Towards a method for detecting the potential genotoxicity of nanomaterials

Interim Report

Milestone 2

Determination of acute toxicity of TiO_2, SiO_2, and CNT nanomaterials of the NANOGENOTOX Joint Action Plan

Version 3
June, 2012

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.

Health Programme 2008-2013

Executive Agency for Health and Consumers

NANOGENOTOX

Grant Agreement n° 2009 21 01

Grant Agreement n° 2009 21 01

WP 7 : Toxicokinetics and tissue distribution of MNs for specification of organs at risk for genotoxicity testing (toxicokinetics)

Milestone 2 : Identification of doses for use of *in vivo* studies

Milestone leader : RIVM

Wim H De Jong

National Institute for Public Health and the Environment

Antonie van Leeuwenhoeklaan 9, 3721 MA, Bilthoven, The Netherlands

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

www.nanogenotox.eu

Workflow	
Author(s) Wim H De Jong (RIVM) Margarita Apostolova (IMB-BAS) Francesco Cubadda, Francesca Maranghi, Federica Aureli (ISS) Vincent Dive (CEA) Nicklas R Jacobsen (NRCWE)	**Reviewer(s)** WP Leader: Wim H De Jong (RIVM) Coordinator: Nathalie Thieriet (ANSES)

Document status: draft Creation date: 29/03/2012

Confidentiality level of the deliverable		
PU	Public	
CO	Confidential, only for members of the consortium (including the Commission Services)	CO

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Introduction

This report presents an overview of the results obtained in the acute toxicity studies with various nanomaterials as used within the NANOGENOTOX Joint Action Plan. *In vivo* studies will be performed for identification of possible target organs for genotoxicity after oral and intravenous exposure to nanomaterials. In addition, also in vivo genotoxicity studies need to be performed for confirmation of possible genotoxicity as demonstrated in *in vitro* assays.

One of the aims of the toxicokinetics work package is to identify relevant organs for genotoxicity testing based on the determination of organ exposure to MNs. To identify organs at risk the choice was made for intravenous administration as for other application routes (inhalation, oral, dermal) cellular barriers are present that may limit uptake of nanomaterials. Such limited uptake may result in rather low undetectable organ levels of the nanomaterials.

Before conducting toxicokinetic studies and determination of tissue distribution, a dose for the *in vivo* studies has to be selected. The dose administered should be sufficiently high to be able to detect the nanomaterials (CNTs), or its main chemical constituent (Ti or Si), but also be sufficiently low to cause no or minimal toxicity. The OECD (Organisation for Economic Co-operation and Development, Paris, France) has developed guidelines for the testing of chemicals. OECD Guideline 420 describes a fixed dose procedure for determination of the LD50 (the dose causing death in 50% of the treated animals). By using this fixed dose approach the number of animals needed for the determination of the LD50 can be reduced to the absolute minimum.

Titanium dioxide (TiO_2).

Oral acute toxicity studies. NM-101, NM-102, NM-103, NM-104, NM-105

Nanomaterial preparation

Five TiO_2 materials used in the NANOGENOTOX project were evaluated for oral acute toxicity.

Table 1. TiO_2 nanomaterials.

Nanomaterial	Size	Characteristics
NM-101	7-10 nm	spherical
NM-102	15-25 nm	spherical
NM-103	20 nm	hydrophobic, spherical
NM-104	20 nm	hydrophilic, spherical
NM-105	22 nm	spherical

Particle suspensions were prepared fresh every day. A 2.56 mg/ml stock dispersion was prepared by pre-wetting the TiO2 powder in 0.5 vol % ethanol (96% purity) followed by dispersion in 0.05 wt% Rat Serum Albumin (Sigma Aldrich, #A6272) in ultra pure water. The suspensions were sonicated for 16 minutes on ice using a Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a disruptor horn (Model number: 101-147-037).

Health Programme 2008-2013

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Executive Agency for Health and Consumers

www.nanogenotox.eu

The prepared stock suspensions were diluted 9:1 with 10x concentrated sterile filtered phosphate buffer pH 7.4 (702 mg NaH2PO4 x 2H2O, 4155 mg Na2HPO4 x 7H2O, dissolved in 1L) making the exposure suspension. Vehicle suspension were also sonicated and diluted according to above description. The final solution for administration to the animals was 2.3 mg/ml.

