Conference Report

Experimental Assessment of the Toxicological Effects of Inhaled Complex Mixtures on the Respiratory System – Feasibility and Limitations

April 23–25, 2005, Barcelona, Spain

(in: *Experimental and Toxicologic Pathology*, Vol. 57, S1, July 2005)

Anja Thielen

Verband der Cigarettenindustrie, Neustädtische Kirchstraße 8, 10117 Berlin, Germany

The vast majority of toxicological studies and risk evaluations deal with single chemicals. The scientific evaluation of complex airborne mixtures, such as atmospheres produced by combustion or thermolysis, e.g., diesel and gasoline exhausts, tobacco smoke and polluted ambient air, remains a challenge for experimental toxicologists. Accurate hazard characterization is essential for the meaningful risk assessment of inhaled materials. The potential health hazards posed by a variety of complex mixtures have become increasingly prominent in recent years (e.g., fine particulate matter). However, no international standards currently exist in the field of toxicity testing of cigarette smoke or other complex inhaleable mixtures.

The Workshop “Experimental Assessment of the Toxicological Effects of Inhaled Complex Mixtures on the Respiratory System – Feasibility and Limitations” was organized and made possible by Professor Ulrich Mohr of the Fraunhofer Institute of Toxicology and Experimental Medicine in Hannover (Germany) and held in Barcelona (Spain) on April 23–25, 2005. Internationally recognized experts were brought together representing various approaches to the experimental assessment of the toxicological effects of complex mixtures *in vivo* and *in vitro*. About 70 participants from academia, industry and regulatory bodies followed the invitation and discussed the topics based on 19 presentations given during the three days of the Workshop. The main goal of the Workshop was to review and critique current toxicological methods, identify promising new approaches and make recommendations concerning the toxicological assessment of complex mixtures. Besides tobacco smoke, the presentations mainly dealt with air pollution and diesel exhaust. The Workshop was divided into three sessions: 1) composition of mixtures and bio-

monitoring, 2) *in vivo* animal studies and 3) *in vitro* studies. In July 2005, a special issue of the journal *Experimental and Toxicological Pathology* was published which contains the presentations of the three sessions of the Workshop. In addition, the issue includes the “Summary and Conclusions of the Review Committee”, which were put together by a selected group of experts immediately after the Workshop. There are different routes that can be taken to assess the toxic effects of single chemicals and complex mixtures. These include: epidemiological studies, human clinical studies, animal studies, *in vitro* studies and, as a base, the knowledge of the chemical composition. These approaches can be depicted as a pyramid in relation to their relative importance:

Each of these approaches has its strengths and limitations. In the following, the contributions and comments made at the Workshop are discussed with the focus on issues relevant for tobacco products and are dealt with from the bottom to the top of the pyramid.

Knowledge of the *chemical composition* of complex inhaleable mixtures is an indispensable requirement for assessing toxic potency and may help to identify some of the most toxic constituents of a mixture.

Cigarette smoke was the most complex mixture discussed at the Workshop. Michael Borgerding (Winston-Salem, NC, USA) and Hubert Klus (St. Poelten, Austria) gave an overview of the physical properties and the chemical composition of mainstream and sidestream smoke. They emphasized that special care must be taken in sample preparation for testing because fresh un-aged cigarette smoke is extremely complex and reactive. Mainstream smoke is inhaled fresh and un-aged by the smoker, whereas inhaled sidestream smoke is aged and diluted. The sampling conditions and facilities for collecting particulate matter are different for the evaluation of mainstream smoke, sidestream smoke and ETS (environmental tobacco smoke). The so-called "Hoffmann Analytes" (a list of about 40 toxic/carcinogenic substances in cigarette smoke, which are considered to be involved in the induction of smoking related diseases) are commonly measured and represent one possibility for assessing relative smoke toxicity. However, there is little information about the influence of individual Hoffmann Analytes on *in vivo* or *in vitro* effects. While additives were found to induce some minor changes in the composition of mainstream smoke, no differences were observed in the biological response of various *in vitro* and *in vivo* test systems (1–7).

In the past, there have been several attempts to apply toxicological risk assessment methods to cigarette smoke and to establish hazard ranking for chemical constituents of cigarette smoke (8–10). Potency factors were determined for many of the over 4,000 components of cigarette smoke in acute or chronic exposure studies of single substances in ambient air. There is some, but limited, value in using these data for the evaluation of a complex mixture, such as tobacco smoke, and the special exposure conditions of human smoking. Another topic in the presentation of Borgerding and Klus was the different machine-based smoking regimens and the comparison of 'tar' yields to personal smoking behaviour. Daily average 'tar' intake by smokers is generally overestimated by the more intense smoking regimens, like "Massachusetts" and "Canadian Intense". The overestimation increases with the nominal 'tar' yields of a cigarette. Yields obtained with whichever standardized machine smoking regimen do not reflect actual exposure of a smoker; much rather, they allow ranking and comparison of different brands.

