United States
Consumer Product Safety Commission

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CPSC[1] Staff Statement on: Versar Report "Review of Toxicity Data and Assessment of Nanosilver, Nano Titanium dioxide, and Carbon Nanotubes"

The report titled, "Review of Toxicity Data and Assessment of Nanosilver, Nano Titanium dioxide, and Carbon Nanotubes" presents toxicity data on three nanomaterials commonly used in consumer products, and was performed by the consulting firm Versar, Inc. under Contract CPSC-D-06-0007, Task Order 009, and was submitted to the CPSC staff in May 2012.

The National Nanotechnology Initiative (NNI) was established in 2001 to provide coordination among federal agencies to responsibly develop and commercialize products enabled by nanotechnology. As part of the NNI, CPSC issued a statement in 2005 to provide manufacturers with guidance on the CPSC approach to regulating nanomaterials, and the data needed to conduct robust assessments of the potential hazards and risks associated with the use of nano-enabled consumer products. Hazard data on the biological effects of nanomaterials on human populations, especially sensitive receptors such as young children, was identified as a key data gap that must be addressed for risk assessment. The CPSC staff selected three nanomaterials (nano silver, nano titanium, and carbon nanotubes) that are likely to be used in consumer products and contracted to have a thorough evaluation of the available literature on the toxicology of these materials.

Nanomaterials represent a wide range of compounds that may vary considerably in their chemical composition and their potential effects on the environment and human body. The report is divided into three sections. Each section focuses on a specific nanomaterial and identifies key toxicological studies and provides an overview of the methods used in the study and the study results. The report demonstrated that there was a considerable amount of toxicity data available for each material. However, the quality of these studies and their data was not uniform, and the report focused on studies that may be used for decision making. The data show that there may be potential health effects associated with each material, and that there is sufficient animal data to derive an acceptable daily intake (ADI) for each nanomaterial reviewed.

[1] This statement was prepared by the CPSC staff and the attached reports were prepared by Versar, Inc. for CPSC staff. The statement and reports have not been reviewed or approved by, and do not necessarily represent the views of, the Commission.

The report cautioned that extrapolating the potential health effects from animals to humans involves a relatively high degree of uncertainty. Although a significant amount of data was available for review, the report emphasized that more high quality studies are needed to fill the identified knowledge gaps.

FINAL REPORT

REVIEW OF TOXICITY DATA AND ASSESSMENT OF NANOSILVER, NANO TITANIUM DIOXIDE, AND CARBON NANOTUBES

Contract No. CPSC-D-06-0007
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LIST OF ABBREVIATIONS AND ACRONYMS

1,2-DSPC	1,2-dipalmitoyl-sn-glycero-3-phospholine choline
3-D-HB	3-D-hydroxybutyrate
ACP	acid phosphatase
ADI	acceptable daily intake
AES	atomic emission spectrometry
AES	auger electron spectroscopy
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AM	alveolar macrophage
AST	aspartate aminotransferase
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BALT	bronchoalveolar lymphatic tissue
BUN	blood urea nitrogen
CAM	chorioallantoic membrane
CAT	catalase
ChE	cholinesterase
CHO	Chinese hamster ovary
CK	creatine phosphokinase
CMD	count median diameter
CNT	carbon nanotube
CuZnSOD	copper-zinc-containing SOD
DNA	deoxyribonucleic acid
DOTA	1,4,7,19-tetraazacyclododecane-1,4,7,10-tetracacetate
DTPA	diethylenetriaminepentaacetate
EDX	energy-dispersive x-ray
eNOS	endothelial nitric oxide synthase
FOB	functional observational battery
GAPDH	glyceraldehyde-3-phospahe dehydrogenase
GD	gestation day
GDNF	glial cell line-derived neurotrophic factor
GI	gastrointestinal
GSD	geometric standard deviation
GSH	glutathione
γ-GT	γ-glutamyltransferase
γ-GGT	γ-glutamyltransferase
GPX	glutathione peroxidase
GSD	geometric standard deviation
Hb	hemoglobin
α-HBDH	α-hydroxybutyrate dehydrogenase
HCT	hematocrit
ho-1	heme oxygenase-1
HPMC	hydroxypropylmethylcellulose
hsp 70	heat shock protein 70kDa

HYP	hydroxyproline
ICP	inductively coupled plasma
ICP-MS	inductively-coupled plasma mass spectrometry
IL	interleukin
IL-1β	interleukin-1β
i.p.	intraperitoneal
IR	infrared
i.v.	intravenous
LALN	lung-associated lymph node
LDH	lactate dehydrogenase
L-NAME	N-nitro-L-arginine methyl ester
LOAEL	lowest-observed-adverse-effect level
LPS	lipopolysaccharide
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MDA	malondialdehyde
MDD	manufacturer designated diameters
MEF	mouse embryo fibroblast
microSPECT	microsingle photon emission tomography
MIP-2	macrophage inhibitory protein
MMAD	mass median aerodynamic diameter
MN	micronucleus
MnSOD	manganese-containing superoxide dismutase
MPO	myeloperoxidase
MPV	mean platelet volume
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	mass spectrometry
mt-2A	metallothionein-2A
MTS	3-(4,5, dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphopheyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCNT	multi-walled CNT
NADPH	nicotinamide adenine dinucleotide phosphate
NAG	N-acetyl-β-glucosaminidase
β-NAG	β-N-acetyl-β-glucosaminidase
NCE	normochromatic erythrocyte
NIOSH	National Institute for Occupational Safety and Health
NK	natural killer
NO	nitric oxide
NOAEL	no-observed-adverse-effect level
NQO1	NADPH oxidoreductase 1
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
OES	optical emission spectrometry

OVA	ovalbumin
PBS	phosphate-buffered saline
PCE	polychromatic erythrocyte
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDW	platelet distribution width
PEG	polyethylene glycol
PET	positron emission tomography
PMN	polymorphonuclear neutrophil
PND	postnatal day
RBC	red blood cell
RDW	red (cell) distribution width
RLE	rat liver epithelial
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
s.c.	subcutaneous
SCE	sister chromatid exchange
SD	standard deviation
SE	standard error
SEM	scanning electron microscopy
SHE	Syrian hamster embryo
SiO_2	silicon dioxide
SOD	superoxide dismutase
SPF	specific-pathogen-free
SWCNT	single-walled CNT
TEM	transmission electron microscopy
TGF	transforming growth factor
TiO_2	titanium dioxide
TMAO	trimethylamine-N-oxide
TNFα	tumor necrosis factor α
TWA	time-weighted-average
U.S. EPA	U.S. Environmental Protection Agency
WBC	white blood cell
XRD	x-ray diffraction
XTT	sodium 3,3'-[1- (phenylaminocarbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate

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EXECUTIVE SUMMARY

This report provides a summary of the current data for physical and chemical properties, use, exposure, and toxicity on three nanomaterials: elemental silver nanomaterials, titanium dioxide (TiO_2) nanoparticles, and carbon nanotubes (CNTs). In addition, data gaps and future research needs are also included, where possible, in this document.

Nanomaterials represent a wide range of compounds that are being used in consumer products with the stated purpose of improving the performance and durability of these products. Nanomaterials are defined as materials/particles that range from 1 to 100 nanometers (nm) in length. Although these materials may have the same chemical composition as non-nanomaterials, these new materials may vary significantly in their structure, physical and chemical properties, and potentially in their behavior in the environment and in the human body.

The U.S. Consumer Product Safety Commission (CPSC) is currently evaluating information on nanomaterials in consumer products to determine if there are any potential safety and health risks to the public. As with other consumer products, the CPSC can assess a product's potential risk to the public under existing CPSC statutes, regulations, and guidelines. Neither the Consumer Product Safety Act (CPSA) nor the Federal Hazardous Substances Act (FHSA) requires the pre-market registration or approval of products. Thus, it is usually not until a product has been distributed in commerce that the CPSC would evaluate a product's potential risk to the public (http://www.cpsc.gov/library/cpscnanostatement.pdf). In the conference report on H.R. 4040, Consumer Product Safety Improvement Act (CPSIA) of 2008, in its discussion on the authorization of appropriations (Section 201), Congress stated that it recognizes nanotechnology as a new technology utilized in the manufacture of consumer products, and that the Conferees expect the Commission to review the utilization and safety of its application in consumer products consistent with the Commission's mission. Therefore, the CPSC is interested specifically in reviewing toxicity and exposure information to assess the potential health concerns of nanomaterials that can be used in consumer products.

The main focus of this document is to assess the most recent toxicological data; however, potential health risks of using nanomaterials depend on the possible hazards of the nanomaterials in these products and the resulting potential exposure. The main characteristics important for human exposure to nanomaterials from consumer products include physical and chemical properties, concentration of the nanomaterials, the consumer products where used, and the exposure route. Therefore, these characteristic data are provided in the document, as well. A brief summary on the data for use, physical and chemical properties, and exposure for nanomaterials is provided below followed by the results of the toxicity review for each nanomaterial.

The use of nanomaterials has been reported in various consumer products. These products include: cosmetics, cleaning products, personal care products, sporting goods, textiles and clothing, electronics, food and beverages, home and garden products, and sunscreens. Reported uses for nanosilver, nano TiO_2 and carbon nanotubes are discussed to follow.

Nanosilver has been reported to be used in a number of products including spray disinfectants, medical products, hair dryers, make up, soaps and dish detergents, cooking utensils, food preservative film, filters of air and water purifications systems, dietary supplements, household appliances, textiles, clothes, shoes, and personal care products. An inventory of nanotechnology-based consumer products currently on the market has been developed by The Woodrow Wilson Center Project on Emerging Nanotechnologies and contains more than 1,300 manufacturer-identified nanotechnology products entering the commercial market worldwide. Approximately 24 percent (313) of the 1,300 consumer products in the inventory contain nanosilver particles. Thus, nanosilver is believed to be one of the most widely used nanomaterials in consumer products due to its antimicrobial properties.

TiO_2 nanoparticles have been used in various products including air filtration and purification systems, motor vehicle coatings (anti-scratch/stick), household products and home improvement products. Nano TiO_2 is also a popular ingredient in cosmetics and sunscreens because it blocks harmful ultraviolet rays.

CNTs are used in a variety of products due to their desirable electrical, mechanical, and thermal properties that are useful in developing lightweight, strong building materials and computers. The following products have been reported to contain CNTs: sporting good equipment (e.g., tennis rackets, bicycles), textiles and shoes, transparent electrodes for computers and electronics. Other uses include bio-medical applications such as enhanced electron-scanning microscopy imaging techniques and in pharmaceutical biomedical devices for bone grafting, tissue repair, drug delivery, and medical diagnostics.

The physical and chemical properties vary widely for each nanomaterial. Thus, the appropriate physicochemical characterization of nanomaterials used in toxicity testing is essential for data interpretation in relation to the material properties, intercomparisons between studies and conclusions drawn regarding hazard. The dependence of nanomaterial behavior on physical and chemical properties includes assessing a range of properties such as shape, crystal structure, particle size distribution, agglomeration state, surface area, surface chemistry, surface charge and porosity. Chemical properties such as vapor pressure, boiling point, water solubility, molecular weight and other properties, usually included for discrete chemical substances, may not be important for some nanomaterials, but properties such as those reported in the previous sentence are expected to be important for most nanomaterials.

Potential exposure to nanomaterials in consumer products is difficult to assess because of the many uncertainties related to what data are required for the assessment and the lack of extensive information such as the content of the nanomaterial in the product. Exposure to nanomaterials depends on the properties of the nanomaterial in the consumer product, chemical entity and the shape of the nanomaterial in the product, concentrations of the nanomaterial in the product, application of the product (where, how much, how long, and number of people using the product) and exposure routes (inhalation, ingestion or via the skin).

Results from available toxicity studies for elemental silver nanomaterials identified mild liver and gastrointestinal effects with oral exposure and pulmonary inflammation and liver effects with inhalation exposure as potential health hazards in Wistar rats exposed to uncoated

silver nanoparticles. A potential inhalation ADI value was developed using the no-observed-adverse-effect level (NOAEL) approach based on the lung and liver effects supporting data. For oral exposure, a potential ADI was derived using the NOAEL for liver and gastrointestinal data. No chronic inhalation exposure carcinogenicity/toxicity studies or reproductive or developmental toxicity studies are available, although the National Toxicology Program is currently planning chronic toxicity/carcinogenicity testing for silver nanoparticles.

The available repeated-exposure toxicity data clearly identified pulmonary inflammation as a critical effect in rodents following inhalation exposure to aerosols made from TiO_2 nanoparticles. A potential ADI value for inhalation exposure to TiO_2 nanoparticles was derived using the NOAEL approach. However, there are several areas of uncertainty to consider in extrapolating the observed effects in rodents to possible human exposure scenarios. ADI values for oral or dermal exposure to TiO_2 nanoparticles were not derived due to inadequate data to identify possible health hazards from these routes of exposure.

Available repeated-exposure toxicity studies clearly identified pulmonary effects as the most sensitive effect in rats exposed to respirable aerosols of multi-walled CNTs (MWCNTs) for 13 weeks and provide adequate data to demonstrate increased incidence, severity, and variety of adverse lung responses with increasing exposure levels. Although the available data are adequate to derive a potential inhalation ADI for MWCNTs, several areas of uncertainty are associated with extrapolating the observed effects in rodents to possible human exposure scenarios, and in using the recommended ADI for other types of CNTs, other than the MWCNTs used in the principal study. Potential ADIs were not derived for CNTs by oral or dermal exposure due to the lack of repeated-dose oral or dermal toxicity data for any types of CNTs.

Overall, the results of this review, as well as authors of peer reviewed journal articles and published reports, note that more research and/or data on nanomaterials are needed in, but not limited to, the following areas: (1) physical chemical characterization of nanomaterials, (2) effect of the physical chemical properties on toxicity and exposure (3) concentration of nanomaterials in consumer products, (4) possible routes of exposure, (5) methods for accurately measuring and detecting nanomaterial concentrations, (6) information on how many consumers use products containing nanomaterials, (7) exposure factor data (frequency of use, duration of use), and (8) toxicity testing.

1.0. INTRODUCTION

1.1. PURPOSE

The primary purpose of this document is to provide a summary of the most recent toxicological data on elemental silver nanomaterials, titanium dioxide (TiO_2) nanoparticles, and carbon nanotubes (CNTs). It also provides potential acceptable daily intake (ADI) values if adequate data were available to identify possible health hazards from a specific route of exposure. Pertinent toxicological and mechanistic studies are presented in Sections 3, 4, and 5 and include summaries of inhalation, oral, and dermal studies. Summaries of cytotoxicity and genotoxicity studies are also included for each of the nanomaterials under consideration. Background information such as the use of each nanomaterial, key physical and chemical properties, potential exposure concerns and sources, and future research needs are included in this document.