Animals

Female (N=9) and male (N=36) Wistar rats ((CRL:WI (WU)) were obtained from Charles River, Germany. They were delivered as 7 weeks old animals, weighing between 151 and 175g for females and 201 and 225g for males. The rats were randomly divided into groups of two males or three females and housed in polycarbonate cages with a bottom area of 905 cm^2 and a height of 21.5 cm, with pinewood sawdust bedding (Jeluxyl HW 300/500), enrichment (rodent tunnels (Brogaarden, Denmark), Enviro Dri and rat aspen wood blocks from Lillico). Standard feeding consisted of chow diet (Altromin no. 1324, Christian Petersen, Denmark) and water *ad libitum*. The cages were kept in rooms with a 12 h light period from 6 a.m. to 6 p.m., and the temperature and relative humidity in the animal room were 21 ± 2°C and 50 ± 5%, respectively. The cages were sanitized twice weekly.
The rats were kept under pathogen-limited conditions and were allowed to acclimatize for 2 weeks before they entered the experimental protocol. All rats were 9 weeks old at the time of the experiment. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government, and the Animal Experiment Inspectorate under the Ministry of Justice, approved the study.

Experimental design

All particle suspensions were used immediately after preparation (between 0-1 h). Oral dosing was performed according to the guidelines described in Handbook of Laboratory Animal Science 2nd Edition by Jann Hau and Gerald Hoosier. The rats were dosed using a straight metal feeding needle with bulbed tip. The distance of the needle should be from lips to the last rib of the animal. Once the needle is in place, the animal is observed to be properly breathing before dosing 1 ml. The rats were not sedated or deprived of feed before or after administrations of the test sub-stance.
Animals were dosed orally with exposure suspension (1 ml per exposure) either once (3 males per group, 5 particles and controls) or during five consecutive days (3 males per group, 5 particles and controls; 3 females per group 2 particles NM-101 or NM-105 and controls) (Table 2). The experiment was terminated 24 h after last exposure. Each rat received either 0 or 2.304 mg per dose, the dose in mg/kg body weight (b.w.) varying depending on the actual weight of the animals. The doses administered ranged from 7.7 – 8.4 mg/kg b.w. and 38.4 – 41.9 mg/kg b.w. for the male rats for single and multiple dosing, respectively. For the female rats the doses ranged from 11.5 – 13.2 and 56.0 – 65.8 mg/kg b.w. for single and multiple dosing, respectively.

Table 2. Experimental design for oral acute toxicity studies.

Nanomaterial	Treatment Single	Treatment Repeated (5x)	Treatment Repeated (5x)
NM-101	3 males	3 males	3 females
NM-102	3 males	3 males	
NM-103	3 males	3 males	
NM-104	3 males	3 males	
NM-105	3 males	3 males	3 females
Control	3 males	3 males	3 females

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Results

The animals were observed several times each day during the exposure period for signs of discomfort and acute toxicity. No unusual changes in behavior or in locomotor activity were observed. No ataxia, no pilo erection and no signs of intoxication were observed during the 6 day period. As no signs of toxicity was observed using the maximum obtainable dose (NANOGENOTOX protocol), lower doses were not tested.

Conclusions

For all five TiO_2 nanomaterials (NM-101, NM-102, NM-103, NM-104, NM-105) tested the maximum obtainable exposure dose with the NANOGENOTOX dispersion protocol (approximately 12.5 mg/kg body weight) did not induce toxicity after a single or five times repeated oral administrations.

Intravenous acute toxicity studies. NM-100 and NM-102

Nanomaterial preparation.

A stock dispersion was prepared with RSA (rat serum albumin). RSA was used in contrast to the protocol prepared in WP4 (characterization) that uses BSA (bovine serum albumin). As in the toxicokinetic experiments the nanomaterial suspension had to be administered repeatedly (on five consecutive days) the compatible RSA was chosen to avoid the induction of a possible immune response to the BSA present in the solutions. RSA was obtained form Sigma-Aldrich (A6272 albumin from rat serum, ≥ 96%, agarose gel electrophoresis).

A TiO_2 nanomaterial solution was prepared according to the protocol of WP4. A 2.56 mg/ml stock dispersion was prepared by prewetting the amount of nanomaterial powder needed in 0.5 vol% ethanol (≥ 96% purity) followed by dispersion in 0.05 wt% RSA-ultra pure water during 16 minutes of probe sonication on ice (Branson Sonifier S-450D, Branson Ultrasonics Corp., Danbury, CT, USA, equipped with a disruptor horn Model number: 101-147-037). The stock solution was diluted for 10% using 10x concentrated Phosphate buffer resulting in a final solution for administration to the animals of 2.3 mg/ml.

Animals

Six-week-old male Wistar rats (HsdCpb:WU) were purchased from Harlan Nederland BV (Horst, The Netherlands) and allowed an acclimatization period for at least one week before starting the experiment. Animals were bred under specific pathogen-free (SPF) conditions and barrier maintained during the entire experiment in Macrolon cages at a room temperature of 23 ± 1 ^{o}C, a relative humidity of 50 ± 5% and a 12-h light/dark cycle. Drinking water and conventional feed were provided *ad libitum*. The experiment was approved by an independent Ethical Committee on Animal Experimentation and conducted in compliance with all applicable provisions of the national laws, i.e. the Experiments on Animal Decree and the Experiments on Animal Act.

