad libitum, except during exposure, when food pellets were removed.

### 2.4. Inhalation exposure

Mice were exposed in whole-body stainless steel and glass inhalation units (2.3 $m^3$, Hazleton Systems, Aberdeen, MD) to WS, PP, or GVP or to fresh conditioned air (sham exposure). Because the A/J mouse lung tumor model has been shown to be sensitive to stress (Stinn et al., 2005b), whole-body exposure was chosen to avoid confounding of the results by the restraint-related stress of nose-only exposure. The total air flow through the inhalation units was between 61 and 89 l/min. Before the start of the inhalation period, the homogeneity of the WS distribution in the chamber was confirmed for TPM, nicotine, and aldehydes. In the sham exposure chambers, the average daily minimum and maximum temperatures ranged from 23.5 to 25.6, with a relative humidity between 37% and 64%; these conditions are considered representative of the other chambers as well.

The mice were adapted slowly to the smoke exposure by increasing the daily exposure duration. Starting with exposure day 13, the full 6-h exposure period was used. The composition of the WS, PP, GVP, and the air in the sham exposure group were routinely analyzed at designated time intervals in order to determine the reproducibility of the test atmosphere generation. TPM concentrations