1.2. BACKGROUND

The U.S. Consumer Product Safety Commission (CPSC) is currently evaluating information on nanomaterials in consumer products to determine if there are any potential safety and health risks to the public. As with other consumer products, CPSC can assess a product's potential risk to the public under existing CPSC statutes, regulations, and guidelines. Neither the Consumer Product Safety (CPSA) nor the Federal Hazardous Substances Act (FHSA) requires the pre-market registration or approval of products. Thus, it is usually not until a product has been distributed in commerce that the CPSC would evaluate a product's potential risk to the public (http://www.cpsc.gov/library/cpscnanostatement.pdf).

Nanomaterials represent a wide range of compounds that are being used in consumer products with the stated purpose of improving the performance and durability of these products. Nanomaterials are defined as materials/particles that range from 1 to 100 nanometers (nm) in length. Although these materials may have the same chemical composition as non-nanomaterials, these new materials may vary significantly in their structure, physical and chemical properties, and potentially in their behavior in the environment and in the human body

In the conference report on H.R. 4040, Consumer Product Safety Improvement Act (CPSIA) of 2008, in its discussion on the authorization of appropriations (Section 201), Congress stated that it recognizes nanotechnology as a new technology utilized in the manufacture of consumer products, and that the Conferees expect the Commission to review the utilization and safety of its application in consumer products consistent with the Commission's mission. Therefore, the CPSC is interested in reviewing toxicity and exposure information to assess the potential health concerns of nanomaterials that can be used in consumer products.

The CPSC addresses these concerns by reviewing available toxicity studies for each nanomaterial under consideration and determining whether the substance meets the definition of "toxic" under the FHSA. The FHSA is risk-based and addresses both acute and chronic hazards. If a substance meets the definition of toxic under the FHSA due to chronic toxicity, then a

quantitative assessment of exposure and risk will follow to determine if a substance may be considered a "hazardous substance" under the FHSA.

The CPSC is currently collaborating with several federal agencies to obtain information on the use of nanomaterials. These federal agencies include the National Institute for Occupational Safety and Health (NIOSH), the Environmental Protection Agency (EPA), and the National Institute of Standards and Technology (NIST). The CPSC initiated an Interagency Agreement (IAG) with NIOSH and EPA to study the release of nanosilver from a variety of products, including those used by young children. In fiscal year 2008, the CPSC also initiated an IAG with NIOSH to evaluate the particulate aerosol generated during use of an antimicrobial spray product containing TiO_2 nanoparticles. The tests have been completed and confirmed the presence of nanoparticles in the aerosol spray. In addition, the CPSC signed an IAG with NIST in 2009 to quantify releases of CNTs that are used as flame retardants from treated mattresses and upholstered furniture components.

1.3. METHODOLOGY/APPROACH

The objectives of this effort were to review and provide the most recent data relating to the toxicity of elemental silver nanomaterials, TiO_2 nanoparticles, and CNTs. In addition, efforts were performed to obtain the available physical and chemical, use, and exposure data for the nanomaterials. To achieve these objectives, the following sequential steps occurred:

- A literature search was conducted to identify available data from the year 2000 to 2010 on the three nanomaterials for acute, repeated-dose, chronic, and reproductive and developmental toxicity studies. The primary databases searched were PubMed and the Agency for Toxic Substance and Disease Registry (ATSDR) toxicological profiles and chemical evaluation documents. The ATSDR toxicological profiles and chemical evaluation documents were used as a starting point for the search. Other databases, such as TOXLINE, were used to augment the primary data search.

- The literature search results (titles, authors, abstracts, and other citation information) were incorporated into a separate EndNote database for each selected nanomaterial. Approximately 1,734 toxicity references were identified.

- The abstracts in the EndNote databases were reviewed to determine if they included data that potentially could be used to derive ADI values. The references were primarily grouped in the nanomaterials' respective EndNote database and provided below is the amount of references identified for each nanomaterial.

Table 1.1 Number of Toxicity References Identified by Nanomaterial

Nanomaterial	All References
Silver Nanoparticles	1,045
Titanium Dioxide Nanoparticles	346
Carbon Nanotubes	343

- Copies of the full references were retrieved for any reference that was determined to provide information on ADI values, and PDFs of the references were incorporated into the EndNote database.

- An additional literature search was conducted to identify physical chemical property, use, and exposure data from 2000-2011. The primary databases searched were PubMed, Science Direct, and Google Scholar. Copies of the full journal articles, reports, or other publications were retrieved for all references that provided exposure or consumer exposure related information.

- The toxicity references obtained were reviewed to determine if they were appropriate to evaluate/assess pertinent toxicokinetic, toxicological, and mechanistic studies, including in vitro studies into potential mechanisms of action for nanomaterials, to determine NOAELs and LOAELs for pertinent toxicology studies, provide conclusions about the weights of evidence associating exposure with health hazards, and make conclusions about the suitability of the available data for deriving ADIs for specific substances within the three classes of materials. This review also included examining the apparent critical effects identified from the available data and what might be prospective principal studies for ADI development. Where there was derivation of ADIs, the rationales are provided for selection of the critical effect(s), principal study(ies), and points of departure (POD). Database deficiencies such as the lack of specific toxicity data are provided and a qualitative discussion of the available information regarding characterization of the test materials (size, shape, contaminants, coatings, etc.) and address information on routes of exposure are provided, where possible.

- The references for physical chemical properties, use and exposure data were reviewed to determine if relevant data were contained that could be used to characterize properties of nanomaterials that affect toxicity and exposure and provide information on their use in consumer products. The available data to address these parameters are also provided, where possible.

1.4. RESEARCH NEEDS/DATA GAPS

Overall, more research and/or data on nanomaterials are needed in, but not limited to, the following areas: (1) physical chemical characterization of nanomaterials, (2) effect of the physical chemical properties on toxicity and exposure (3) concentration of nanomaterials in consumer products, (4) possible routes of exposure, (5) methods for accurately measuring and detecting nanomaterial concentrations, (6) information on how many consumers use products containing nanomaterials, (7) exposure factor data (frequency of use, duration of use), and (8) toxicity testing (Aschberger et al., 2011; Hansen et al., 2008; Oberdorster et al., 2005; U.S. EPA, 2007; U.S. EPA, 2010a,b; Wijnhoven et al., 2009a,b.).

Based on the available literature, specific data gaps were identified for elemental silver nanomaterials, TiO_2 nanoparticles, and CNTs. A summary of the data gaps for each of the three

nanomaterials are discussed below.

- *Nanosilver* - Studies were not located for: (1) quantitative data on the rate or extent of absorption of inhaled, ingested, or dermally applied elemental silver nanoparticles for humans or animals, (2) examining the oral absorption of silver nanoparticles in humans were not located, (3) examining the tissue distribution of silver following inhalation exposure of humans to silver nanoparticles, (4) examining the tissue distribution of silver following oral exposure of humans to silver nanoparticles, (5) on the distribution of silver among tissues following dermal exposure of humans or animals to silver nanoparticles, (6) metabolism studies regarding the extent or rates of possible transformation of elemental silver nanoparticles to other forms of silver (e.g., silver oxides or silver ion) in human or animal tissues (7) acute inhalation toxicity studies of silver nanoparticles, (8) on the possible carcinogenicity of repeated exposure to silver nanoparticles by any route of exposure, and (9) on standard mammalian tests of reproductive or developmental toxicity for silver nanoparticles.

- *Nano TiO_2* - Studies were not located for: (1) examining lung deposition and clearance kinetics following exposure to nanosized aerosols of TiO_2, (2) data regarding the route, rate, or extent of excretion of absorbed TiO_2 nanoparticles following dermal exposure, and (3) acute dermal toxicity of TiO_2 nanoparticles. In addition, currently available data are inadequate to assess whether the toxic potency of TiO_2 nanoparticles is significantly influenced by the degree of agglomeration within airborne aerosols (i.e., aerosol size).

- *Carbon nanotubes* - Studies were not located for: (1) the possible toxicity of repeated exposure to CNTs by the oral route, (2) the possible toxicity of repeated exposure to CNTs by the dermal route, (3) oral studies on the possible carcinogenicity of repeated exposure to CNTs, (4) inhalation studies on the possible carcinogenicity of repeated exposure to CNTs, (5) standard mammalian tests of reproductive or developmental toxicity, (6) the possible toxicity of repeated exposure to CNTs by the oral route, and (7) the possible toxicity of repeated exposure to CNTs by the dermal route.

1.5. ORGANIZATION

The sections of this document are organized as follows:

- Section 2 provides information on the use, physical and chemical properties, and potential exposure concerns associated with nanomaterials.

- Sections 3, 4, and 5 provide the available and most recent toxicity information on silver nanomaterials, TiO_2 nanoparticles, and CNTs, respectively.

2.0. USE, PHYSICAL AND CHEMICAL PROPERTIES, AND EXPOSURE

2.1. USE OF NANO MATERIALS

Many consumer products have been reported to contain nanomaterials. For example, nanomaterials are being developed for, but not limited to, use in cosmetics, cleaning products, personal care products, sporting goods, electronics, food and beverage, home and garden products, and sunscreens. An inventory of nanotechnology based consumer products currently on the market has been developed by The Woodrow Wilson Center Project on Emerging Nanotechnologies (PEN). PEN (2011) notes in its most recent update that the use of nanotechnology in consumer products continues to grow. In 2006, the inventory contained 212 products and currently has more than 1,300 manufacturer-identified nanotechnology products entering the commercial market worldwide (PEN, 2011). The PEN consumer products inventory includes products that have been identified by their manufacturer or other credible sources as being nanotechnology based, however, PEN (2011) reports that not all uses may have been captured in their inventory. Some of the uses of the assessed nanomaterials that have been reported in the available literature are briefly described in the sections to follow.

2.1.1. Use of Elemental Silver Nanomaterials

Nanosilver is believed to be one of the most widely used nanomaterials in consumer products due to its antimicrobial properties (Benn et al., 201; U.S. EPA 2010 a,b,c). PEN (2011) has reported that that 24 percent (313 products) of the more than 1,300 consumer products in the inventory contain nano-silver particles. Products that have been reported to possibly contain nanosilver include spray disinfectants, medical products (wound dressings, contraceptives, burn creams, nasal sprays, surgical instruments, cardiac catheters, bone prosthesis, contact lenses, implants, pharmaceuticals, catheters), hair dryers, make up, soaps and dish detergents, cooking utensils, food preservative film, filters of air and water purifications systems, dietary supplements, household appliances (refrigerators and washing machines), electronics (computer hardware, mobile phones), textiles, clothing, shoes, toys, and personal care and cosmetic products (Wijnhoven et al., 2009a; U.S. EPA, 2010a,b,c; PEN 2011).

2.1.2. Use of Titanium Dioxide Nanoparticles

Nano TiO_2 is used in air filtration and purification systems, motor vehicle coatings (anti-scratch/stick), household products and home improvement products, cleaning products, and personal care products (Wijnhoven et al., 2009b). Nano TiO_2 is a popular ingredient in cosmetics and sunscreens because it blocks harmful ultraviolet rays (Sass, 2007: U.S. EPA, 2010b). Other reported uses are self-cleaning surface coatings, light emitting diodes, solar cell, disinfectant sprays, sporting goods and drinking water treatment agents (U.S. EPA, 2010b).

2.1.3. Use of Carbon Nanotubes

Carbon-based nanomaterials have desirable electrical, mechanic, and thermal properties. They are useful in developing lightweight, strong building materials, and computers (Sass, 2007). PEN (2011) has reported that CNTs are used in sporting good equipment (e.g., tennis

rackets, bicycles), textiles and shoes, and transparent electrodes for computers and electronics. Single-walled CNTs (SWCNTs) are also used in in aerospace devices and in the chemical, polymer, and pharmaceutical industries (Shvedova et al., 2008). CNTs are also currently being used in bio-medical applications such as enhanced electron-scanning microscopy imaging techniques and in pharmaceutical biomedical devices for bone grafting, tissue repair, drug delivery and medical diagnostics (NIOSH, 2010).

2.2. PHYSICAL AND CHEMICAL PROPERTIES

In general, the appropriate physicochemical characterization of nanomaterials used in toxicity testing is essential for data interpretation in relation to the material properties, intercomparisons between studies, and conclusions drawn regarding hazard (Oberdorster et al., 2005). The dependence of nanomaterial behavior on physical and chemical properties includes assessing a range of properties which include shape, crystal structure, particle size distribution, agglomeration state, surface area, surface chemistry, surface charge and porosity (Oberdorster, et al., 2005). The U.S. EPA (2007) reported that understanding the physical and chemical properties of nanomaterials is a requirement in the evaluation of all routes of exposure and hazard, both toxicological and ecological. Chemical properties such as vapor pressure, boiling point, water solubility, molecular weight and other properties usually included for discrete chemical substances may not be important for some nanomaterials, but properties such as those reported by Oberdorster et al. (2005) are expected to be important for most nanomaterials (U.S. EPA, 2007).

2.3. EXPOSURE

The possible exposure to nanomaterials in consumer products is difficult to assess because of the many uncertainties related to what data are required for the assessment, the lack of extensive information such as the content/concentration of the nanomaterial in the product, and characteristics of the particles (Wijnhoven 2009b; Hansen et al., 2008; U.S. EPA 2010a,c; Aschberger et. al, 2011). There are three main categories of characteristics important for the assessment of exposure Wijnhoven, 2009b). The first category includes: (1) the properties of the nanomaterial in the consumer product, (2) the chemical entity and the shape of the nanomaterial in the consumer product, both important for the identification of the nanomaterial used in the product, (3) the concentrations of the nanomaterial in the product. The second category is a description for the application of the product (where, how much, how long, and number of persons using the product). The third category is the exposure route (inhalation, ingestion or via the skin). The main characteristics reported by Wijnhoven (2009b) for evaluating human exposure to nanomaterials from consumer products are shown in Table 2-1. Wijnhoven (2009b) noted that the aim of her study was to identify consumer products containing nanomaterials with a high priority for exposure studies in the future. However, the information from product inventories and market reports was not sufficient to perform a detailed exposure assessment for this identification. The Dutch National Institute for Public Health and the Environment (RIVM) created an expert panel to identify the most important exposure characteristic and to categorize the products into low, medium, and high exposure. The expert panel concluded that personal care products and cosmetics (sun cosmetics, oral hygiene products, and supplements and health products) are expected to lead to high potential exposure. Other products such as do it yourself

(DIY) coatings, adhesives, and cleaning products and fuel from motor vehicles (after combustion) are also expected to lead to high potential exposure. The

Table 2.1 Main characteristics for human exposure to nanomaterials from consumer products

Important characteristics for the exposure assessment		Comments
Nanomaterial in consumer product	*Chemical entity of nanomaterial*	*Actual composition of material*
	Shape of nanomaterial	Thin films and coatings Composite Solid particle Hollow particle Other particle Aggregates Agglomerates
	Product form	Spray Powder Liquid Suspension Solid/ coating
	Free / fixed	Free particles Fixed inside matrix
	Concentration	Mostly unknown (based on mass?)
Applications	Direct/ indirect exposure	Direct exposure to nanomaterials in the product or indirect via release of particles out of the product
	Indoor/ outdoor use	Inside or outside a small space
	Event duration	< 5 min 5 min-1 hr 1 hr - 1 day
	Frequency of events	> 1x/ day 1x/ ay- 1x/ week 1x/ week - 1x/ month 1x/ month –-1x/ year
	Number of users in population	<10% 10-50% 50-90% > 90%
Exposure route		Inhalation Dermal Oral Combination

Source: Wijnhoven et al. (2009b)

RIVM panel also noted that possible exposure is high when the nanomaterial is/ nanoparticles are free and the possible exposure is expected to be low (or not existing) when the nanomaterial is fixed inside a matrix and not expected to be able to migrate, leach, or evaporate from the product.