Experimental design

The OECD Guideline 420 for a fixed dose procedure was used to determine the acute toxicity (LD50) of the TiO_2 nanomaterials NM-101 and NM-102. By using this fixed dose approach the number of animals needed for the determination of the LD50 can be reduced to the absolute minimum. The maximum dose administered was approximately 9.2 mg/kg b.w. (calculated using a weight for the rats of 250 g). The schedule used is presented in Figure 1.

Animals were observed at 30 min, and 1, 2, 3, 4, 8, and 24 hour after administration for signs of toxicity. As no signs of toxicity were observed animals were autopsied at day 14 after exposure. Several organs were collected as a pilot study for determination of the Ti content with ICP-MS (Inductively Coupled Plasma-Mass Spectroscopy).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

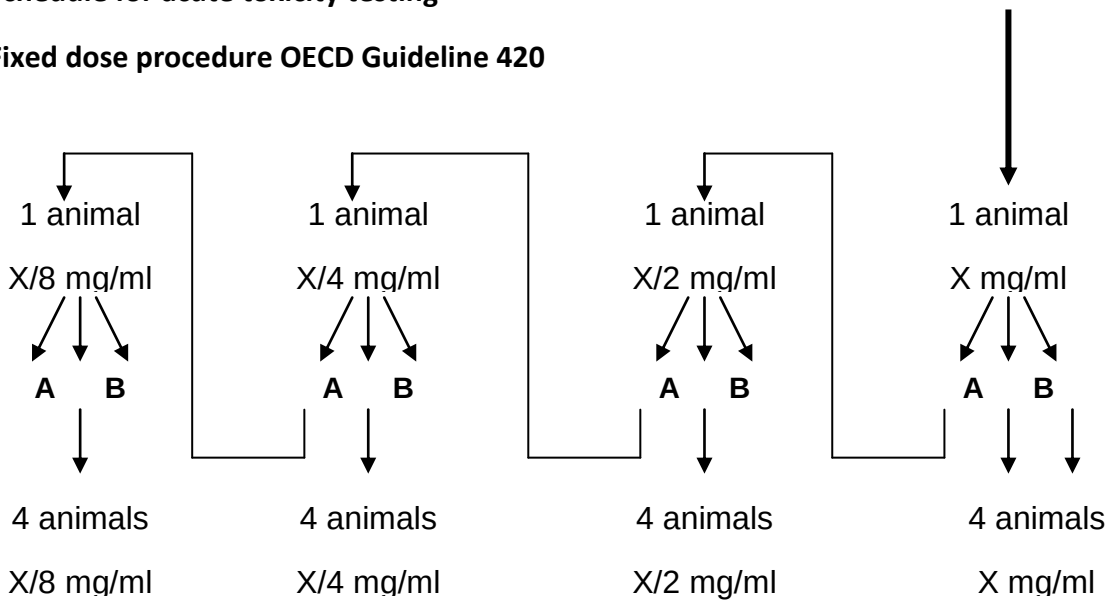
Health Programme 2008-2013

Executive Agency for Health and Consumers

www.nanogenotox.eu

Schedule for acute toxicity testing

Fixed dose procedure OECD Guideline 420



A Lethality

B Toxicity

C No effect

X= highest possible concentration

Figure 1. Schedule for the fixed dose procedure for the evaluation of the LD50 dose.

Results

First one animal was injected intravenously via the tail vein with 1 ml solution containing 2.3 mg/ml of TiO_2 (~ 9.2 mg/kg). As no signs of toxicity were observed an additional four animals were treated with the same dose. During the observation period of 14 days no signs of toxicity were observed for the two preparations (NM-100 and NM-102) investigated. No further dilutions of TiO_2 were investigated. The weight of the animals before and after the TiO_2 administration is presented in Table 3.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Table 3. Animal weight before and after intravenous administration of TiO_2 NM-100 and NM-102 (single dose of 9.2 mg/kg b.w.).

Treatment	Animal	Weight before (g)	Weight after (g)
NM-100	201000255-1	229	265
	201000255-2	262	293
	201000255-3	241	291
	201000255-4	262	301
	201000255-5	266	317
NM-102	201000255-11	224	282
	201000255-12	262	288
	201000255-13	265	296
	201000255-14	260	298
	201000255-15	251	281

The mean growth of the animals during the 14 days of observation was for NM-100 and NM-102 treated animals respectively, 41.4 ± 8.8 gram and 36.6 ± 12.7 gram.

Conclusions

It was concluded that the LD50 value was above the highest possible dose of TiO2 that could be obtained using the NANOGENOTOX dispersion protocol. The dose for the *in vivo* toxicokinetics studies using NM-100 and NM-102 was decided to be 2.3 mg/ml intravenously administered which was approximately 9.2 mg/kg body weight, considering a mean weight of the animals of about 250 g.

