A common European approach to the regulatory testing of nanomaterials

NANoREG

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Provide histological samples from chronic study

(extended for lung, standard for other organs)

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1 Description of task

The task is part of a project that serves to experimentally study hypotheses on the assumed mode of action of selected nanomaterials. It aims at clarifying essential questions in the risk assessment of granular biopersistent particulate nanomaterials (GBP). As inhalation is considered to be the most relevant route of exposure a chronic study was performed using inhalation. An OECD TG 453 compliant study, conducted with OECD depository materials (NM-212 CeO_2 and NM-220 $BaSO_4$) under GLP, focuses on investigating a putative inhalation carcinogenicity of GBP nanomaterials in low dose exposures. This study offers relevant information not only for occupational but also for environmental and consumer health. Moreover, systemic distribution and systemic toxicity will be studied as well.

2 Description of work & main achievements

2.1 Summary

A combined chronic/carcinogenicity whole body inhalation study was performed according to OECD TG 453 with several protocol extensions. Female rats (n=100/group) were exposed to cerium dioxide (NM-212, 0.1; 0.3; 1; 3 mg/m³) and barium sulfate (NM-220; 50 mg/m³) for 24 months. A control (n=100) was exposed to clean, filtered air in parallel. The aim is to investigate lung carcinogenicity and putative systemic effects of low-dose exposures to biopersistent nanoparticles. The analysis of particle deposition and systemic distribution complemented the study. The 24-month exposure period was successfully terminated and 50 animals per dose group sacrificed. The remaining animals were kept exposure-free for maximally 6 additional months. This post-exposure period successfully ended December 11, 2015. After sacrifice of the remaining animals, the organs required according to OECD 453 were collected and stored, and fixed in formalin. The sampled organs of the 24-month exposure period have been sent to Fraunhofer ITEM in Hannover in July 2015. Histological analysis from these slices is ongoing. The samples of the post-exposure period animals will be sent to Fraunhofer ITEM in Hannover (Germany) in the third week of January. Slices for histological analyses will be prepared from these organs at Fraunhofer. The histological examinations will be performed subsequently at Fraunhofer ITEM. The histological analyses will cover standard evaluations according to OECD protocols for all organs except for lung. For lung, an extended analysis will be performed where 60 instead of up to 6 slices per organ will evaluated.

2.2 Background of the task

In the last years, there has been an emphasis on the experimental testing of nanomaterials using in vitro and in vivo approaches. However, several essential questions on nanomaterial toxicology cannot yet be clarified by such approaches. One of these questions is a putative carcinogenicity of nanomaterials. Due to reasons of feasibility it is not possible to test each single nanomaterial for carcinogenicity. Grouping approaches for safety testing can be chosen in case a common mode of action is known. A relevant group of nanomaterials are likely to share a common mode of carcinogenic action. These nanomaterials belong to a group of materials named poorly soluble, low toxicity particles (PSLT) (Dankovic et al. 2007), poorly soluble particles of low cytotoxicity (PSP) (Oberdorster 2002) or respirable granular biodurable particles without known significant specific toxicity (GBP) (Roller and Pott 2006). All terms describe the same type of materials. Industrial-relevant nanomaterials like carbon black or titanium dioxide belong to this group. There is a current scientific controversy, whether the lung tumours detected in chronic rat inhalation studies induced by PSLT only appear at high exposure concentrations (i.e. so-called dust 'overloading' of the lungs) associated with inflammation. According to the overload hypothesis, in lower (and real-life) exposure levels there is no dust overloading and no inflammation in the lung, and consequentially no tumour risk in case an exposure threshold is not exceeded. Several authors (e.g. (Morrow 1992)

describe that dust overloading in the rat becomes evident in respirable dust concentrations higher than 1 mg/m³ in a chronic study.

Further up to now unclarified aspects with respect to putative health hazards of nanomaterials can and will also be studied. This comprises the systemic distribution of particles after chronic inhalation exposure and a putative accumulation in tissues like brain or the cardiovascular system and putative adverse effects associated with this chronic accumulation. As the study focuses on investigating a putative inhalation carcinogenicity of PSLT nanomaterials in a range of exposure concentrations (including low concentrations), this study offers relevant information not only for occupational but also for environmental and consumer health.

Long-term exposure to biopersistent, poorly soluble nanomaterials and possible carcinogenicity induced thereof, has been identified as one of the major data gaps for regulatory decision process in the field of nanomaterials (Becker *et al.* 2011). While an increasing number of short-term data becomes available, long-term inhalation studies according to GLP and OECD TG guidelines in rodents are technically demanding and the resources needed require a high investment. Only two nanomaterials, titanium dioxide and carbon black, have been tested so far in rodent inhalation carcinogenicity studies. In these studies tumors in rats were obtained at higher doses (Heinrich *et al.* 1994; 1995; Nikula *et al.* 1995).