Hansen et al. (2008) used the PEN database in an attempt to carry out an exposure assessment from use of consumer products containing nanomaterials. The U.S. EPA (2010a) reported that

based on the results of the Hansen et al. (2008) analysis, nano silver was determined to have the highest possible consumer exposure of all the nano materials considered, based on the types of products in which the nanomaterial is used and the exposure scenarios relating to these products. The U.S. EPA (2010a) has reported possible exposure routes to humans from use of products containing nano-silver and the expected exposure routes range from multi-routes to a single exposure route depending on the product type. For example, the expected exposure routes for household cleaning products are listed as dermal, inhalation and oral.

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3.0 TOXICITY DATA ON ELEMENTAL SILVER NANOMATERIAL

3.1. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Quantitative data on the rate or extent of absorption of inhaled, ingested, or dermally applied elemental silver nanoparticles are not available for humans or animals. Qualitative evidence of absorption and distribution is available in reports of elevated silver concentrations in non-portal-of-entry tissues in animals following repeated exposure to silver nanoparticles via the inhalation (Sung et al., 2009; Ji et al., 2007a) or oral (Kim et al., 2010, 2008) routes. The degree to which elevated silver concentration in tissues is due to absorption of dissolved silver ions from elemental silver nanoparticles deposited on the epithelia of the respiratory or gastrointestinal tract or due to direct absorption of elemental silver nanoparticles is unknown.

Following repeated inhalation or oral exposures of rats to elemental silver nanoparticles, elevated silver concentrations were detected in extra-portal-of-entry tissues, but concentrations were very much lower than portal-of-entry tissue concentrations (Sung et al., 2009; Ji et al., 2007a; Takenaka et al., 2001). For example, in female rats exposed to 2.85×10^6 particles/cm^3 in air for 90 days, mean tissue concentrations (ng/g wet weight) showed the following order: lung (20,585) >>> liver (71) > kidney (38) > olfactory bulb (33) > brain (20) > blood (7) (Sung et al., 2009). Following 28 days of oral exposure of female rats to silver nanoparticles at a dose of 1,000 mg Ag/kg-day, tissue concentrations showed the following order (µg/g wet weight): stomach (176) > kidney (69) > liver (65) > lungs (27) > brain (2) > blood (0.7) (Kim et al., 2008). The findings suggest that only limited amounts of inhaled or ingested silver nanoparticles are distributed to extra-portal-of-entry tissues. Tissue distribution studies in animals did not quantitatively determine the chemical form of silver in the tissues. Studies on the extent or rate of possible transformations of elemental silver nanoparticles to other forms of silver (silver ion or silver oxides) in human or animal tissues are not available.

Results from in vitro studies with human skin samples mounted in diffusion cells (Larese et al., 2009) and studies of burn patients treated with a commercial dressing containing silver nanoparticles (Vlachou et al., 2007) provide qualitative evidence of limited dermal absorption, which is increased when skin is damaged. Transmission electron microscopy (TEM) of skin from dermally exposed pigs showed silver nanoparticles only in the surface stratum corneum and not in lower dermal layers (Samberg et al., 2010). Limited evidence from a study of rats subcutaneously injected with single 62.8 mg Ag/kg doses of suspensions of silver nanoparticles indicated that the fecal route of elimination may be more important than urinary elimination (Tang et al., 2009). Amounts of silver collected in 24-hour feces were much higher than amounts in 24-hour urine samples through 24 weeks after dose administration. No other studies examining elimination routes or kinetics following exposure to silver nanoparticles were located.

3.1.1. Absorption, Inhalation

In contrast to fine particles with diameters in the 1–2.5 µm range, which are deposited mainly in the peripheral lung, inhaled nanoparticles (with at least one dimension <100 nm) can be deposited in the oral and nasal cavities, the tracheal/bronchiole region of the lung, and the alveolar region of the lung (Kreyling et al., 2002). Nanoparticles deposited in the alveolar region

of the respiratory tract may be cleared from the alveolar region by: (1) macrophage phagocytosis and mucociliary transport along the tracheobronchial tree to the gastrointestinal tract; (2) translocation into interstitial tissue; (3) translocation to the lymphatic system; (4) particle dissolution with subsequent absorption into lung cells and transport into the blood; and (5) translocation of the particles into lung cells from lung surfaces and possible transport into the blood (Chen and Schluesener, 2008; Geiser et al., 2008; Kreyling et al., 2002; Oberdorster, 1988). Clearance from tracheal/bronchiole regions may occur by similar pathways. Nanoparticles deposited in the nasal mucosa also may be subject to particle dissolution and absorption into the blood or direct translocation of silver ions or elemental silver nanoparticles into the olfactory bulb of the brain via the olfactory nerve (Oberdorster et al., 2004).

Although studies of the absorption of inhaled elemental silver nanoparticles (i.e., transport across epithelial cell membranes and entry into the lymph or blood circulatory systems) in humans were not located, the deposition of inhaled silver nanoparticles has been examined in human subjects. Three male volunteers were exposed to air containing silver particles with an average diameter of 9 nm (geometric SD = 2.0) at a concentration of 2×10^5 particles/cm^3 (Muir and Cena, 1987). Particle deposition (in the respiratory tract as a whole) was determined by relating the concentrations of particles inhaled to the concentration of particles exhaled by the subjects. Particle deposition increased from 20 to 90% as the breathing cycle (duration of breath including both inspiration and expiration) increased from 2 to 10 seconds, but was not affected by tidal volume (volume of air inhaled and exhaled at each breath, 0.5-3 L tested). These results suggest that deposition of nanoparticles in the respiratory tract increases with residence time of particles in the respiratory tract.

In another deposition study of 10 human subjects exposed to aerosols of silver particles of various sizes generated from silver wool (99.9% pure) via an evaporation-condensation method, deposition efficiencies (in the respiratory tract as a whole) for 4 nm particles ranged from about 25 to 65% with nose-in/mouth-out breathing and 25–62% with mouth-in/nose-out breathing (Cheng et al., 1996). For 8 nm particles, respective deposition efficiencies were 14–49 and 14–45%. The respective ranges for 20 nm particles were 2–35 and 3–28%. The results suggest that respiratory tract deposition efficiencies of nanoparticles increase with decreasing particle diameters. The inter-individual variability of deposition efficiencies was correlated with variability in nasal dimensions, such as total surface area, minimum cross-sectional area, and complexity of the airway shape (Cheng et al., 1996).

In rats, the detection of elevated silver concentrations in extrarespiratory tissues (e.g., liver, kidney, brain, olfactory bulb, and blood) following inhalation exposure provides qualitative evidence of the absorption of silver from inhaled elemental silver nanoparticles at concentrations in the range of $1–3 \times 10^6$ particles/cm^3, but quantitative data on the rate or extent of absorption of inhaled silver nanoparticles have not been collected. Section 3.1.4 of this document provides detailed descriptions of these studies, which involved 6-hour (Takenaka et al., 2001), 28-day (Ji et al., 2007a), or 90-day (Sung et al., 2009) exposures. Also described are results showing that lung clearance of silver following intratracheal instillation of agglomerated suspensions of silver nanoparticles is impaired, compared with lung clearance of silver following 6-hour inhalation exposures to non-agglomerated silver nanoparticles (Takenaka et al., 2001).

3.1.2. Absorption, Oral

Studies examining the oral absorption of silver nanoparticles in humans were not located. In rats, the detection of elevated silver concentrations in non-portal-of-entry tissues (e.g., testes, liver, kidney, brain, and blood) following repeated oral exposure to aqueous suspensions of silver nanoparticles provides qualitative evidence of absorption by the gastrointestinal tract at doses in the range of 30–1,000 mg/kg-day (Kim et al., 2008). Section 3.1.5 describes this study in detail. Quantitative data on the rate or extent of absorption of ingested silver nanoparticles in animals, however, have not been collected.

3.1.3. Absorption, Dermal

Results from human studies indicate that absorption of silver from silver nanoparticles can occur, and that rates of absorption are higher in damaged skin than in intact skin (Larese et al., 2009; Vlachou et al., 2007). Indicative of dermal absorption, elevated serum silver levels were detected 9 days after treatment of 30 burn patients with a commercial dressing containing nanocrystalline silver (maximum mean serum silver concentration = 56.8 µg/L); serum silver levels were very low (0.8 µg/L) 6 months after treatment was stopped (Vlachou et al., 2007).

Silver absorption was detected across intact human skin samples mounted in Franz static diffusion cells and exposed to silver nanoparticles, but when the skin was abraded, rates were about five fold higher (Larese et al., 2009). The silver nanoparticles used in this experiment were coated with polyvinylpyrolidone to prevent aggregation in an aqueous suspension. Intact and abraded human abdominal skin samples (stored for <4 months at -25°C) were exposed to 70 µg/cm^2 silver nanoparticles in aqueous 0.14% (w/w) ethanol diluted 1:10 with synthetic sweat for up to 24 hours. TEM examination indicated that particle diameters ranged from 9.8 to 48.8 nm, with a median of 25 ± 7.1 nm and 25th and 75th percentiles of 19.5 and 29.3 nm, respectively. Samples of receptor solutions were analyzed for silver concentration by atomic absorption spectrophotometry. With intact skin samples exposed for 24 hours, silver was detected in the receptor solution at a median level above the detection limit (median = 0.46 ng/cm^2, range = less than the detection limit [0.1 µg/L] to 2.23 ng/cm^2); absorption rates of silver through abraded skin were about fivefold higher (median = 2.32 ng/cm^2; range = 0.43–11.6 ng/cm^2). TEM of skin samples following exposure showed silver nanoparticles in the stratum corneum (Larese et al., 2009).

In pigs dermally exposed to washed silver nanoparticles suspended in deionized water for 14 days, TEM of skin samples revealed silver nanoparticles only in the stratum corneum and not in lower epidermal layers (Samberg et al., 2010). Sites (surface area unspecified) on the back skin of female weanling pigs (n = 2; 20–30 kg) were dosed with 500 µL of suspension concentrations ranging from 0.34 to 34 µg/mL for 14 days. The test materials were commercial silver nanoparticles (purchased from nanoComposix, San Diego, California) including washed particles with 20 or 50 nm manufacturer designated diameters (MDD), unwashed particles with 20 nm or 50 nm MDD, and carbon-coated nanoparticles with 25 or 35 nm MDD. All tested materials were reported to be localized by TEM within or on top of the stratum corneum following the 14-day exposure period. Although macroscopic evidence of skin irritation was not found in this study, light microscopic examination showed focal inflammation and edema in skin

layers under the stratum corneum, where particles were not detected by TEM. Samberg et al. (2010) hypothesized that the lesions were caused by silver ion flux into the lower layers from the particles in the stratum corneum. Possible penetration of silver into the blood or other tissues was not examined in this study.

3.1.4. Distribution, Inhalation

No studies were located examining the tissue distribution of silver following inhalation exposure of humans to silver nanoparticles. Several rat studies provide evidence that silver is distributed to extrarespiratory tissues following inhalation exposure to silver nanoparticles (Sung et al., 2009; Ji et al., 2007a; Takenaka et al., 2001).

Takenaka et al. (2001) examined silver concentrations in tissues of female F344 rats (150–200 g, n = 16) exposed for 6 hours to elemental silver nanoparticles at a concentration of 3×10^6 particles/cm^3 (reported mass concentration = 133 µg/m^3). Eight control rats were exposed to clean air in another chamber. The particles were generated by spark discharging through an argon atmosphere. Size distribution and number concentrations were determined with a differential mobility analyzer and a condensation nucleus counter. Chemical analysis to document the chemical identity of the particles (e.g., elemental versus silver oxides or salts) was not reported, but TEM of generated particles indicated that they were compact, spherical, and electron-dense, but were not agglomerated. Reported results from particle size distribution analysis were: modal diameter = 14.6 ± 1.0 nm; median diameter = 17.1 ± 1.2 nm; and geometric standard deviation = 1.38. Groups of four rats were sacrificed on days 0, 1, 4, and 7 following exposure, and the following tissues were collected for determination of silver concentrations via inductively-coupled plasma mass spectrometry (ICP-MS): lung; liver; kidney; heart; tracheobronchiolar lymph nodes; mediastinal tissues including mediastinal lymph nodes; anterior and posterior nasal cavities; olfactory portion and the remaining portion of brain; and blood (note that not all of the sampled tissues were analyzed; see Table 1).

Immediately after exposure, silver was detected at elevated concentrations in the lungs, nasal cavity, liver, and blood (Takenaka et al., 2001). The data are shown in Table 1. With increasing time after exposure (1, 4, and 7 days), silver concentrations in these tissues generally declined (Table 3.1). For example, the mean lung concentration on day 7 was about 4% of the mean concentration immediately after exposure. One day after exposure, silver was detected in the kidney, lymph nodes, and brain; on subsequent sampling days, tissue concentrations generally declined (Table 1). The results demonstrate absorption of inhaled silver nanoparticles by respiratory tissues, followed by distribution of silver to other organs and tissues. They also provide evidence of partial clearance of silver from these tissues within 4–7 days after exposure.