Intravenous acute toxicity study NM-105

Nanomaterial preparation

NM-105 was prepared as stock dispersion of 2.56 mg/ml TiO_2 wetted with EtOH followed by dispersion in with 0.05% RSA in water. The stock solution was diluted in endotoxin free 10x concentrated phosphate buffer, or 10x concentrated physiological saline solution to a final concentration of 2.3 mg/ml.

Animals

Ten two-months old Wistar rats (5 male, 5 female, EBBA-BAS, Bulgaria) initially weighting 202 ± 13 g were housed under constant conditions of temperature, humidity and a 12:12 h day/night cycle, with food and water *ad libitum*. The experiments were approved by the Animal Research Committee of the Institute of Neuroscience – BAS (Protocol N 27/02.06.2011).

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Experimental design
The rats were randomly divided into 4 groups and treated intravenously with either NM-105 or a phosphate solution (Table 4). The dose administered was approximately 11.5 mg/kg body weight (mean body weight of the animals approximately 200 g).

Table 4. Experimental design acute toxicity testing NM-105 (single dose of 11.5 mg/kg b.w.).

Group	Number	Sex	Treatment
1	3	male	NM-105
2	2	male	Phosphate solution
3	3	female	NM-105
4	2	female	Phosphate solution

Results
Twenty four hours following the injection of NM-105 there was no lethality. Two injected animals of each sex were sacrificed and went for autopsy. No abnormalities were registered. The health statuses of animals were followed for 5 days and no adverse effects were registered.
A similar experiment was performed for NM-105 prepared in sterile physiological saline solution. There were no lethality and no adverse effects.

Conclusions
The LD50 value for TiO_2 NM-105 was above 12.8 mg/kg body weight.

Overall conclusions TiO_2
The results obtained with the various TiO_2 nanomaterials show a relative low toxicity. With the maximal concentration that could be obtained using the NANOGENOTOX dispersion protocol no overt toxicity was noted for the various TiO_2 nanomaterials after oral or intravenous administration. Although for the intravenous route not all TiO_2 nanomaterials were investigated based on the obtained results it can be assumed that the dose of 12.5 mg/kg body weight will not induce overt toxicity. Indeed follow up toxicokinetic studies with NM-100 and NM-102 showed that also a five times consecutive intravenous administration was well tolerated by the animals.

Silicon dioxide (SiO_2)

Nanomaterials preparation
The NANOGENOTOX dispersion protocol, as modified for silica nanomaterials, was used throughout. The highest possible concentration for SiO_2 according to the findings of WP4 was 6.0 mg/ml (expressed as nanomaterial) in NaCl 0.90% w/v. In the studies presented below the nanomaterials were dispersed at this concentration.

The two silica nanomaterials tested were NM-200 (amorphous precipitated silicon dioxide) and NM-203 (fumed silicon dioxide). From the bottles provided by the JRC, 0.1800 g of each material were accurately weighted on an analytical balance (readability 0.0001 g) in a 50 ml polypropylene Falcon® tube, then added with 30 mL of sterile physiological solution (Normal saline - NaCl 0.90% w/v) to

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Health Programme 2008-2013

Executive Agency for Health and Consumers

www.nanogenotox.eu

obtain a concentration of 6.0 mg/ml expressed as nanomaterial. The tube containing the dispersion was transferred into a 500 ml glass beaker equipped with a polystyrene support to hold the tube in upright position; the beaker was filled up to 90% volume with ice and placed close to the sonication device that consisted of a Bandelin Sonopuls Ultrasonic Homogenizer HD3200 series equipped with a SH 213 G booster horn and a sterile KE 76 tapered tip (recommended for nanoparticles dispersion); the probe was embedded between the upper third and the second third of the dispersion, carefully avoiding any contact with the tube walls or any deflection from the vertical position (perpendicular to the bench), which may affect the efficiency of the procedure. The sonication was run for 16 minutes at 10% amplitude and the dispersion was thus used for intra-venous (IV) or oral administration as detailed below. Particle suspensions were freshly prepared before use and dosing of animals took place within 10 min after sonication of the dispersions. All the described procedures were accomplished in a VBH cabinet, to minimize the contamination of the dispersion. In order to avoid any possible contamination, all the instruments intended for the experimental procedures were sterilized before use and kept under the VBH cabinet.

Intravenous route - Single dose study

Animals and Treatment

Intravenous administration: Twenty-two Sprague-Dawley rats (approx 150 g b.w.), 11 males and 11 females, were obtained from Harlan (Italy). They were kept under standard laboratory conditions (22 ± 0.5 °C room temperature, 50-60% relative humidity, 12 hrs dark-light alternation with 12-14 air changes *per* hour). During and after the two-week acclimatization period, all the rats were fed with the Harlan Teklad 2016 rodent diet, which was found to contain the lowest amount of Si compared to the other standard rodent diets. Water and food were available *ad libitum*. Animals were divided into the three treatment groups: NM-200 5 rats/sex, NM-203 5 rats/sex and control 1 rat/sex. All experiments on animals were performed according to the European Community Council Directive 86/609/EEC and National Law on Animal Experimentation (D. Lvo 116/92).