The limited data as well as the uncertainty of the role of inflammation and overload (ILSI 2000) in the process of tumor development when exposed to poorly soluble nanomaterials hinder any regulatory decision on how to evaluate the long-term risk of nanomaterials. Uncertainty on mode of action in the respiratory tract and the possibility of translocation to extra-pulmonary organs do not allow a final evaluation using short-term studies only. Insight into mechanistic hypothesis, i.e. the role of inflammation and / or overload has to be addressed with a long-term study to gain regulatory insight into model substances and allow a proper future risk evaluation. Well characterized material and study design selection is crucial for the success of the project to serve as basis for regulatory decisions. Furthermore, based on the rare incidence of lung tumors the study design has to be adapted to gain sufficient statistical power. An OECD TG 453 compliant study conducted with OECD depository material under GLP was carried out and will provide inside into general principles and allow an exemplary risk assessment to be conducted as well as evaluation of the role that inflammation and overload play in the formation of tumors in the lung.

The study serves to experimentally verify the 'overload' hypothesis. It aims to clarify an essential question in the risk assessment of an industrial relevant group of particulate nanomaterials. Only few representative materials have to be tested and the results can be cross-read to other PSLT nanomaterials. For the first time, relevant results for low dose exposures will be available for nanomaterials for which the mode of possible carcinogenic action is determined exclusively by dust toxicity. As the study focuses on investigating a putative inhalation carcinogenicity of PSLT nanomaterials in low dose exposures, this study offers relevant information not only for occupational but also for environmental and consumer health.

The chosen approach will help to clarify additional aspects with respect to putative health hazards of nanomaterials, i.e. the systemic distribution of particles after chronic inhalation exposure and a putative accumulation in tissues like brain or the cardiovascular system and putative adverse effects associated with this chronic accumulation.

2.3 Description of the work carried out

2.3.1 Study objectives

The objective of this combined chronic inhalation toxicity and carcinogenicity study is to determine the effects of two nanoparticles NM-212 CeO_2 and NM-220 $BaSO_4$ in female Wistar rats following prolonged and repeated whole-body inhalation exposure. The application of this guideline should generate data which identify the majority of chronic and carcinogenicity effects and determines concentration-response relationships. The design and conduct should

allow determination the carcinogenic potential as well as general toxicity, including physiological, biochemical, and hematological effects and exposure-related morphological (pathology) effects after chronic exposure (12 month).

CeO_2 was selected as model substance at low, mid and high dose level based on its effects in kinetic and short-term studies, its inert properties and its commercial relevance (Keller *et al.* 2014; Konduru *et al.* 2014; Molina *et al.* 2014). $BaSO_4$ was initially selected as negative control at one high dose level. Data for $BaSO_4$ were available from the NanoCare project using a short-term inhalation study (NanoCare, 2009; Landsiedel *et al.* 2014). $BaSO_4$ is considered to serve as example substance for low inflammation effects and positive control for overload, based on preliminary data, which indicate no or very low inflammation (Landsiedel *et al.* 2014; Cullen et al. 2000; Tran et al. 2000) even at overload concentrations.

2.3.2 Study protocols

The conduct of inhalation exposures will be performed according to the following test guideline concerning repeated dose inhalation toxicity studies:

- Organization for Economic Cooperation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, No. 413 "Sub-Chronic Inhalation Toxicity: 90-day Study" adopted 07 September 2009.

In addition the study was carried out taking into account the following guidelines:

- Organization for Economic Cooperation and Development (OECD), OECD Guidelines for Testing of Chemicals, Section 4: Health Effects, Method 453 "Combined Chronic Toxicity/Carcinogenicity Study in Rodents" adopted 07 Sep 2009.
- Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Part B.33.: Combined Chronic Toxicity/Carcinogenicity Test
- US Environmental Protection Agency (EPA), Health Effects Test Guidelines OPPTS870.4300, Combined Chronic Toxicity/Carcinogenicity, EPA 712-C-98-212, August 1998

In deviation to the guidelines, only females were exposed, because female rats are considered to be slightly more sensitive concerning carcinogenicity after inhalation exposure to dust aerosols (Nikula et al. 2000).

The chronic study was started with 100 rats per dose group. 50 animals per dose group were sacrificed after 24 months. The remaining animals were kept exposure-free till natural death or till month 30, i.e. December 2015. The intention for this extension was to enhance study sensitivity. It is known that a relevant portion of particle induced tumours become detectable first rather late in rats. The study sensitivity to detect lung tumours will be further enhanced by an extended lung histopathology as 60 instead of 6 sliced will be studied per lung.

Satellite groups were sacrificed after 12 months (chronic group with 10 animals per dose for histopathology) and after 3 months, 12 months, and 24 months (for kinetic/organ burden evaluations).

Main exposure groups are used for histopathology examinations (carcinogenicity groups). Groups of 50 animals (of each exposure level) were sacrificed and examined after 24 months of exposure. Additional groups of 50 animals were kept without exposure up to 30 months and animals are sacrificed and examined after 30 months or if the only 25% or less animals are still alive. Animals of each group which died during the exposure or post-exposure period are examined as well.