Table 3.1. Silver concentrations in tissues following inhalation exposure of female F344 rats to silver nanoparticles at a concentration of 3×10^6 particles/cm^3 for 6 hours

Tissues/organs	Mean (SD) concentration, ng Ag/g wet weight			
	Day 0	Day 1	Day 4	Day 7
Exposed rats (n = 4)				
Lung	2,375 (171)	904 (31)	199 (41)	98 (19)
Liver	33 (13)	24 (5.1)	5.6 (1.8)	3.0 (1.1)
Kidney	ND	39 (8.1)	4.7 (4.4)	ND
Heart	ND	2.8 (0.5)	0.7 (0.1)	ND
Lymph, tracheobronchial	ND	21 (7.7)	72 (63)	ND
Lymph, mediastinal	ND	6.8 (0.7)	1.6 (0.1)	ND
Anterior nasal cavity	59.2 (22.6)	13.9 (2.2)	ND	ND
Posterior nasal cavity	96 (20.4)	68.3 (4.5)	ND	ND
Brain, olfactory portion	1.9 (1.1)	3.1 (1.3)	ND	ND
Brain, residual	1.4 (0.5)	1.2 (0.2)	ND	ND
Blood	8.9 (6.2)	6.2 (0.8)	2.9 (1.5)	1.0 (0.2)
Unexposed rats (n = 8)				
Lung	<1.2 (0.2)	ND	ND	ND
Blood	<0.6 (0.1)	ND	ND	ND

SD = standard deviation; ND = not determined

Source: Takenaka et al. (2001).

Takenaka et al. (2001) also examined silver contents of lungs and livers in female F344 rats 1, 4, or 7 days following intratracheal instillations of aqueous suspensions of 50 µg silver nanoparticles/rat or aqueous solutions of silver nitrate (4.4 µg Ag/rat). TEM of the suspensions of elemental silver nanoparticles indicated that particles were agglomerated into structures with diameters >100 nm. Estimated silver contents of lungs and liver did not display marked time-dependent changes on days 1, 4, and 7 following instillation of agglomerated nanoparticles (Table 2). In contrast, declines in tissue concentrations were seen between day 1 and 7 after instillation of silver nitrate solutions (Table 2). The results indicate that lung clearance of instilled agglomerated suspensions of silver nanoparticles is impaired compared with instilled silver nitrate solutions. They also indicate that clearance of intratracheally instilled silver nanoparticles may be slower than inhaled silver nanoparticles. Mean silver concentrations in lung tissue significantly declined within 7 days after a 6-hour inhalation exposure of rats (Table 1), whereas silver concentrations in lung tissue did not decline within 7 days after intratracheal dose administration of silver nanoparticles (Table 3.2).

Table 3.2. Estimated mean silver content (µg/rat) in lung and liver of rats following intratracheal instillation of aqueous suspensions of silver nanoparticles or aqueous solutions of silver nitrate

Tissue	Day after instillation		
	Day 1	**Day 4**	**Day 7**
	Aqueous suspensions of silver nanoparticles (50 µg Ag/rat) Estimated mean silver content, µg/rat (individual values in parentheses)		
Lung	12.9 (16.4, 9.4)	16.3 (16.5, 16.3, 16.2)	13.6 (15.7, 11.4)
Liver	0.35 (0.55, 0.14	0.42 (0.58, 0.39, 0.28)	0.41 (0.51, 0.31)
	Aqueous solutions of silver nitrate (4.4 µg Ag/rat) Estimated mean silver content, µg/rat[a] (SD in parentheses)		
Lung	1.04 (0.45)	0.33 (0.09)	0.26 (0.06)
Liver	0.36 (0.01)	0.19 (0.08)	0.07 (0.04)

[a]The values reported in this table for silver content following silver nitrate instillation are 1000-fold lower than those reported in Table 3 of the report by Takenaka et al. (2001). Based on comparison to other data presented in the study, it appears likely that the units in Table 3 of Tanaka et al. (2001), although labeled as µg/rat, were actually for ng/rat,.

Source: Takenaka et al. (2001).

Ji et al. (2007a) examined silver concentrations in liver, lung, and brain in Sprague-Dawley rats (10/sex/group) following exposure for 4 weeks (6 hours/day, 5 days/week) to silver nanoparticles at three concentrations or clean air. Silver nanoparticles were generated by evaporation/condensation using a ceramic heater. Particles generated by this method were determined to be elemental silver (not silver oxides or salts) by energy-dispersive x-ray (EDX) analysis and x-ray diffraction (XRD) (Ji et al., 2007b; Jung et al., 2006). Concentrations and size distributions were measured with a differential mobility analyzer and ultrafine condensation particle counter. Mean concentrations in the low, medium, and high exposure chambers were 1.73×10^4, 1.27×10^5, and 1.32×10^6 particles/cm^3, respectively. Respective geometric mean diameters (and geometric standard deviations) were 11.93 nm (0.22), 12.40 nm (0.15), and 14.77 nm (0.11); the higher diameter at the high concentration was explained as being due to some agglomeration of particles. Mean mass concentrations were 0.48, 3.48, and 61.24 µg Ag/m^3. Following exposure, the lungs, liver, brain, olfactory bulb, and blood were collected for determination of silver concentrations by atomic absorption spectrometry. Tissue concentrations were increased at the high exposure level, with the lung showing much higher silver concentrations than the other tissues (Table 3). At the high exposure level, silver concentrations showed the following order: lung >>> olfactory bulb > liver > brain > blood. At lower exposure levels, silver concentrations were not significantly increased in the examined tissues, compared with control values (Table 3.3).

Table 3.3. Silver concentrations in tissues following inhalation exposure of Sprague-Dawley rats to silver nanoparticles for 4 weeks[a]

	Mean silver concentration ± SE, ng/g wet weight[b]				
Exposure group	**Lung**	**Liver**	**Brain**	**Olfactory bulb**	**Blood**
Control					
Male	0.89 ± 0.20 (5)	Not detected	0.15 ± 0.05 (5)	Not detected	Not detected
Female	0.27 ± 0.21 (3)	1.15 (1)	Not detected	Not detected	Not detected
Low					
Male	0.32 ± 0.20 (5)	0.49 (1)	Not detected	0.20 (1)	2.59 (1)
Female	0.45 ± 0.16 (4)	0.11 (1)	0.56 ± 0.24 (3)	0.12 (1)	0.24 ± 0.06 (2)
Middle					
Male	1.25 ± 0.16 (5)	0.61 ± 0.17 (2)	0.15 ± 0.01 (2)	0.93 ± 0.28 (5)	Not detected
Female	1.19 ± 0.07 (5)	0.06 ± 0.03 (2)	0.38 ± 0.13 (3)	1.12 ± 0.39 (5)	Not detected
High					
Male	1,180.76 ± 110.97[c] (5)	5.91 ± 2.61 (5)	2.20 ± 0.14[b] (5)	6.96 ± 0.23[d] (5)	Not detected
Female	1,496.64 ± 384.72[c] (5)	6.89 ± 1.46 (5)	3.10 ± 0.46[c] (5)	9.05 ± 2.93[d] (4)	1.58 ± 0.36 (4)

[a]Rats were exposed to 0, 1.73×10^4, 1.27×10^5, or 1.32×10^6 particles/cm^3, 6 hours/day, 5 days/week for 4 weeks.
[b]n = 5, number of detected samples given in parentheses.
[c]Significantly different from control, $p < 0.05$.
[d]Significantly different from low and/or middle groups, $p < 0.05$.

SE = standard error

Source: Ji et al. (2007a).

Sung et al. (2009) examined silver concentrations in liver, kidneys, lung, olfactory bulb, and brain in Sprague-Dawley rats (10/sex/group) exposed for 90 days (6 hours/day, 5 days/week) to silver nanoparticles at three concentrations or clean air. Silver nanoparticles were generated and concentrations and size distributions were measured as described by Ji et al. (2007a, b) and Jung et al. (2006). As reported by Sung et al. (2008), mean concentrations in the low, medium, and high exposure chambers were 6.64×10^5, 1.43×10^6, and 2.85×10^6 particles/cm^3, respectively. Respective geometric mean diameters (and geometric standard deviations) were 18.30 nm (1.10), 18.71 nm (1.78), and 18.93 nm (1.59). Mean mass concentrations were 48.94, 133.19, and 514.78 µg Ag/m^3, respectively. Following exposure, the lungs, liver, kidneys, brain, olfactory bulb, and blood were collected for determination of silver concentrations by atomic absorption spectrometry. In males, silver concentrations showed the following order among the tissues: lung >>> liver > olfactory bulb > brain > kidneys > whole blood (Table 4). In females, the order was: lung >>> liver > kidneys > olfactory bulb > brain > whole blood (Table 4). In both sexes, mean silver concentrations increased with increasing exposure concentration, and statistically significantly elevated concentrations were observed in all tissues at the highest exposure level (Table 4). At the medium exposure level, statistically significantly elevated silver concentrations were observed in lungs, kidneys (males only), brain, and whole blood (Table 3.4). Concentrations of silver in the kidney were three- to fourfold higher in females than in males. This was seen in oral exposure studies as well (see Section 3.1.5).

Table 3.4. Silver concentrations in tissues following inhalation exposure of Sprague-Dawley rats to silver nanoparticles for 90 days[a]

Exposure group	Mean silver concentration ± SE, ng/g wet weight (n = 3–5 for tissues, n = 5–10 for blood)					
	Liver	Kidneys	Olfactory bulb	Brain	Lungs	Whole blood
Control						
Male	0.7 ± 0.2	0.9 ± 0.2	0.5 ± 0.4	1.1 ± 0.3	0.8 ± 0.3	0.1 ± 0.0
Female	0.9 ± 0.1	0.9 ± 0.2	2.3 ± 0.7	0.7 ± 0.3	1.0 ± 0.1	0.1 ± 0.0
Low						
Male	3.5 ± 1.0	1.6 ± 0.3	6.4 ± 0.8	3.5 ± 0.7	613.6 ± 66.0	0.7 ± 0.1
Female	4.6 ± 1.4	2.6 ± 0.6	7.4 ± 0.8	4.1 ± 0.5	295.9 ± 78.5	0.9 ± 0.1
Medium						
Male	13.8 ± 2.3	3.6 ± 0.4[b]	17.1 ± 1.6	7.9 ± 1.0[b]	5,450.3 ± 904.2[b]	1.8 ± 0.2[b]
Female	12.1 ± 2.5	11.8 ± 4.3	13.8 ± 1.3	10.2 ± 1.2[b]	4,241.2.29 ± 641.1[b]	2.1 ± 0.2[b]
High						
Male	133.0 ± 22.9[c]	9.5 ± 0.9[c]	30.5 ± 2.6[c]	18.6 ± 1.2[c]	14,645.4 ± 2,630.2[c]	4.3 ± 0.4[c]
Female	71.1 ± 24.5[c]	37.7 ± 7.0[c]	32.8 ± 2.7[c]	20.0 ± 2.4[c]	20,585.6 ± 1,880.3[c]	6.9 ± 0.6[c]

[a]Rats were exposed to 0, 6.64×10^5, 1.43×10^6, or 2.85×10^6 particles/cm^3, 6 hours/day, 5 days/week for 90 days.
[b]Statistically significantly different ($p < 0.01$) from low and control groups, by analysis of variance and Duncan's multiple range test.
[c]Statistically significantly different ($p < 0.01$) from other groups (control, low, medium), by analysis of variance and Duncan's multiple range test.

Source: Sung et al. (2009).

In all of these studies (Sung et al., 2009; Ji et al., 2007a; Takenaka et al., 2001), the finding of elevated silver concentrations in extrapulmonary tissues and blood provides qualitative evidence of absorption by the respiratory tract, followed by distribution to other tissues. The finding of very high silver concentrations in the lungs, compared with other organs, suggests that, at the tested concentrations, considerable amounts of deposited silver nanoparticles are not rapidly absorbed and translocated to other tissues.

3.1.5. Distribution, Oral

No studies were located examining the tissue distribution of silver following oral exposure of humans to silver nanoparticles. Rat toxicity studies provide evidence that silver is distributed to non-portal-of-entry tissues following oral exposure to aqueous suspensions of silver nanoparticles (Kim et al., 2010, 2008).

Kim et al. (2008) exposed male and female Sprague-Dawley rats (10/sex/group) to suspensions of commercial silver nanoparticles in 0.5% aqueous carboxymethylcellulose at oral doses of 30, 300, or 1,000 mg/kg-day. Dosing volumes were 10 mL/kg. The test material was purchased from NAMATECH Co., Ltd. (Korea) and was reported to be 99.98% pure with particle diameters ranging from 52.7 to 70.9 nm, with an average of 60 nm. The report did not specify the methods used to determine these characteristics of the test material, and no information about the possible agglomerization of particles in the suspension was reported. Following exposure, the rats were sacrificed and silver concentrations were determined by

atomic absorption spectrometry in the following tissues: testes, kidneys, liver, brain, lungs, stomach, and blood. In all tissues examined, mean silver concentrations increased with increasing exposure level (Table 3.5). Statistically significant ($p < 0.05$) elevations in mean silver concentrations, compared with control values, were observed in all analyzed tissues at 300 and 1,000 mg/kg-day in either males or females or both. At 30 mg/kg-day, significantly elevated concentrations were observed in kidneys, brain, and blood in both sexes, testes in males, and lungs in females. The results provide qualitative evidence of absorption of the administered silver nanoparticles by the gastrointestinal tract, followed by distribution to non-portal-of-entry tissues and organs.

Table 3.5. Tissue silver concentrations (mean ± SD or SE, µg/g wet weight) in Sprague-Dawley rats orally exposed to suspensions of silver nanoparticles in 0.5% aqueous carboxymethylcelluose for 28 days[a]

Exposure group	Testes	Kidneys	Liver	Brain	Lungs	Stomach	Blood
Control							
Male	0.1 ± 0.1	0.02 ± 0.01	0.02 ± 0.0	0.03 ± 0.0	0.04 ± 0.0	0.2 ± 0.1	0.01 ± 0.01
Female	–	0.01 ± 0.00	0.01 ± 0.0	0.02 ± 0.0	0.02 ± 0.0	0.2 ± 0.1	0.00 ± 0.00
Low							
Male	1.2 ± 0.4[b]	1.3 ± 0.6[b]	0.5 ± 0.3	0.3 ± 0.0[b]	0.2 ± 0.1	10.7 ± 8.8	0.18 ± 0.0[b]
Female	–	2.8 ± 0.9[b]	0.5 ± 0.3	0.2 ± 0.1[b]	0.1 ± 0.0[b]	6.9 ± 3.6	0.16 ± 0.0[b]
Middle							
Male	3.6 ± 0.6[b]	6.0 ± 2.4[b]	8.7 ± 3.7[b]	0.8 ± 0.1[b]	4.3 ± 0.9[b]	56 ± 25[b]	0.43 ± 0.1[b]
Female	–	16.8 ± 2.8[b]	9.6 ± 3.6[b]	0.6 ± 0.1[b]	6.1 ± 2.5[b]	37 ± 19	0.44 ± 0.1[b]
High							
Male	7.4 ± 1.7[b]	24 ± 11[b]	71 ± 56	2.0 ± 0.3[b]	17 ± 4[b]	228 ± 79[b]	0.80 ± 0.2[b]
Female	–	69 ± 22[b]	65 ± 32[b]	1.8 ± 0.3[b]	27 ± 8[b]	176 ± 44[b]	0.70 ± 0.3[b]

[a]Rats were given doses of 0, 30, 300, or 1,000 mg Ag/kg-day. The report indicated that the units were mg/g wet weight, but a personal communication with one of the authors indicated that the actual units were µg/g wet weight. The report by Kim et al. (2008) does not specify if the means are accompanied by SD or SE.
[b]Significantly different from control, $p < 0.05$ by analysis of variance and Duncan's multiple range test. Kim et al. (2008) did not report the number of tissue samples analyzed per exposure group.