Experimental design

About 2-2.5 ml/kg b.w. of the aqueous dispersion was administered in order to achieve a dose of 20 mg/kg b.w. At day 1, animals were intravenously treated on the tail with 0 mg/kg b.w. (control, vehicle only, sterile physiological solution) and 20 mg/kg b.w. of both NM-200 and NM-203. Twenty-four hours after the treatment (day 2), 3 males and 3 females/group and controls were sacrificed by CO_2 inhalation. Organs were excised for SiO_2 nanoparticle determination (as Si by ICP-MS). The other 2 treated rats sex/group were checked twice a week for general signs of toxicity as well as for body weight and food consumption variations until the sacrifice at day 14.

Table 5. Experimental design for intravenous SiO_2 acute toxicity studies (single dose of 20 mg/kg b.w.).

Nanomaterial	Treatment IV day 1	Treatment IV day 1	Evaluation Day 2	Evaluation Day 14
NM-200	5 males	5 females	3 males + 3 females	2 males + 2 females
NM-203	5 males	5 females	3 males + 3 females	2 males + 2 females
Control	1 male	1 female	1 male + 1 female	

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Results

No clinical signs of toxicity were recorded during the treatment or the recovery period. At sacrifice rats treated with both silica nanomaterials showed no gross alterations in target organs.

Intravenous route - Repeated dose study

Intravenous repeated-dose acute toxicity testing was performed in the frame of the toxicokinetic study.

Animals and Treatment

Forty-eight Sprague-Dawley rats (approx 150 g b.w.), 30 males and 18 females, were purchased from Harlan (Italy). They were kept under standard laboratory conditions (22 ± 0.5 °C room temperature, 50-60% relative humidity, 12 hrs dark-light alternation with 12-14 air changes per hour). During and after the acclimatization period, all the rats were fed with the Harlan Teklad 2016 rodent diet, which was found to contain the lowest amount of Si compared to the other standard rodent diets. Water and food were available *ad libitum*. After approx. five days, they were divided into the treatment groups (NM-200 and NM-203) and intravenously treated via the tail vein. Body weight and food consumption were recorded daily during the five treatment days and once a week for the follow-up period until sacrifice. All experiments on animals were performed according to the European Community Council Directive 86/609/EEC and National Law on Animal Experimentation (D. Lvo 116/92).

Experimental design

About 2-2.5 ml/kg b.w. of the aqueous dispersion was administered in order to achieve a dose of 20 mg/kg b.w. At day 1-5, animals were intravenously treated in the tail vein with 0 mg/kg b.w. (control, vehicle only, sterile physiological solution) and 20 mg/kg b.w. of both NM-200 and NM-203. At day 6, 14, 30 and 90 after the treatment, male and female rats were anaesthetized with gaseous solution of isofluorane and blood samples were collected by intracardiac puncture. Subsequently, animals were sacrificed by CO_2 inhalation and organs were excised for SiO_2 nanoparticle determination (as Si by ICP-MS).

Table 6. Experimental design for intravenous SiO_2 toxicokinetic study (repeated dose of 20 mg/kg b.w.).

Nanomaterial	Treatment IV day 1-5	Evaluation day 6	Evaluation day 14	Evaluation day 30	Evaluation day 90
NM-200	12 males+6 females	3 males+3 females	3 males	3 males	3 males+3 females
NM-203	12 males+6 females	3 males+3 females	3 males	3 males	3 males+3 females
Vehicle control	6 males+6 females	3 males+3 females			3 males+3 females

Results

No clinical signs of toxicity were recorded for NM-200 during the treatment period. Rats treated with NM-203 showed necrotic lesions at the site of injection (the tail); in some cases the tip of the tail was cannibalized. No clinical signs of toxicity were recorded during the recovery period. At sacrifice rats

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Health Programme 2008-2013

Executive Agency for Health and Consumers

www.nanogenotox.eu

treated with silica NM-200 and controls showed no gross alterations in the various organs evaluated. NM-200 treated males and NM-203 treated males and females showed reduced growth between day 1 and day 5 of treatment when compared to the animals treated with vehicle control (Table 7). No pathological alterations in spleen, liver, lungs were observed in NM-200 treated male and female rats as well as in controls. Rats treated with NM-203 and sacrificed at day 6, 14, 30 and 90 exhibited markedly enlarged spleen (splenomegalia) and discolored liver.