2.4 Results

The 24-month exposure period was successfully terminated and 50 animals per dose group sacrificed (see deliverable 4.1). The remaining animals were kept exposure-free for maximally 6 additional months. This post-exposure period successfully ended December 11, 2015. After sacrifice of these remaining animals, the organs required according to OECD 453 were collected and stored, and fixed in formalin. The sampled organs of the 24-month exposure period have been sent to Fraunhofer ITEM in Hannover in July 2015. Histological analysis from these slices is ongoing.

In the post-exposure groups, the body weight development was not significantly impaired by substance treatment (Figure 1) indicating the absence of excess treatment mediated toxicity.

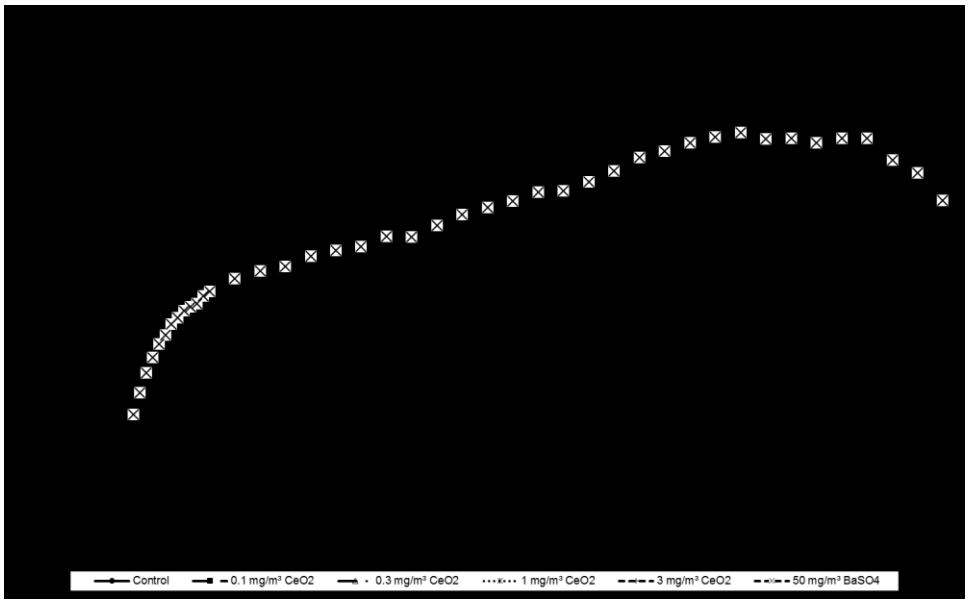

Figure 1: Body weight development during of the recovery group animals (24 months exposure and kept up to 30 months)

Table 1 Lethality rates in the different study groups

	Control	**CeO_2**				**$BaSO_4$**
				mg/m³		
Exposure concentration	**0**	**0.1**	**0.3**	**1**	**3**	**50**
Main Group (24 months)						
total	50	50	50	50	50	50
lethality	12	9	16	16	11	14

Post-exposure group (30 months)						
total	50	50	50	50	50	50
entering recovery period	39	37	35	36	34	38
sacrificed, end of recovery period	22	14	16	18	18	15
lethality	28	36	34	32	32	35

Table 1 shows the lethality rates of the different study groups including the respective data from the groups sacrificed after 24 months (also see deliverable 4.1). 34 to 39 of 50 (68% to 78%, respectively) animals had entered the post-exposure period 15 to 22 out of 50 animals (30% to 44%, respectively) were at the end of the 30-month period and sacrificed then. The maximum lethality rate of all 24-month test groups was below 30 % (also see deliverable 4.1). In average over all 24-month test groups, it was 25.5% which is slightly higher than the average BASF historical control (21.5% which is, however, obtained with feeding studies).

The samples of the post-exposure period animals will be sent to Fraunhofer ITEM in Hannover (Germany) in the third week of January. Slices for histological analyses will be prepared from these organs at Fraunhofer. The histological examinations will be performed subsequently at Fraunhofer ITEM. The histological analyses will cover standard evaluations according to OECD protocols for all organs except for lung. For lung, an extended analysis will be performed where 60 instead of up to 6 slices per organ will evaluated.

2.5 Evaluation and conclusions

The post-exposure phase of the chronic study has been successfully terminated. The total in-life phase of the chronic inhalation study has therefore been successfully finalized. The main phase to generate results from the study samples has begun but will take a considerable effort and time. Thus, results crucial to NANoREG are not yet available. Currently, final or even interim evaluations cannot yet be made nor can conclusions be drawn at this moment.

2.6 Data management

Excel files for the ISA-TAB-Nano templates have been prepared to report the available results from the histological analyses and submitted to be included into the database. Results are not available yet but will be included in the final report in 2016 and will be then put into the database.

3 Deviations from the work plan

No major or relevant deviations from the work plan were necessary. Thus, there is nothing to report.

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