Source: Kim et al. (2008).

Kim et al. (2008) noted that mean silver concentrations in kidneys of females were two- to threefold higher than those in male kidneys (Table 5). In a subsequent study of male and female F344 rats exposed for 28 days to the same test material via the same methods at dose levels of 0, 30, 125, and 500 mg/kg-day, accumulation of silver in kidney regions was histologically examined using an enhanced silver staining method (Kim et al., 2009a). Kim et al. (2009a) reported that female rat kidneys, compared with male rat kidneys, showed higher silver staining in all regions of the kidney, especially in the basement membranes of the renal tubules in the cortex and the inner and outer medulla, but a quantitative morphometric analysis of the photomicrographs was not conducted.

Similar results to those reported by Kim et al. (2008) were reported in a study of silver concentrations in tissues following oral exposure of male and female Sprague-Dawley rats to silver nanoparticles at doses of 0, 30, 125, or 500 mg/kg-day for 90 days (Kim et al., 2010). Dose-dependent increased silver concentrations were observed in all tissues examined (testes,

liver, kidney, brain, lungs, and blood), and, at each dose level, silver concentrations in kidneys were considerably higher in females than males.

3.1.6. Distribution, Dermal

No studies were located on the distribution of silver among tissues following dermal exposure of humans or animals to silver nanoparticles.

3.1.7. Distribution, Other Routes

Tang et al. (2009) compared the tissue distribution, translocation, and accumulation of silver in female Wistar rats for up to 24 weeks following subcutaneous injection of single 62.8 mg/kg doses of commercial elemental silver nanoparticles or microparticles suspended in Dulbecco's modified eagle medium. The test materials were purchased from Aldrich Sigma Co. (Beijing, China). Although particle size distributions were not explicitly specified in the study report, TEM examination of administered materials showed dispersed spherical nanoparticles with 50–100 nm diameters and occasional agglomerations of 2–5 particles; microparticles were irregularly shaped obtuse cubes of varying sizes with 2–20 µm diameters. Groups of five rats were sacrificed at 2, 4, 8, 12, 18, and 24 weeks after dose administration for determination of silver content (by ICP-MS) in injection sites, brain, heart, liver, spleen, lung, kidney, femur, adrenal gland, ovary, and uterus. At each sacrifice, 24-hour urine and feces samples and blood samples were collected and determined for silver content.

Following injection of either nanoparticles or microparticles, most of the detected silver remained at the injection sites, but a relatively greater proportion of the injected silver was in organs following nanoparticle injection (average = 0.15%), compared with microparticle injection (average = 0.02%) (Tang et al., 2009). Tissue silver contents (µg) were not significantly ($p > 0.05$) different between control rats and rats injected with silver microparticles, but silver contents in kidney, liver, spleen, brain, lung, and blood were significantly ($p < 0.05$) higher in rats injected with silver nanoparticles, compared with control rats. Following injection with nanoparticles, the liver and kidney showed the highest silver contents. For example, at 12 weeks, when total tissue silver contents peaked, mean silver contents in liver and kidney were about 9.3- and 17.3-fold higher than brain contents, respectively. TEM examination of thin section samples of brain, liver, spleen, lung, and kidney from rats injected with nanoparticles detected electron-dense substances (assumed to be silver nanoparticles) in the lumen of blood vessels in the kidney, liver, spleen, brain, and lung, and in other regions including the renal corpuscle, hepatocytes, lymphocytes in the splenic cord, cerebral neurons, vascular endothelial cells of the blood-brain barrier, and alveolar epithelial cells. The results indicate that a small amount of silver, injected subcutaneously as silver nanoparticles, is translocated into the blood and distributed to tissues. The detection of silver nanoparticles in the tissues by TEM indicates that at least some of the elevation in silver concentration in tissues is due to distribution of the nanoparticles themselves to the tissues, as opposed to distribution of dissoluted silver ions.

3.1.8. Metabolism

No studies were located regarding the extent or rates of possible transformation of elemental silver nanoparticles to other forms of silver (e.g., silver oxides or silver ion) in human or animal tissues. Tissue distribution studies in animals exposed to elemental silver nanoparticles by various routes did not quantitatively determine the chemical form of silver in the tissues (Kim et al., 2010, 2008; Sung et al., 2009; Tang et al., 2009; Ji et al., 2007a; Takenaka et al., 2001), although Tang et al. (2009) provided TEM evidence that some degree of elevated silver concentrations in tissues is due to movement of the particles themselves, as opposed to dissoluted silver ions.

3.1.9. Elimination

Limited evidence from a study of rats subcutaneously injected with single 62.8 mg Ag/kg doses of suspensions of silver nanoparticles indicates that fecal elimination may be more important than urinary elimination (Tang et al., 2009). Amounts of silver collected in 24-hour feces were much higher than amounts in 24-hour urine samples collected through 24 weeks after injection. For example, at 2 weeks after injection, silver contents in 24-hour feces and urine were 6.3 and 0.004 µg, respectively; at 12 weeks, respective contents were 0.86 and 0.096 µg. No other studies of the kinetics or routes of whole body elimination of silver nanoparticles were located in the available literature.

3.2. ACUTE TOXICITY

3.2.1. Inhalation

No acute inhalation toxicity studies of silver nanoparticles were located.

3.2.2. Oral

Hepatic responses were evaluated in unspecified numbers of male balb/c mice 3 days after oral exposure to 2.5 g of silver nanoparticles or 2.5 g of silver microparticles (Cha et al., 2008). The only reported particle characteristic is size, which was 13 nm for the nanoparticles and 2–3.5 µm for the microparticles. The silver nanoparticles were produced by reduction of "Aldrich grade" $AgNO_3$ with $NaBH_4$, which yielded a solution containing "colloid" particles with a typical size of about 13 nm as determined by TEM. Information on production of the silver microparticles was not provided. The mice were starved for 24 hours before exposure by apparent gavage (silver particles were fed "directly to the stomach"). Inclusion of a control group is not indicated in the study report. Histology was examined, but results were only qualitatively reported in minimal detail.

Histopathology findings included focal lymphocyte infiltration in liver portal tracts in both nano- and microparticle-exposed mice, which suggests induction of inflammation by both sizes of silver particles (Cha et al., 2008). Non-hepatic histopathology apparently only occurred in mice exposed to silver nanoparticles and consisted of focal lymphocyte infiltration in the intestine (region not specified), nonspecific focal hemorrhages in the heart, and nonspecific

medullary congestion in the spleen. Additional histopathology information, including incidences and severity of lesions and other tissues that may have been examined, was not reported.

Gene expression profiles in livers from mice exposed to silver nanoparticles were compared with profiles from livers of microparticle-exposed mice (Cha et al., 2008). Genes related to apoptosis that were differentially perturbed by nanoparticle exposure (relative to microparticle exposure) included upregulated (cyclin-dependent kinase inhibitor 1A, alpha synuclein, Bcl2 modifying factor) and downregulated genes (chemokine [C-X-C motif] ligand 1, heat shock 70kDa protein 2, interleukin 7, and ATP-binding cassette subfamily B [MDR/TAP]). Inflammation-related genes that were upregulated by exposure to silver nanoparticles (relative to microparticles) were keratin 16, chemokine (C-X-C motif) ligand 13, and macrophage receptor with collagenous structure; downregulated genes were nuclear receptor subfamily 1 group D member 2 and chemokine (C-X-C motif) ligand 7.

3.2.3. Dermal

A case study of a patient with 30% mixed depth burns treated with dressings containing silver nanoparticles reported that the patient gradually developed a grayish argyria-like discoloration of the face and that when tested after 6 days, serum levels of silver and enzymes indicative of hepatotoxicity (aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transferase) were elevated (Trop et al., 2006). Clinical symptoms and liver enzymes returned to normal after the dressing was changed to a different type. A case study of 30 less extensively burned patients (median 7% total burned surface area) treated with the same dressing for 3–19 days (average = 10.6 days) reported that serum silver levels were increased in relation to total burned surface area, but were not significantly correlated with changes in serum enzyme levels (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, or gamma-glutamyl transferase), although marginal correlations with three hematological variables were found: platelet count ($p = 0.054$), hemoglobin (Hb) level ($p = 0.055$), and eosinophil count ($p = 0.024$) (Vlachou et al., 2007)

No mortality, clinical signs of toxicity, or effects on body weight or gross pathology were observed in Sprague-Dawley rats within 14 days of dermal exposure for 24 hours to an antimicrobial gel at a dose level of 2 g gel/kg body weight (Jain et al., 2009). The gel contained 0.1 mg Ag/g (equivalent dose was 0.2 mg Ag/kg body weight) from spherical particles showing an average diameter of 16.6 nm (range = 6.5–43.8 nm).

3.3. REPEATED-DOSE TOXICITY

3.3.1. Inhalation

Ji et al. (2007a) conducted a 28-day inhalation toxicity study of uncoated silver nanoparticles in 8-week-old specific-pathogen-free (SPF) Sprague-Dawley rats following Organisation for Economic Co-operation and Development (OECD) Test Guideline 412. Groups of 10 male and 10 female rats were chamber exposed to fresh air or three concentrations of silver nanoparticles for 6 hours/day, 5 days/week for 4 weeks. Silver nanoparticles were generated by evaporation/condensation using a ceramic heater. Particles generated by this method were nearly

spherical, nonagglomerated, and determined to be elemental silver (not silver oxides or salts) by XRD and EDX analysis (Ji et al., 2007b; Jung et al., 2006). Concentrations and size distributions were measured with a differential mobility analyzer and ultrafine condensation particle counter, and diameters of randomly selected particles were determined by TEM. Mean total mass concentrations (and standard errors [SEs]) in the low, medium, and high exposure chambers were 0.48 (0.25), 3.48 (0.49), and 61.24 (1.52) μg Ag/m^3; rounded values are 0.5, 3.5, and 61 μg Ag/m^3. Respective mean total number concentrations (and SEs) were 1.73×10^4 (6.64×10^3), 1.27×10^5 (8.45×10^3), and 1.32×10^6 (1.79×10^4) particles/cm^3. Respective geometric mean diameters (and geometric standard deviations) were 11.93 nm (0.22), 12.40 nm (0.15), and 14.77 nm (0.11); the higher diameter at the high concentration was explained as being due to some agglomeration of particles. The reader is referred to Ji et al. (2007a) for surface area concentration (nm^2/cm^3) and volume concentration (nm^3/cm^3) values.

Animals were examined daily on weekdays for clinical signs, including respiratory, nasal, dermal, behavioral, or genitourinary changes suggestive of irritancy (Ji et al., 2007a). Body weights (weekly) and food consumption (presumably weekly) were measured throughout the study. Evaluations of hematology (20 indices), serum chemistry (22 indices), organ weights, and histopathology were conducted at the end of the study. The organ weight measurements and histology examinations were conducted on tissues including the nasal cavity, trachea, lungs, tongue, esophagus, liver, kidneys, bladder, spleen, thymus, adrenal glands, pancreas, thyroid gland, heart, brain, testes, prostate, and seminal vesicles.

The only remarkable findings were changes in two serum chemistry indices and a liver histopathologic effect (Ji et al., 2007a). The serum chemistry changes were small but statistically significant ($p < 0.05$) increases in calcium in both sexes at 61 μg Ag/m^3 and total protein in males at 61 μg Ag/m^3 (Table 6). These are considered to be not biologically significant changes. No effects on other serum chemistry variables were observed, including activities of alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase. Histological examination of the liver showed marginally increased incidences of cytoplasmic vacuolation in females at 3.5 and 61 μg Ag/m^3, but cytoplasmic vacuolation in males and hepatic necrosis in either sex were not increased (see Table 3.6). No distinct histopathological changes were found in the nasal cavity, lungs or other non-hepatic tissues. The highest exposure level in this study, 61 μg Ag/m^3, is designated as a no-observed-adverse-effect level (NOAEL), due to the lack of a clear increase in incidences of liver lesions or activity levels of serum liver enzymes with increasing exposure, and the determination that the changes in serum calcium and total protein at this exposure level are not biologically significant.

Table 3.6. Effects on serum chemistry and liver histopathology in rats exposed to silver nanoparticles by inhalation for 28 days[a]

Effect	Exposure concentration (µg Ag/m^3)			
	0	0.48	3.48	61.24
Serum chemistry[b]				
Calcium (mg/dL)				
Male	10.22 ± 0.19	10.87 ± 0.39	10.87 ± 0.26	11.58 ± 0.33[c]
Female	10.82 ± 0.29	10.59 ± 0.31	10.82 ± 0.35	11.96 ± 0.44[c]
Total protein (g/dL)				
Male	5.92 ± 0.05	5.87 ± 0.05	5.96 ± 0.09	6.17 ± 0.10[c]
Female	6.35 ± 0.11	6.40 ± 0.09	6.27 ± 0.07	6.48 ± 0.10
Liver histopathology (incidence)				
Cytoplasmic vacuolization				
Male	1/10	4/10	1/10	1/10
Female	2/10	2/10	6/10[d]	7/10[e]
Hepatic necrosis				
Male	0/10	0/10	0/10	2/10
Female	0/10	0/10	0/10	1/10

[a]Rats were exposed for 6 hours/day, 5 days/week for 4 weeks.
[b]Mean ± SE.
[c]Significantly different from control group, $p < 0.05$, by analysis of variance and Duncan's multiple range test.
[d]Marginal statistical difference from control group by Fisher's exact test (two-tailed) conducted for this review ($p = 0.17$).
[e]Marginal statistical difference from control group by Fisher's exact test (two-tailed) conducted for this review ($p = 0.07$).

Source: Ji et al. (2007a).

Hyun et al. (2008) also evaluated the rats from the 28-day inhalation study (Ji et al., 2007a) for histological effects in the lungs and nasal cavity, as well as for histochemical changes in mucins (high molecular weight mucous glycoproteins) in the goblet cells of the nasal respiratory mucosa. Mucins help protect the epithelial lining from chemical, enzymatic, and mechanical damage, and play an important role in first-line host defense mechanisms as a physiochemical barrier preventing bacteria and viruses from gaining access to the underlying mucosa (Hyun et al., 2008). As detailed above (Ji et al., 2007a), exposure levels (mass concentrations) were 0.5, 3.5, and 61 µg Ag/m^3, and mean particle diameters were in the range of 12–15 nm. Hyun et al. (2008) assessed general histological structure of the nasal cavity and lungs; qualitative amounts (absent, trace, weak, moderate, intense, or very intense) of mucins (neutral, acid, sulfated [sulfomucins], or nonsulfated [sialomucins]) in the nasal respiratory mucosa; and size, number, and density of nasal respiratory mucosal goblet cells containing each mucin. Specific histochemical staining procedures were used to detect and distinguish between the mucins, intensity of staining was used to qualitatively determine amounts of mucins, and photographs of stained tissues were used to quantitatively determine goblet cell numbers by manual counting and size and density measurements with an electronic image analyzer.