Table 7. Animal weight (grams) before and after intravenous administration of SiO_2 NM-200 and NM-203 (repeated dose of 20 mg/kg b.w.).

Treatment	Males Day 1	Males Day 5	Females Day 1	Females Day 5
NM-200	186 ± 8	203 ± 9*	165 ± 9	172 ± 9
NM-203	188 ± 9	174 ± 6***	164 ± 6	160 ± 10*
Vehicle control	189 ± 11	214 ± 7	167 ± 4	180 ± 3

* p<0.05, *** p<0.001 according to Students t-test.

Conclusions

After single intravenous administration with the two investigated SiO_2 nanomaterials no toxicity was observed at a dose of 20 mg/kg body weight. On the other hand, after 5 times repeated IV exposure, NM-200 male group showed a slight reduction in weight gain compared to control animals. For NM-203 treated rats, necrotic lesions at the site of injection were observed, accompanied by a decrease in body weight (males and females) and alterations in liver and spleen.

Oral route - Single dose study

Animals and Treatment

Twenty-two Sprague-Dawley rats (approx 150 g b.w.), 11 males and 11 females, were obtained from Harlan (Italy). They were kept under standard laboratory conditions (22 ± 0.5°C room temperature, 50-60% relative humidity, 12 hrs dark-light alternation with 12-14 air changes *per* hour). During and after the two-week acclimatization period, all the rats were fed with the Harlan Teklad 2016 rodent diet, which was found to contain the lowest amount of Si compared to the other standard rodent diets. Water and food were available *ad libitum*. Animals were divided into the three treatment groups: NM-200 5 rats/sex, NM-203 5 rats/sex and control 1 rat/sex. All experiments on animals were performed according to the European Community Council Directive 86/609/EEC and National Law on Animal Experimentation (D. Lvo 116/92).

Exeprimental design

About 2-2.5 ml/kg b.w. of the aqueous dispersion was administered in order to achieve a dose of 20 mg/kg b.w. At day 1, animals were orally treated by gavage with 0 mg/kg b.w. (control, vehicle only, sterile physiological solution) and 20 mg/kg b.w. of both NM-200 and NM-203. Twenty-four hours after the treatment (day 2), 3 males and 3 females/group and controls were sacrificed by CO_2 inhalation. Organs were excised for SiO_2 nanoparticle determination (as Si by ICP-MS). The other 2 treated rats sex/group were checked twice a week for general signs of toxicity as well as for body weight and food consumption variations until the sacrifice at day 14.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Table 8. Experimental design for oral SiO_2 acute toxicity studies (single dose of 20 mg/kg b.w.).

Nanomaterial	Treatment Oral day 1	Treatment Oral day 1	Evaluation Day 2	Evaluation Day 14
NM-200	5 males	5 females	3 males + 3 females	2 males + 2 females
NM-203	5 males	5 females	3 males + 3 females	2 males + 2 females
Control	1 male	1 female	1 male + 1 female	

Results

No clinical signs of toxicity were recorded during the treatment or the recovery period. At sacrifice rats treated with both silica nanomaterials showed no gross alterations in target organs.

Oral route - Repeated dose study

The results presented hereby for repeated-dose acute toxicity were obtained in the frame of the toxicokinetic oral study.

Animals and Treatment

Thirty Sprague-Dawley rats (approx. 150 g b.w.), 16 males and 14 females, were obtained from Harlan (Italy). They were kept under standard laboratory conditions (22 ± 0.5°C room temperature, 50-60% relative humidity, 12 hrs dark-light alternation with 12-14 air changes *per* hour). During and after the two-week acclimatization period as well as during the treatment, all the rats were fed with the Harlan Teklad 2016 rodent diet, which was found to contain the lowest amount of Si compared to the other standard rodent diets. Water and food were available *ad libitum*. Animals were divided into the three treatment groups: NM-200 6 rats/sex, NM-203 6 rats/sex and control (2 females and 4 males). All experiments on animals were performed according to the European Community Council Directive 86/609/EEC and National Law on Animal Experimentation (D. Lvo 116/92).

Experimental design

About 2-2.5 ml/kg b.w. of the aqueous dispersion was administered in order to achieve a dose of 20 mg/kg b.w./day. Animals were orally treated by gavage for five consecutive days with 0 mg/kg b.w. (control, vehicle only, sterile physiological solution) and 20 mg/kg bw of both NM-200 and NM-203. Twenty-four hours after the last treatment (day 6), 3 males and 3 females/group and controls were sacrificed by CO_2 inhalation. Organs were excised for SiO_2 nanoparticle determination (as Si by ICP-MS). The other 2 treated rats sex/group were checked twice a week for general signs of toxicity as well as for body weight and food consumption variations until the sacrifice at day 14.

Table 9. Experimental design for oral SiO_2 acute toxicity studies (repeated dose of 20 mg/kg b.w).