The use of *in vitro toxicology tests* has gained increased attention. Several presentations at the Workshop described the advantages and limitations of *in vitro* toxicology in comparison with epidemiological, human and animal studies. Well-conducted *in vitro* studies have the potential to characterize the underlying mechanisms, by which substances damage cells, and elucidate the underlying pathophysiological processes. *In vitro* toxicology test methods that are sensitive and reproducible and show dose-

effect relationships would be ideally suited for the screening of many pollutants, because they are inexpensive and rapid compared to *in vivo* studies. However, it may be difficult to expose cells in a way that mimics *in vivo* exposure. Data were shown at the Workshop demonstrating that the response of cultured non-tumorigenic human bronchial epithelial cells (BEAS-2B) exposed to air pollution particles was similar to the response seen in humans following exposure to the same particles (Devlin et al., Research Triangle Park, NC, USA). On the other side, whole body exposure of mice to diesel exhaust and exposure of A549 cells in suspension to diesel exhaust showed little congruence with relative toxicity (Seagrave et al., Albuquerque, NM, USA). Obviously, there are relevant parallels but also discrepancies between the exposure of cells and living animals.

Toxicological investigations should be carried out under conditions resembling the *in vivo* situation as far as possible. For simulating inhalation and the exposure of the respiratory tract a technique was introduced where cells are directly exposed *in vitro* to test atmospheres composed of complex gases and particulate matter. Michaela Aufderheide (Hannover, Germany) gave an overview of the different *in vitro* techniques and introduced a promising approach, which is based on a biphasic cell culture technique. Cells are grown on microporous membranes at an air-liquid interface and nurtured from the basal side while the apical part is in direct contact with the test atmosphere. One major limitation of *in vitro* studies is that cells have been removed from their normal environment (neighbouring cells, tissues, blood vesicles and their signals). The origin of the cells (permanent or primary cells, species and tissue) and their metabolic competence should be considered. The lung is a primary target organ for the toxicity of inhaled compounds. José V. Castell (Valencia, Spain) gave an overview of the metabolism and bioactivation of toxicants in the lung. The final goal of metabolizing foreign compounds is to facilitate the elimination of these substances. Frequently, the activity of metabolizing enzymes results in reactive intermediates, which are more toxic than the original substance. The classic example for this process, called metabolic bioactivation, is benzo[a]pyrene. The activity of xenobiotic-metabolizing enzymes in eight individual human lung tissues showed a wide range. The comparison between the metabolic competence of human lung tissues and the adenocarcinoma A549 cell line, derived from type II pneumocytes, showed that the basal P450-specific activities in A549 cells were far below those present in the human lungs.

Animal studies are an important tool for assessing health implications of chemicals in humans. They have all the advantages of human clinical studies in terms of assessing causality, biological plausibility and underlying mechanisms and they are relatively inexpensive to perform compared to human studies, although highly standardized and controlled studies are required. Different inhalation exposure techniques have their strengths and weaknesses, like problems of high-to-low dose extrapolation following inhalation exposures. All rodents are nose-breathers; therefore, inhalation of cigarette smoke is different between rodents and human smokers. Most carcinogenic studies with cigarette smoke in mice, rats, hamsters, dogs and monkeys

yield no apparent positive results. Hanspeter Witschi (Davis, CA, USA) discussed an animal model for tobacco carcinogenesis, namely A/J mice. Many tobacco smoke inhalation studies with A/J mice suggest that tobacco smoke is a comparatively weak mouse lung carcinogen. The author mentioned that this model has met with various criticisms and is far from being generally recognized. For example, strain A/J mice are very susceptible to certain important classes of carcinogens found in tobacco smoke, such as polycyclic aromatic hydrocarbons, nitrosamines and carbamates but show little sensitivity towards aromatic amines, naphthalene or metals. Lung tumours in mice look and behave differently from lung tumours in humans. Nevertheless, there are many similarities, particularly with regard to signal transduction, changes in gene expression and oncogene mutations.

Human clinical studies represent a logical complement to epidemiological studies. The controlled exposure to the pollutant of interest establishes causality and minimizes confounders. For example, biomarkers represent steps in an exposure-disease continuum, and biomonitoring is a promising tool in human clinical and epidemiological studies. Biomonitoring of the exposure to complex mixtures poses a particular challenge as these exposures have many constituents in common and many people are exposed to more than one of these mixtures. Gerhard Scherer (Munich, Germany) gave a detailed overview of biomonitoring data on the exposure to ambient air, diesel exhaust and tobacco smoke. Many biomonitoring studies have been performed for all three complex mixtures (ambient air, diesel exhaust and cigarette smoke). However, specific biomarkers, which would allow the identification of the source, are only available for tobacco smoke (cotinine and NNAL/NNAL-glucuronide).