No histopathological changes were found in the lungs or nasal cavity (Hyun et al., 2008). Foamy alveolar macrophages were observed in rats exposed to 3.5 and 61 µg Ag/m^3 (incidences, severity, and other data were not reported), but the authors did not deem this finding to be abnormal or related to exposure. The histochemical evaluation of the nasal mucosa showed

increases in amounts of neutral mucins in the goblet cells at all exposure concentrations (≥0.5 µg Ag/m^3) that were dose-related but not statistically significant (*p* values not reported). There were statistically significant ($p < 0.05$) increases in number and size (but not density) of goblet cells containing neutral mucins at 3.5 and 61 µg Ag/m^3. No clear changes in goblet cells containing acid mucins, sulfomucins, or sialomucins were observed. The authors concluded that exposure to silver nanoparticles had an influence on neutral mucins in the nasal respiratory mucosa that was not toxicologically significant. Based on the lack of adverse histological effects in the nasal cavity and lungs or meaningful histochemical changes in the nasal mucosa, this study identified a subchronic NOAEL of 61 µg Ag/m^3 and no lowest-observed-adverse-effect level (LOAEL) for respiratory tract effects in rats.

Sung et al. (2009, 2008) conducted a 90-day inhalation study of silver nanoparticles that followed OECD Test Guideline 413, and was designed to identify possible adverse effects not detected in the 28-day study (Hyun et al., 2008; Ji et al., 2007a). Groups of 10 male and 10 female 8-week-old SPF Sprague-Dawley (Slc:SD) rats were chamber exposed to fresh air or three concentrations of silver nanoparticles for 6 hours/day, 5 days/week for 13 weeks. Silver nanoparticles were generated and concentrations and size distributions were measured as described by Ji et al. (2007a, b) and Jung et al. (2006). As reported by Sung et al. (2008), mean total mass concentrations (and SEs) in the low, medium, and high exposure chambers were 48.94 (0.47), 133.19 (1.05), and 514.78 (3.74) µg Ag/m^3; rounded values were 49, 133, and 515 µg Ag/m^3. Respective mean total number concentrations (and SEs) were 6.64×10^5 (1.44×10^3), 1.43×10^6 (4.58×10^3), and 2.85×10^6 (7.36×10^3) particles/cm^3; rounded values were 0.6, 1.4, and 3.0×10^6 particles/cm^3. Respective geometric mean diameters (and geometric standard deviations) were 18.12 nm (1.42), 18.33 nm (1.12), and 18.93 nm (1.59). The reader is referred to Sung et al. (2008) for surface area (nm^2/cm^3) concentrations.

Animals were examined daily on weekdays for clinical signs, including respiratory, nasal, dermal, behavioral, or genitourinary changes suggestive of irritancy. Body weights and food consumption were measured weekly throughout the study. Pulmonary function was evaluated weekly in 4 rats/sex/group using a ventilated bias flow whole-body plethysmograph to determine tidal volume, minute volume, respiratory frequency, inspiration and expiration times, and peak inspiration and expiration flow (Sung et al., 2008). Lung inflammatory responses were evaluated at the end of the exposure period in the animals tested for lung function by analysis of bronchoalveolar lavage (BAL) fluid for numbers of total cells, macrophages, polymorphonuclear (PMN) cells, and lymphocytes, and levels of albumin, total protein, and lactate dehydrogenase (LDH) (Sung et al., 2008). Evaluations of hematology (22 indices), serum chemistry (20 indices), organ weights and histopathology were performed in all animals at the end of the study (Sung et al., 2009). Histology examinations were conducted on adrenal glands, bladder, testes, ovaries, uterus, epididymis, seminal vesicle, heart, thymus, thyroid gland, trachea, esophagus, tongue, prostate, lungs, nasal cavity, kidneys, spleen, liver, pancreas, and brain. Relative organ weights were reported for testes, ovaries, kidney, spleen, liver, adrenal glands, heart, thymus, lungs, brain, and olfactory bulbs. Lung histopathology findings were first reported by Sung et al. (2008) and then finalized in Sung et al. (2009).

Results of the 90-day study indicate that the lungs and liver were target organs for inhaled silver nanoparticles in rats (Sung et al., 2009, 2008). Both functional and inflammatory

changes were observed in the lungs. Lung function testing at most testing intervals throughout exposure showed statistically significant ($p < 0.05$) decreases in tidal volume, minute volume, and/or peak inspiration flow in males at 515 µg Ag/m^3 (Sung et al., 2008). The graphed results indicated that mean values were decreased by about 30–35, 35–60, and 18–55% for tidal volume, minute volume, and peak inspiration flow in high-exposure males, compared with controls. Statistical decreases in these endpoints were also observed at a few time points in low- and mid-exposure males, but the changes at these exposure concentrations were small and inconsistent across testing intervals and likely not due to treatment. There were no consistent exposure-related changes in lung function endpoints in female rats.

BAL fluid cell counts were not significantly increased in males or females (Sung et al., 2009, 2008). BAL fluid inflammatory markers (LDH, total protein) were significantly ($p < 0.05$) increased in females at 515 µg Ag/m^3 (Table 7). Albumin in BAL fluid was also increased in this group, although the difference from controls was not statistically significant. The magnitudes of the differences in means for inflammation markers between 515 µg Ag/m^3 females and controls ranged from about 1.5- to 4-fold (see Table 7) and are indicative of a biologically significant change. There were no noticeable increases in BAL inflammatory markers in males. Assessment of the lung function and inflammation marker responses is complicated by small numbers of animals (4 rats/sex/dose group).

Histopathological examination of the lungs (10 rats/sex/dose) found alterations in both sexes at 515 µg Ag/m^3, particularly increased incidences of minimal severity chronic alveolar inflammation, alveolar macrophage accumulation, and a mixed cell perivascular infiltrate, as shown in Table 7 (Sung et al., 2009, 2008). No apparent exposure-related histopathological effects were observed in the nasal cavity or at any other site except the liver (Sung et al., 2009). In the liver, incidences of minimal bile duct hyperplasia were significantly increased in both sexes at 515 µg Ag/m^3 (Table 3.7). The results indicate that 515 and 133 µg Ag/m^3 were the LOAEL and NOAEL in this study for increased incidences of male and female rats with lung lesions (chronic alveolar inflammation, alveolar macrophage accumulation, and/or mixed cell perivascular infiltrate) and liver lesions (bile duct hyperplasia). The LOAEL was also associated with increased mean values for markers of inflammation (total protein, albumin, and LDH activity) in BAL fluid from female rats and impaired pulmonary function (decreased tidal volume, minute volume, and peak inspiration flow) throughout the study in male rats.

Table 3.7. Lung and liver effects in rats exposed to silver nanoparticles by inhalation for 90 days[a]

Endpoint	Exposure concentration (µg Ag/m^3)			
	0	49	133	515
Lung inflammation markers[b]				
Females				
LDH (BAL fluid, IU/L)	57.3 ± 7.9	41.8 ± 4.5	41.8 ± 5.4	116.7 ± 20.0[c]
Total Protein (BAL fluid, µg/mL)	13.9 ± 1.9	10.4 ± 0.5	10.9 ± 1.0	20.33 ± 3.5[c]
Albumin (BAL fluid, µg/mL)	9.3 ± 1.8	11.3 ± 1.5	11.8 ± 1.0	37.75 ± 20.1
Lung histopathology[d] (incidence)				
Males				
Alveolar macrophage accumulation	3/10	5/10	5/10	8/9[f]
Chronic alveolar inflammation[e]	2/10	3/10	2/10	8/9[f]
Mixed cell perivascular infiltrate	3/10	4/10	6/10	7/9[g]
Females				
Alveolar macrophage accumulation	7/10	4/10	4/10	6/10
Chronic alveolar inflammation[e]	3/10	2/10	0/10	8/10[g]
Mixed cell perivascular infiltrate[e]	0/10	0/10	1/10	7/10[f]
Liver histopathology[d] (incidence)				
Males				
Bile duct hyperplasia[e]	0/10	0/10	1/10	4/9[f]
Females				
Bile duct hyperplasia[e]	3/10	2/10	4/10	9/10[f,h]
Hepatocellular necrosis, single-cell[e]	0/10	0/10	0/10	3/10

[a]Rats were exposed for 6 hours/day, 5 days/week for 13 weeks.
[b]Mean (±SE) levels in BAL fluid, n = 4 females/group; no effects observed in males.
[c]Significantly different from control group, $p < 0.05$ (total protein) or $p < 0.01$ (LDH), by analysis of variance and Duncan's multiple range test.
[d]Lesions were minimal severity unless otherwise specified.
[e]Significant difference reported for treated rats versus control group, $p < 0.05$ (lung lesions) or $p < 0.01$ (liver lesions), by χ^2 analysis.
[f]Pairwise significant difference from control group, $p \leq 0.05$, by Fisher's exact test (two-sided) conducted for this review.
[g]Pairwise marginal difference from control group, $0.05 \leq p \leq 0.1$, by Fisher's exact test (two-sided) conducted for this review.
[h]Includes minimal severity (8/10) and moderate severity (1/10) hyperplasia.

Sources: Sung et al. (2009, 2008).

3.3.2. Oral

Kim et al. (2008) conducted a 28-day oral toxicity study of silver nanoparticles in 6-week-old SPF Sprague-Dawley rats following OECD Test Guideline 407. Groups of 10 male and 10 female rats were exposed to suspensions of commercial silver nanoparticles in 0.5% aqueous carboxymethylcellulose by gavage at doses of 0 (vehicle control), 30, 300, or 1,000 mg/kg-day for 28 consecutive days. Dosing volumes were 10 mL/kg. The test material was purchased from NAMATECH Co., Ltd. (Korea) and reported to be 99.98% pure with particle diameters ranging from 52.7 to 70.9 nm with an average of 60 nm. The method(s) used to determine these characteristics of the test material was not reported, and no information about the possible agglomerization of particles in the suspension was reported. Clinical signs

(presumed daily) and body weight and food consumption (presumed weekly) were evaluated throughout the study. Hematology (20 indices), coagulation time (two measures), blood chemistry (22 indices), organ weights and histopathology were evaluated at the end of the exposure period. The organ weight measurements and histology examinations were conducted on tissues including the tongue, esophagus, nasal cavity, trachea, lungs, liver, kidneys, bladder, spleen, thymus, adrenal glands, pancreas, thyroid gland, heart, brain, testes, epididymis, seminal vesicles, prostate, ovaries, and uterus. Additionally, femur bone marrow smears were prepared to determine numbers of normochromatic erythrocytes (NCEs), polychromatic erythrocytes (PCEs) and micronucleated PCEs. The frequency of micronucleated erythrocytes was used to assess bone marrow deoxyribonucleic acid (DNA) damage in a micronucleus assay based on OECD Guideline 474 (results in Section 3.6.2., Genotoxicity), and the ratio of PCEs among total erythrocytes [PCE/(PCE + NCE)] was used to assess toxicity to bone marrow cells.

Results of this 28-day study indicate that liver was the main target organ in orally exposed rats (Kim et al., 2008). The most consistently observed effects were changes in liver-associated blood biochemical endpoints and liver histopathology in both sexes at 1,000 mg/kg-day. As summarized in Table 8, dose-related and statistically significant increases were observed for blood levels of alkaline phosphatase in males at 300 mg/kg-day and both sexes at 1,000 mg/kg-day, and for total cholesterol in females at 300 mg/kg-day and both sexes at 1,000 mg/kg-day. No increases were observed in other liver enzymes (aspartate aminotransferase, alanine aminotransferase, LDH, or gamma-glutamyl transpeptidase). Other statistically significant blood findings included a small decrease in total protein in males at 1,000 mg/kg-day, and small increases in mean corpuscular volume (MCV) in males at 1,000 mg/kg-day and red blood cells (RBCs), Hb, and hematocrit (HCT) in females at ≥300 mg/kg-day (Table 8). Coagulation time was affected in females exposed to 1,000 mg/kg-day as indicated by a statistically significant decrease in active partial thromboplastin time, although there were no changes in prothrombin time in females or in either measure of clotting time in males (Table 3.8).

Reported histopathology findings were limited to the liver and described in minimal detail, with no incidence data or specification of lesion severity (Kim et al., 2008). The liver examinations were reported to show dose-dependent increased incidences of bile duct hyperplasia around the central vein to the hepatic lobule in both sexes, with dilatation of the central vein and infiltration of inflammatory cells in the central vein, hepatic lobule and portal tract. In a personal communication with SRC, Inc. (August 30, 2010), Dr. Il Je Yu provided the following incidence data for bile duct hyperplasia in the control through high-dose groups: 0/9, 1/10, 0/5, and 3/8 in males, and 1/10, 0/6, 1/5, and 3/8 in females (Yu, 2010). The incidence for high-dose males is marginally ($p = 0.08$) increased, compared with control incidence by Fisher's exact test (two-tailed). The combined incidence for bile duct hyperplasia in male and female high-dose rats (6/16) is significantly increased (Fisher's exact test: $p = 0.03$, two-tailed), compared with the control incidence (1/19).

Supporting evidence for hepatotoxicity at 1,000 mg/kg-day is provided by the increases in blood alkaline phosphatase and total cholesterol levels in both sexes at 1,000 mg/kg-day, particularly the nearly twofold elevation in alkaline phosphatase in high-dose males. Kim et al. (2008) concluded that the findings for increased alkaline phosphatase activity in males and

increased cholesterol in females in the 300 mg/kg-day group (see Table 8) indicated that this dose may result in slight liver damage. The hematological effects (increased RBC, Hb, and HCT in females at ≥300 mg/kg-day and MCV in males at 1,000 mg/kg-day) and coagulation time (decreased active partial thromboplastin time in females at 1,000 mg/kg-day) are of unlikely toxicological significance due to inconsistent responses in males and females, the small magnitude of the hematological alterations, and the direction of the coagulation effect (clotting time was decreased rather than increased). The results indicate that 300 and 30 mg/kg-day were the LOAEL and NOAEL in this study for minimal liver toxicity (increased alkaline phosphatase activity and increased cholesterol). Increased combined incidence of bile duct hyperplasia in male and female rats was observed at 1,000 mg/kg-day in this study.