Nanomaterial	Treatment Oral days 1-5	Treatment Oral days 1-5	Evaluation Day 6	Evaluation Day 14
NM-200	6 males	6 females	3 males + 3 females	2 males + 2 females
NM-203	6 males	6 females	3 males + 3 females	2 males + 2 females
Control	4 male	2 female	2 male + 1 female	2 male + 1 female

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Results

No clinical signs of toxicity were recorded during the treatment or the recovery period. Mean body weights are reported in Tables 10 and 11. At sacrifice rats treated with both silica nanomaterials showed no gross alterations in target organs.

Table 10. Animal weight (grams) after five-day oral administration of SiO_2 NM-200 and NM-203 (repeated dose of 20 mg/kg b.w.).			
Males	Controls	NM-203	NM-200
Mean body weight day 1	333 ± 9	303 ± 18	329 ± 6
Mean body weight day 5	332 ± 7	298 ± 18	340 ± 7
Body weight gain (mean±SD)	-0.9 ± 1.7	-4.8 ± 16.8	11.0 ± 6.0

Table 11. Animal weight (grams) before and after five-day oral administration of SiO_2 NM-200 and NM-203 (repeated dose of 20 mg/kg b.w.).			
Females	Controls	NM-203	NM-200
Mean body weight day 1	213 ± 8	215 ± 9	236 ± 4
Mean body weight day 5	216 ± 9	213 ± 14	238 ± 6
Body weight gain (mean±SD)	2.9 ± 6.6 g	-2.8 ± 4.8 g	1.5 ± 6.3 g

Conclusion

After single or repeated oral administration with the two investigated SiO_2 nanomaterials no toxicity was observed at a dose of 20 mg/kg body weight either as single or repeated exposure.

Overall conclusions SiO_2

The results obtained with two SiO_2 nanomaterials show that a single intravenous and single or repeated oral administration did not induce overt toxicity in rats at a dose of 20 mg/kg b.w. Follow up toxicokinetic studies did detect some toxic effects. Repeated (five consecutive days) intravenous administration induced reduced body weight gain for NM-200, whereas for NM-203 weight loss and pathological alterations at the administration site (necrotic lesions in the tail) and at autopsy (markedly enlarged spleen and discolored liver) were observed.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Carbon Nanotubes (CNT)

MWCNT preparation

The dispersion protocol involved the preparation of a 2,56 mg/ml solution of MWCNT in dispersion medium followed by sonication. Dispersions were prepared of NM-400, NM-401, NM-402, and NRCWE-006 multi wall carbon nanotubes (MWCNT).

Dispersion medium was 0.05% w/v rat serum albumin (Sigma #A4538) in water, MWCNT were pre-wetted in 0,5% ethanol before sonication (Branson Sonifier 450, with 10% amplitude, during 15min). During sonication, heat was dissipated by placing samples on ice. Dispersed ^{14}C-MWCNT solutions were then observed by optical microscopy to control sample homogeneity. A 1:10 dilution was performed to obtain a final concentration of 256 µg/ml in 0.05% w/v rat serum albumin for the injection solution. MWCNT were immediately used after their dispersion for intravenous and oral administration in rats. Depending on the MWCNT, the dispersion solutions were stable from one to several hours as indicated by visible observations.

Animals

Male and female Wistar rats were obtained from Charles River, France. The rats weight was between 226 and 234g for females and 256 and 266g for males.

Experimental design

For both the intravenous and oral studies the same protocol was used. For each nanoparticle preparation 1ml was administrated either as a single dose or as repeated doses either orally or intravenously

Two different exposure protocols have tested (NANOGENOTOX protocol):

1°: a single dosage and organ analyses at day 1 and day 90;

2°: repeated doses (one per day during 5 consecutive days) and evaluation of organs at different times (day, 6, day 14, day30 and day 90). For each CNT nanomaterial 6 animals were included per group, except for D14 and D30 for repeated dosages for which we had 3 animals were evaluated.

For each protocol, 30 rats were injected with 12 single dose (6 males and 6 females) and 18 rats for repeated doses (12 males and 6 females). The experimental design for the CNT nanomaterials is presented in Table 12.

Table 12 Experimental design for intravenous and oral CNT acute toxicity studies (single and repeated dose of 1.1 mg/kg b.w. for females, and 0.98 mg/kg b.w. for males)

Nanomaterial	Treatment Day 1	Treatment Day 1	Evaluation Day 2 (24h)	
CNT	3 males	3 females	3 males + 3 females	
Nanomaterial	Treatment Days 1-5	Treatment Days 1-5	Evaluation Day 6	Evaluation Day 14
CNT	6 males	3 females	3 males + 3 females	3 M

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Results

For all four CNT nanomaterials investigated (NM-400, NM-401, NM-402 and NRCWE-006) no indications for toxicity were observed after either single or repeated, or intravenous or oral administration of a dose of 256 µg/ml per animal or 1 mg/kg body weight. A representative example of body weight and body weight gain after intravenous and oral administration of NM-400 is presented in Table 13. For NM-401, NM-402 and NRCWE similar results were obtained (data not shown).