Different susceptibility factors, which include age, gender, ethnicity, health status and genetic factors can modify biomarkers responses. The genetic background and inter-individual variations in human responses to air pollutants were described using the example of ozone (Kleeberger, Research Triangle Park, NC, USA).

Epidemiological studies investigate people in natural settings and measure "real world" exposure scenarios. End points measured in epidemiology studies are highly relevant and include mortality and various types of morbidity. In addition, potentially susceptible populations or acute and chronic effects can be studied. Epidemiological studies have several limitations. Causality is often difficult to demonstrate. People are often exposed to more than the pollutant or mixture that is being studied. The accurate measurement of the individual exposure is, besides time and cost, one of the most important limitations of epidemiological studies. Yuzo Hayashi (Sagamihara, Kanagawa, Japan) illustrated some basic strategies for the hazard characterization of inhaled complex mixtures using the example of tobacco smoke. He discussed representative epidemiological and experimental data from published papers concerning the association of cancer, chronic obstructive pulmonary disease (COPD), cardiovascular disease and low birth weight with tobacco smoking. Epidemiology is considered the "gold standard", but can in practice not be expected to be a requirement in a test battery for cigarettes.

With the three sessions finished, a Review Committee produced a summary and the conclusions of the Workshop. Their impression was that the development of a unified approach is possible implying the need to generate uniform protocols for both *in vivo* and *in vitro* testing approaches for complex mixtures, while the use of *in vitro* models is gaining increased attention because of recent advances in technology that allow more realistic exposure scenarios. For instance, mechanistically based, controlled exposure studies using an air liquid cell culture system will surely gain acceptance in the future. Essential concepts are required to define exposure and determine appropriate and relevant effects. There is also a need to better understand dosimetry and extrapolation, especially for *in vitro* data.

At the end of her presentation, JeanClare Seagrave quoted: "All models are wrong, but some are useful." No single approach covers the broad range of risks and health hazards caused by complex inhaleable mixtures but, hopefully, different approaches will work together and result in a strategy for meaningful risk assessment of complex inhaleable mixtures.

It was not expected that this Workshop would produce a "recipe" for the toxicological evaluation of complex inhaleable mixtures. The Workshop was intended as an effort to develop strategies and discuss methodological approaches for toxicity testing. The Review Committee recommends that an empirical strategy be developed in a targeted workshop of experts in *in vitro* studies.

In comparison to diesel exhaust and ambient air pollution, cigarette smoke is a well-analyzed and documented complex mixture with a lot of research already done on toxicity testing. Currently, a wide range of approaches is in use for testing the toxicity of cigarette smoke but these testing methods are not validated for complex inhaleable mixtures. In the context that the tobacco industry is increasingly subjected to national and international regulations focused on consumer protection, this Workshop was a step in the direction of developing, in a co-operative way, a unified approach for toxicity testing for tobacco smoke and other complex inhaleable mixtures.

REFERENCES

1. Baker, R.R., E.D. Massey, and G. Smith: An overview of the effects of tobacco ingredients on smoke chemistry and toxicity; Food Chem. Toxicol. 42 Suppl. (2004) S53–S83.
2. Carmines, E.L.: Evaluation of the potential effects of ingredients added to cigarettes. Part 1: Cigarette design, testing approach, and review of results; Food Chem. Toxicol. 40 (2002) 77–91.
3. Carmines, E.L. and C.L. Gaworski: Toxicological evaluation of glycerin as a cigarette ingredient; Food Chem. Toxicol. 43 (2005) 1521–1539.
4. Carmines, E.L., R. Lemus and C.L. Gaworski: Toxicologic evaluation of licorice extract as a cigarette ingredient; Food Chem. Toxicol. 43 (2005) 1303–1322.
5. Paschke, T., G. Scherer, and W.D. Heller: Effects of ingredients on cigarette smoke composition and biological activity: A literature overview; Beitr. Tabakforsch. Int. 20 (2002) 107–247.

6. Rodgman, A. Some studies of the effects of additives on cigarette mainstream smoke properties. III. Ingredients reportedly used in various commercial cigarette products in the USA and elsewhere; *Beitr. Tabakforsch. Int.* 21 (2004) 47–104.
7. Roeper, W., R. Wieczorek, and M. Intorp: "Hoffmann" Analytes and cigarette smoke in vitro toxicity revisited – How do the data compare? CORESTA Congress, 3–7 October 2004, Kyoto, Program and Abstracts APSSPT03.
8. Fowles, J. and E. Dybing: Application of toxicological risk assessment principles to the chemical constituents of cigarette smoke; *Tob. Control* 12 (2003) 424–430.
9. Levasseur, G., J. Fillion, and M.J. Kaiserman (Health Canada): Determination of the exposure levels of smokers to chemicals present in cigarette smoke; 58th Tobacco Science Research Conference, 2004.
10. Rodgman, A. and C.R. Green: Toxic chemicals in cigarette mainstream smoke – hazard and hoopla; *Beitr. Tabakforsch. Int.* 20 (2003) 481–545.