Table 3.8. Effects on blood biochemistry, hematology, and clotting time in rats following oral exposure to silver nanoparticles for 28 days[a]

Endpoint	Dose (mg/kg-day)			
	0	30	300	1,000
Blood biochemistry[b]				
Males				
Alkaline phosphatase (IU/L)	427.20 ± 70.61	475.40 ± 98.54	613.70 ± 128.61[c]	837.70 ± 221.53[c]
Total cholesterol (mg/dL)	69.00 ± 8.23	73.10 ± 16.03	82.10 ± 21.68	87.50 ± 13.05[c]
Total protein (g/dL)	5.79 ± 0.21	5.61 ± 0.25	5.77 ± 0.24	5.53 ± 0.20[c]
Females				
Alkaline phosphatase (IU/L)	367.60 ± 125.67	335.40 ± 78.98	403.30 ± 75.93	499.00 ± 107.34[c]
Total cholesterol (mg/dL)	89.60 ± 9.07	85.60 ± 12.19	102.90 ± 17.74[c]	117.50 ± 14.08[c]
Total protein (g/dL)	5.79 ± 0.22	5.83 ± 0.22	5.74 ± 0.23	5.79 ± 0.24
Hematology[b]				
Males				
RBCs (M/µL)	9.05 ± 1.81	9.34 ± 0.38	9.66 ± 0.68	9.65 ± 0.59
Hb (g/dL)	14.84 ± 3.14	15.37 ± 0.44	15.60 ± 1.01	16.03 ± 0.93
HCT (%)	65.20 ± 13.59	66.66 ± 2.33	67.83 ± 4.76	71.20 ± 3.23
MCV (fl)	71.96 ± 3.63	70.19 ± 3.34	70.30 ± 3.18	73.88 ± 2.61[c]
Females				
RBCs (M/µL)	7.84 ± 0.58	7.75 ± 1.01	8.48 ± 0.55[c]	8.53 ± 0.45[c]
Hb (g/dL)	13.36 ± 0.93	13.11 ± 1.87	14.43 ± 0.78[c]	14.74 ± 0.68[c]
HCT (%)	65.18 ± 5.32	64.94 ± 9.27	71.69 ± 4.71[c]	73.42 ± 4.35[c]
MCV (fl)	83.09 ± 2.39	83.67 ± 1.89	84.66 ± 3.42	86.08 ± 3.81
Coagulation time[b]				
Males				
Prothrombin time (sec)	15.11 ± 0.96	14.80 ± 0.96	14.95 ± 0.77	15.01 ± 0.91
Active partial thromboplastin time (sec)	9.26 ± 2.14	9.90 ± 2.19	10.87 ± 2.23	11.27 ± 3.19
Females				
Prothrombin time (sec)	13.59 ± 0.73	14.78 ± 1.20	14.46 ± 0.83	14.80 ± 0.93
Active partial thromboplastin time (sec)	12.13 ± 2.13	10.96 ± 2.89	10.28 ± 3.67	8.81 ± 2.48[c]

[a]Rats were exposed to suspensions of commercial silver nanoparticles in 0.5% aqueous carboxymethylcellulose by gavage at doses of 0, 30, 300, or 1,000 mg/kg-day for 28 consecutive days.
[b]Mean (±SD) values, 10/sex/group for biochemistry and hematology indices and 7–10/sex/group for coagulation times.
[c]Significantly different from control group, $p < 0.05$ by multiple variance analysis and Duncan's multiple range test.

Source: Kim et al. (2008).

In a second 28-day oral study of silver nanoparticles in rats by the same researchers, Jeong et al. (2010) investigated effects on the histology and histochemical composition of mucins in the intestinal tract. The research group and general experimental design, including test material and dose levels, are the same as in the Kim et al. (2008) study summarized above. In particular, 6-week-old SPF Sprague-Dawley rats (10/sex/group) were exposed to suspensions of commercial silver nanoparticles (60 nm average diameter) in 0.5% aqueous carboxymethyl-cellulose by gavage at doses of 0 (vehicle control), 30, 300, or 1,000 mg/kg-day for 28 consecutive days. The ileum, colon, and rectum were assessed for general histological structure and amounts (absent, trace, weak, moderate, intense, or very intense) of mucins (neutral, acid, sulfated, or nonsulfated) in the mucosa. Specific histochemical staining procedures were used to detect and distinguish between the mucins, and intensity of staining was subjectively scored to assess amounts of mucins. Lower amounts of neutral, acidic, and sulfomucins were reported to have occurred in goblet cells from the ileum, colon, and rectum mucosa of silver-exposed rats, but the reporting of the data was inadequate to determine whether there were differences between the exposed groups. As such, the reporting of the data is inadequate to support determination of a NOAEL and LOAEL in this study.

Kim et al. (2010) conducted a 90-day oral toxicity study of silver nanoparticles in 5-week-old SPF F344 rats following OECD Test Guideline 408. Groups of 10 male and 10 female rats were exposed by daily gavage to suspensions of commercial silver nanoparticles in 0.5% aqueous carboxymethylcellulose at doses of 0 (vehicle control), 30, 125, or 500 mg/kg-day for 90 days. Dosing volumes were 10 mL/kg. The test material was purchased from NAMATECH Co., Ltd. (Korea) and reported to be 99.98% pure. Count median diameter and geometric standard deviation of the particles in suspension were 56 nm and 1.46, as determined by TEM, but no mention was made of possible agglomerization of the particles in the administered suspensions. Clinical signs (presumed daily) and body weight and food consumption (presumed weekly) were evaluated throughout the study. Hematology (20 indices), blood chemistry (22 indices), organ weights, and histopathology were evaluated at the end of the exposure period. Histology examinations were conducted on adrenal glands, bladder, testes, ovaries, uterus, epididymis, seminal vesicle, heart, thymus, thyroid gland, trachea, esophagus, tongue, prostate, lungs, nasal cavity, kidneys, spleen, liver, pancreas, and brain. Relative organ weights were reported for testes, ovaries, spleen, liver, pituitary gland, adrenal glands, uterus, prostate, lungs, brain, heart, thymus, and kidneys. Tissue samples were prepared for silver content determination using atomic absorption spectrometry.

Statistically significant ($p < 0.05$) changes in body weight and relative organ weights were: decreased body weights in high-dose males at several time points and mid-dose males at 10 weeks; increased relative weight of left testes for high-dose males; and decreased relative weight of the right kidney for low- and middle-dose females. The magnitudes of the changes were within 10% of control values, and are considered to be biologically nonsignificant. Absolute organ weights were not reported. At the highest dose level, total cholesterol was significantly increased by about 1.2-fold in males and females, and alkaline phosphatase activity was significantly increased by about 1.3-fold in high-dose females, compared with controls (see Table 9). Mean activities for other serum liver enzymes in exposed groups (including aspartate aminotransferase, alanine aminotransferase, and LDH) were not significantly different than control means. No exposure-related changes in hematological variables were found.

Histopathological examination showed minimal focal hepatic necrosis of uncertain relationship to treatment (marginally significant increases in low- and high-dose male rats) and significant increases for pigmentation in the villi of the intestine of high-dose male and female rats (see Table 3.9). Overall, the results indicate a NOAEL of 125 mg/kg-day and a LOAEL of 500 mg/kg-day based on evidence for minimal liver toxicity (increased serum cholesterol and alkaline phosphatase without clear evidence for increased liver lesions) and yellow pigmentation in intestinal villi.

Table 3.9. Selected histopathological lesions and serum biochemical variables in F344 rats following oral exposure to silver nanoparticles for 90 days

Endpoint	Dose (mg/kg-day)			
	0	30	125	500
Histopathological lesions				
Hepatic bile duct hyperplasia, minimum				
Male	4/10	7/10	8/10	6/10
Female	3/10	7/10	8/10	7/10
Hepatic focal necrosis, minimum				
Male	0/10	4/10[a]	3/10	4/10[a]
Female	0/10	2/10	2/10	1/10
Intestines: yellow pigment, villi				
Male (yellow)	0/10	0/10	1/10	8/10[b]
Female (faint yellow)	0/10	0/10	0/10	5/10[b]
Serum biochemical variables				
Cholesterol (mg/dL; mean ± SD, n = 10 per group)				
Male	88.50 ± 8.45	94.80 ± 7.54	93.30 ± 5.46[c]	106.00 ± 6.38[c]
Female	107.50 ± 7.35	115.11 ± 12.36	116.30 ± 6.95	126.70 ± 12.39[c]
Alkaline phosphatase (units/L; mean ± SD, n = 10 per group)				
Male	291.60 ± 35.40	279.50 ± 48.78	306.40 ± 35.16	343.80 ± 65.67
Female	237.50 ± 29.05	241.22 ± 22.12	253.50 ± 25.42	314.80 ± 42.13[c]

[a]Marginal statistical difference from control by Fisher's exact test (two-tailed) conducted for this review (p = 0.087).
[b]Significantly different from control by Fisher's exact test (two-tailed) conducted for this review ($p < 0.05$).
[c]Significantly ($p < 0.05$) different from control mean by two-tailed Student's t-test or analysis of variance following multiple comparison tests with Duncan's method.

Source: Kim et al. (2010).

3.3.3. Dermal

Dermal toxicity was evaluated in pigs topically exposed to washed or unwashed commercial silver nanoparticles (with average diameters of 20 or 50 nm, respectively; purchased from nanoComposix, San Diego, California) suspended in deionized water for 14 days (Samberg et al., 2010). Sites on the back skin of female weanling pigs (n = 2; 20–30 kg) were dosed with 500 µL of suspension concentrations ranging from 0.34 to 34 µg/mL for 14 days (surface area of exposed skin was not specified). Macroscopic evidence of skin irritation (using the Draize evaluation system) was not found at the application site, but light microscopic examination

showed focal inflammation and edema in skin layers under the stratum corneum. The effect was dose-related, with slight intracellular and intercellular epidermal edema at 0.34 µg/mL, moderate epidermal edema and focal epidermal and dermal inflammation at 3.4 µg/mL, and severe epidermal edema with severe focal dermal inflammation, epidermal hyperplasia, and parakeratosis at 34 µg/mL. Particle size or washing did not affect the observed skin responses. Samberg et al. (2010) hypothesized that the lesions in layers under the stratum corneum were caused by silver ion flux into the lower layers from the particles in the stratum corneum. TEM examination of skin detected no particles in the skin layers showing lesions; particles were found only on the surface of the stratum corneum or with superficial layers of the stratum corneum. No examinations of nondermal tissues were conducted.

3.4. CHRONIC TOXICITY/CARCINOGENICITY

No studies were located on the possible carcinogenicity of repeated exposure to silver nanoparticles by any route of exposure. The National Toxicology Program (NTP, 2010) selected silver nanoparticles for chronic toxicity/carcinogenicity testing, which is currently in planning stages.

3.5. REPRODUCTIVE/DEVELOPMENTAL TOXICITY

No standard mammalian tests of reproductive or developmental toxicity were located for silver nanoparticles. Several studies have examined the cytotoxicity of silver nanoparticles in cultured mouse embryos, cultured zebrafish embryos, or early life stages of Medaka fish (Li et al., 2010; Bar-Ilan et al., 2009; Wu et al., 2009; Lee et al., 2007), but results from these studies are inadequate to assess the potential reproductive/developmental toxicity of silver nanoparticles in mammals. Apoptosis and cytotoxicity were observed in mouse embryos/blastocytes exposed for 24 hours to culture media containing 50 µM of silver nanoparticles with an average particle size of about 13 nm (Li et al., 2010). When exposed blastocytes were implanted in female mice, percentages of implantations and surviving fetuses were decreased and percentages of resorptions were increased, compared with values in mice implanted with control blastocytes. Developmental abnormalities have been observed following in vitro exposure of zebrafish embryos (Bar-Ilan et al., 2009; Lee et al., 2007) or Medaka fish at early life stages (Wu et al., 2009) to silver nanoparticles.

3.6. MECHANISTIC DATA ON SILVER NANOPARTICLES

3.6.1. Cytotoxicity

The antimicrobial activity of silver has led to its widespread use as an antibiotic in water purification, wound care, and medical devices (Johnston et al., 2010). Ionic silver is generally recognized as the toxic form of silver, but the use of elemental silver nanoparticles for antibiotic activity has many recent applications (Johnston et al., 2010; Chen and Schluesener, 2008). The cytotoxicity of the silver ion to microbial cells is thought to involve silver binding to DNA and proteins, leading to interference with cellular functions (Greulich et al., 2009; Jung et al., 2008). The dissolution of silver ions from silver nanoparticles within cells and in close proximity to cells in aqueous environments has been proposed to be involved in the mode of action for the

antimicrobial activity of silver nanoparticles, but contributions from responses to the particles themselves have also been proposed (Hwang et al., 2008). Currently, the relative importance of direct responses to dissolved ionic silver and elemental silver nanoparticles to the mode of toxic action of silver nanoparticles is uncertain.

The comparative in vitro cytotoxicity of elemental silver nanoparticles and ionic silver has been examined in studies with mammalian cells, including human mesenchymal stem cells (Greulich et al., 2009), human monocytic cells (Foldbjerg et al., 2010, 2009), human HEPG2 hepatoma cells (Kawata et al., 2009; Kim et al., 2009b), human HeLa S3 cells (Miura and Shinohara, 2009), rat alveolar macrophages (Carlson et al., 2008), and mouse spermatogonia stem cells (Braydich-Stolle et al., 2010). The available results suggest that mammalian cytotoxic responses to elemental silver nanoparticles are dependent on particle physical and chemical characteristics and are similar, although not identical, to responses to ionic silver, especially for silver nanoparticles with smaller average diameters (<~30 nm). Additional studies of mammalian cells exposed to silver nanoparticles, but not ionic silver, provide supporting results (Arora et al., 2009, 2008; Rosas-Hernandez et al., 2009; Hsin et al., 2008; Hussain et al., 2006, 2005).

Key findings following exposure to either silver nanoparticles or ionic silver include increased levels of reactive oxygen species (ROS) (Foldbjerg et al., 2010, 2009; Kim et al., 2009b; Carlson et al., 2008; Hussain et al., 2006, 2005), induction of oxidative stress management genes (Kim et al., 2009b; Miura and Shinohara, 2009), increased percentage of apoptotic cells (Foldbjerg et al., 2009; Miura and Shinohara, 2009; Hsin et al., 2008), and attenuation of cytotoxic effects of silver by N-acetylcysteine (a glutathione precursor, ligand for ionic silver, and ROS scavenger) (Kawata et al., 2009; Kim et al., 2009b; Hsin et al., 2008). Across a number of studies, ionic silver from either silver nitrate or silver acetate was consistently more potent than silver nanoparticles in inducing apoptosis and ROS (Foldbjerg et al., 2009) and reducing cell viability (Greulich et al., 2009; Kim et al., 2009b; Miura and Shinohara, 2009; Carlson et al., 2008), but, in another comparison, silver carbonate was not as potent as silver nanoparticles in decreasing cell viability (Kawata et al., 2009).