Table 13 Body weight of rats after IV and oral treatment with MN-400 MWCNT.

MALES	Day 1	Day 2	Day 6	Day 14
Single IV	253 ± 8	254 ± 9		
Repeated IV	252 ± 5		309 ± 62	
Repeated IV	249 ± 7			307 ± 3
Single oral	251 ± 8	251 ± 8		
Repeated oral	250 ± 6		258 ± 4	
Repeated oral	252 ± 7			313 ± 8
FEMALES	Day 1	Day 2	Day 6	
Single IV	209 ± 2	209 ± 1		
Repeated IV	207 ± 3		221 ± 2	
Single oral	206 ± 6	207 ± 6		
Repeated oral	209 ± 9		223 ± 7	

Conclusion

The results obtained with the four CNT nanomaterials show that a single or repeated intravenous and oral administration did not induced toxicity in rats at a dose of 0.98 – 1.1 mg/kg body weight.

Overall conclusions acute toxicity testing

The TiO_2 nanomaterials were evaluated for their acute toxicity at the highest possible concentration using the NANOGENOTOX dispersion protocol. At the concentration of 2.3 mg/ml or 9.2 – 11.5 mg/kg body weight no acute toxicity was observed for either the intravenous or oral route of exposure. It can be concluded that this concentration can be used for the *in vivo* studies with TiO_2 nanomaterials. Indeed experiences with both single and repeated intravenous administration have shown that this dose can be used.

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

For SiO_2 nanomaterials a dose of 20 mg/kg b.w., based on a dispersion concentration of 6.0 mg/ml, could be established as non toxic after single intravenous and single or repeated oral administration of NM-200 and NM-203. After repeated-dose exposure, effects were observed during the toxicokinetic intravenous study. During intravenous administration for 5 consecutive days, a slight toxicity was noted for NM-200 as indicated by the reduced body weight gain in treated animals when compared to controls. For NM-203, the toxicity was overt, as indicated by a weight loss, growth retardation in both males and females, and pathological alterations at the administration site (necrotic lesions in the tail) and at autopsy (markedly enlarged spleen and discolored liver).

For all four MWCNT nanomaterials investigated (NM-400, NM-401, NM-402, NRCWE-006) the dose of 1 mg/kg body weight did not induce toxicity after either intravenous or oral, or single or repeated administration.

Health Programme 2008-2013

The NANOGENOTOX Joint Action is co-funded by the Executive Agency for Health and Consumers (Grant Agreement n°2009 21 01) under the European Union 2nd Health Programme.

Executive Agency for Health and Consumers

www.nanogenotox.eu

• Contact •

Website:
www.nanogenotox.eu

E-mail:
nanogenotox@anses.fr

Coordinator: French Agency for Food, Environmental and Occupational Health & Safety (ANSES)

27-31, avenue du Général Leclerc
94701 Maisons-Alfort Cedex
France

anses
French agency for food, environmental and occupational health & safety

Partners

French Agency for Food, Environmental and Occupational Health Safety (France)	ANSES	anses
Federal Institute of Risk Assessment (Germany)	BfR	BfR
French Atomic Energy Commission (France)	CEA	cea
Institute of Mineralogy and Crystallography (Bulgaria)	IMC-BAS	
Veterinary and Agrochemical Research Centre (Belgium)	CODA-CERVA	
Finnish Institute of Occupational Health (Finland)	FIOH	Finnish Institute of Occupational Health
Roumen Tsanev Institute of Molecular Biology Academy of Sciences (Bulgaria)	IMB-BAS	
Institut national de recherche et de sécurité (France)	INRS	inrs
National Health Institute Doutor Ricardo Jorge (Portugal)	INSA	
Scientific Institute of Public Health (Belgium)	IPH	isp wiv
Institut Pasteur of Lille (France)	IPL	Institut Pasteur de Lille
Istituto superiore di sanità (Italy)	ISS	
The Nofer institute of Occupational Medicine (Poland)	NIOM	
National Research Centre for the Working Environment (Denmark)	NRCWE	
National Institute for Public Health and the Environment (The Netherlands)	RIVM	rivm
Universitat Autònoma de Barcelona (Spain)	UAB	UAB

This document arises from the NANOGENOTOX Joint Action which has received funding from the European Union, in the framework of the Health Programme under Grant Agreement n°2009 21.
This publication reflects only the author's views and the Community is not liable for any use that may be made of the information contained therein.

Health Programme 2008-2013

Executive Agency for Health and Consumers

NANOGENOTOX

Grant Agreement n° 2009 21 01