3.6.1.1. Human Cell In Vitro Cytotoxicity

Results from comparative in vitro studies with various types of human cells suggest that oxidative stress and stimulation of apoptosis (programmed cell death) play roles in the cytotoxicity of both silver nanoparticles and ionic silver (Foldbjerg et al., 2010, 2009; Greulich et al., 2009; Kawata et al., 2009; Kim et al., 2009b; Miura and Shinohara, 2009). These studies are described below.

In a human monocytic cell line, THP-1, the 24-hour EC_{50} values for reduced cell viability (assayed by the annexin V/propidium iodide assay that discerns necrotic from apoptotic cells) were 2.436 ± 0.170 µg Ag/mL for silver nanoparticles and 0.624 ± 0.053 µg Ag/mL for silver nitrate, indicating a fourfold difference in potency (Foldbjerg et al., 2009). Significantly increased percentages of apoptopic cells occurred at concentrations ≥5 µg Ag/mL silver nanoparticles and ≥0.66 µg Ag/mL silver nitrate. Levels of ROS (assayed by dichlorofluorescein fluorescence) were significantly ($p < 0.05$) increased after 24 hours of exposure at concentrations

≥2.5 µg Ag/mL silver nanoparticles and ≥0.66 µg Ag/mL silver nitrate. The silver nanoparticles (which were coated with polyvinylpyrolidone) had an average diameter of 69 nm (SD = 49; assayed by TEM), and were agglomerated in the culture media. Dynamic light scattering of the culture media following exposure of the cells indicated a hydrodynamic diameter of 149 nm (SD = 37). Similar results were reported in studies with human lung cancer cells (Foldbjerg et al., 2010).

In human HeLa S3 cells, 24-hour EC_{50} values for reduced cell viability (assayed with the Alamar Blue reagent) were about 92 and 17 µg Ag/mL for silver nanoparticles and silver nitrate, respectively, indicating a fivefold higher potency for silver nitrate, compared with silver nanoparticles (Miura and Shinohara, 2009). Following 24 hours of exposure, significantly ($p < 0.05$) increased percentages of apoptotic cells were found at concentrations ≥60 and ≥8 µg Ag/mL for silver nanoparticles and silver nitrate, respectively. Four-hour exposure to silver nanoparticles (1–100 µg Ag/mL) or silver nitrate (3–16 µg Ag/mL) significantly ($p < 0.05$) induced genes for metal binding (metallothionein-2A, *mt-2A*) and oxidative stress management (heme oxygenase-1, *ho-1*), but did not markedly alter expression of a heat shock management gene (heat shock protein 70kDa, hsp 70). The maximal induction of these genes by ionic silver was about two- to threefold higher than the maximal induction by silver nanoparticles. For example, at 6 µg ionic Ag/mL, an approximate 40-fold induction of *mt-2A* was observed, compared with an approximate 20-fold induction with 30 µg silver nanoparticles/mL. The commercial (Mitsuboshi Belting Ltd. Japan) silver nanoparticles in this study had reported mean diameters of 5–10 nm and included an unspecified patented material reported to suppress silver ionization. TEM photomicrographs of the particles in the cell media showed some agglomeration, described by Miura and Shinohara (2009) as "polymeric masses."

In HepG2 human hepatoma cells, 24-hour exposure to silver nanoparticles or silver nitrate decreased mitochondrial function (degree of mitochondrial reduction of a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT]), decreased cell viability (Alamar Blue reagent), and impaired membrane integrity (measured by leakage of LDH) (Kim et al., 2009b). Respective EC_{50} values for silver nanoparticles and silver nitrate were 3.38 and 1.37 µg Ag/mL for decreased mitochondrial function, 1.95 and 1.76 µg Ag/mL for cell viability, and 0.53 and 0.78 µg Ag/mL for membrane integrity. ROS levels (assessed by dichloro-fluorescein fluorescence) were increased, compared with control cells, after 1-hour exposures to 0.2 or 0.4 µg Ag/mL silver nanoparticles or 0.1 µg Ag/mL silver nitrate. Dose-dependent DNA damage (double strand breaks assayed using fluorescently labeled mouse monoclonal γ-H2AX antibody) was increased by 24-hour exposure to either silver nanoparticles or silver nitrate at ≥1 µg/mL. Pretreatment of cells with the glutathione precursor, N-acetylcysteine, attenuated the effects of silver nanoparticles and silver nitrate on mitochondrial function, cell viability, membrane integrity, ROS generation, and DNA. Twenty four-hour exposure to 0.2 µg Ag/mL silver nanoparticles or 0.2 µg Ag/mL silver nitrate increased the expression of two genes for oxidative stress management proteins (catalase and superoxide dismutase [SOD] 1), as assayed by real-time reverse transcriptase polymerase chain reaction (PCR) procedures. Silver nitrate exposure also induced expression of genes for metallothionein 1b and glutathione peroxidase 1, but expression of these genes was not influenced by silver nanoparticle exposure. TEM showed the commercial (Nanopoly, Seoul Korea) silver nanoparticles to be well dispersed in aqueous suspension with average diameters about 5–10 nm, but agglomerates, ranging in sizes from

100 to 300 nm, were observed in the culture media after exposure of the cells. TEM indicated the presence of silver nanoparticles within the cells following 24 hours of exposure.

Greulich et al. (2009) compared the effects of 7-day exposures to silver nanoparticles and silver acetate on the viability, chemotaxis, and cytokine release of cultured human mesenchymal stem cells. Cell viability (assessed by calcein-acetoxymethylester fluorescence) and chemotaxis were significantly ($p < 0.05$) decreased at silver acetate concentrations ≥2.5 µg Ag/mL and silver nanoparticle concentrations ≥3.5 µg Ag/mL. Chemotaxis was stimulated, compared with control cells, at silver nanoparticle and silver acetate concentrations between 0.05 and 3 µg Ag/mL and 0.05 and 1 µg Ag/mL, respectively. Significantly ($p < 0.05$) decreased releases of the cytokines IL-6 and IL-8 into the culture media were observed at 5 and 50 µg Ag/mL concentrations of either material. The tested nanoparticles were prepared by the reduction of silver nitrate with heat in the presence of polyvinylpyrolidone (the "polyol process"). The particles were reported to have an average diameter of 100 nm. Agglomeration of the particles in the cell culture medium (as assayed by dynamic light scattering) was prevented by the inclusion of 10% fetal calf serum; the median particle size was about 1,000 nm in the cell culture medium, compared with about 100 nm for the cell culture medium plus 10% fetal calf serum.

Kawata et al. (2009) compared the influence of 24-hour exposures of HepG2 human hepatoma cells to silver nanoparticles or silver carbonate on cell viability, gene expression patterns as assessed by microarray analysis, and induction of micronuclei. Tested concentrations ranged from 0.1 to 3.0 mg Ag/mL for both test materials. The commercial silver nanoparticles (Nano Chemical Co., Ltd, Kyoto, Japan) were reported to be "stabilized with polyethylenimine" and to have diameters of 7–10 nm. Further characterization of the tested material was not supplied in the report. Exposure to silver nanoparticles at concentrations ≥1.5 µg Ag/mL decreased cell viability (assayed by the neutral red uptake assay), whereas exposure to silver carbonate did not decrease cell viability at concentrations as high as 3 µg Ag/mL. At 1 mg Ag/mL, exposure to silver nanoparticles, but not silver carbonate, increased micronuclei frequency, compared with controls; respective mean frequencies were 47.9 and 2.6 per 1,000 cells, compared with 2.1 per 1,000 cells in controls. Inclusion of 5 mM N-acetylcysteine in the culture media with nanosilver particles attenuated the micronuclei response (mean frequency = 29.3 per 1,000 cells). Kawata et al. (2009) hypothesized that the ameliorating effect of N-acetylcysteine on the micronuclei effects was due to the strong binding of ionic silver by this compound.

In the microarray analysis, up- and down-regulated genes were determined as those with ≥twofold change (Kawata et al., 2009). Exposure to silver nanoparticles or silver carbonate altered the expression of 529 genes (236 induction and 293 repression) or 304 genes (162 induction and 142 repression), respectively. One hundred ninety-two common genes showed altered expression by either silver nanoparticles or silver carbonate (101 induction and 91 repression). Classification of gene ontology categories indicated that 521 of the silver-nanoparticle-influenced genes could be classified and 255 biological processes were identified. Among the identified processes associated with silver nanoparticles were several expected to be related to micronuclei formation: cell cycle progression through mitotic (M) phase (31 genes); microtubule-based processes (19 genes); DNA repair (16 genes); DNA replication (24 genes); and intracellular transport (32 genes). Similar processes were identified in the analysis of the

silver carbonate data: M phase (28 genes); microtubule-based processes (17 genes); DNA repair (17 genes); DNA replication (24 genes), and intracellular transport (14 genes). Silver nanoparticles upregulated three metallothionein genes and 3 heat shock proteins, but silver carbonate upregulated only one of these stress management genes (*HSPB1*).

Signs of oxidative stress occurred after 24-hour exposure of human cell lines A431 or HT-1080 (derived from human skin carcinoma and fibrosarcoma, respectively) to silver nanoparticles at concentrations about one-half of EC_{50} values for impaired mitochondrial function (reduction of a tetrazolium salt, sodium 3,3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT)]) (Arora et al., 2008). Respective EC_{50} values for impaired mitochondrial function were 10.6 and 11.6 µg Ag/mL for HT-1080 and A-431 cells. Following exposure to 6.25 µg Ag/mL (one-half of EC_{50} values for impaired mitochondrial function), the following signs of oxidative stress were observed (significant changes from control levels [$p < 0.05$], mean responses for the two cell lines given in parentheses): decreased glutathione levels (2.5- and 2-fold); decreased SOD activity (1.6- and 3-fold); and increased lipid peroxidation (2.5- and 2-fold). Using caspase-3 activity as an indicator of apoptosis, maximally increased activities were reported (about 110% of control values) at 6.25 µg Ag/mL, and decreased activities (about 80–90% of control) occurred at 25 µg Ag/mL. The decreased caspase-3 activity at the higher concentrations were taken as an indicator of cell necrosis. The results suggest that at lower concentrations (up to about 6.26 µg Ag/mL), decreased cell viability (assayed as impaired mitochondrial activity) was due to increased apoptosis, whereas at higher concentrations (above the EC_{50} values), necrosis made a greater contribution to the decrease in cell viability.

In similar tests with primary mouse fibroblasts and liver cells exposed to the same test material, respective EC_{50} values for impaired mitochondrial function were 61 and 449 µg Ag/mL (Arora et al., 2009). Exposure to one-half EC_{50} concentrations did not change glutathione levels, lipid peroxidation, or SOD activities more than twofold, compared with control values. The silver nanoparticles in these studies were synthesized by an unspecified proprietary process. They were reported to be stable in the culture media, with >90% of particles with diameters between 7 and 20 nm. Possible agglomeration of the particles in the culture media was not mentioned.

3.6.1.2. Animal Cell In Vitro Cytotoxicity

Results from a study of rat alveolar macrophages indicate that oxidative stress plays a contributory role in the cytotoxicity of elemental silver nanoparticles and ionic silver, and that the in vitro cytotoxic potency of silver nanoparticles declines with increasing particle diameter and degree of agglomeration (Carlson et al., 2008). Indices of cell viability of rat alveolar macrophages, mitochondrial function, and membrane integrity were significantly decreased after 24-hour exposure to increasing doses (10–75 µg Ag/mL) of hydrocarbon-coated elemental silver nanoparticles, dependent on the average diameters of the particles (Carlson et al., 2008). Nanoparticles with average diameters of 15, 30, and 55 nm were tested in this study. Light microscopy of the macrophages in the incubation medium showed that the nanoparticles in the medium were agglomerated to varying degrees; the 55-nm particles showed the greatest degree of agglomeration. TEM of macrophages exposed to 55-nm nanoparticles showed that

nanoparticles within cells were clustered in vacuoles, indicating that the particles were taken up by the cells and sequestered to some degree in vacuoles.

EC_{50} values for the mitochondrial function variable (degree of mitochondrial reduction of a tetrazolium salt, MTT) were 27.9 and 33.4 µg Ag/mL for the 15- and 30-nm nanoparticles, compared with an EC_{50} value for silver nitrate of 27.4 µg Ag/mL (Carlson et al., 2008). Exposure to 55-nm nanoparticles did not affect mitochondrial function. Another measure of cell viability/cytotoxicity, membrane integrity (measured by leakage of LDH) was significantly ($p < 0.05$) impaired in macrophages in the tested concentration range for the 15- and 30-nm nanoparticles, compared with control macrophages. EC_{50} values were 14.8 and 13.9 µg Ag/mL for 15- and 30-nm particles, whereas significantly decreased membrane integrity from the 55-nm particles was only observed at the highest concentration tested (41% decreased at 75 µg Ag/mL: EC_{50} value >75 µg Ag/mL). An EC_{50} value for decreased membrane integrity from silver nitrate was not determined. However, at 50 µg Ag/mL silver nitrate (the only concentration reported), LDH-assessed cell viability was only 2.5% of control values, compared with about 20–30% for the same concentration of 15-, 30-, or 55-nm silver nanoparticles. These results suggest that silver nitrate had a greater cytotoxic potency than the tested silver nanoparticles.

Reduced glutathione could not be detected in cells exposed to 50 µg Ag/mL concentrations of 15- and 30-nm nanoparticles or silver nitrate, and glutathione levels were decreased in cells exposed to 10 µg Ag/mL of 15- or 30-nm nanoparticles, compared with controls (Carlson et al., 2008). Exposure to 55-nm nanoparticles at 50 µg Ag/mL did not markedly affect reduced glutathione content. Exposure to the 15-nm particles at 10, 30, and 50 µg Ag/mL was also associated with significantly ($p < 0.05$) increased levels of ROS (assayed by dichlorofluorescein fluorescence). ROS levels were not markedly increased in cells exposed to 30- or 55-nm particles (except in cells exposed to 75 µg Ag/mL 55-nm particles). ROS levels were not measured in cells exposed to silver nitrate. The glutathione and ROS results suggest that generation of ROS and depletion of glutathione play roles in the observed cytotoxicity of silver nanoparticles and silver nitrate. Indices of inflammatory responses by the cells (levels of tumor necrosis factor α [TNFα], macrophage inhibitory protein [MIP-2], and interleukin-1β [IL-1β] in the culture media) were significantly ($p < 0.05$) increased after 24-hour exposures to 5, 10, or 25 µg Ag/mL 15-, 30-, or 55-nm particles (similar data for silver nitrate exposed cells apparently were not collected).

Earlier studies with BRL3A rat liver cells (Hussain et al., 2005) and PC-12 rat cells derived from pheochromocytoma cells (Hussain et al., 2006) obtained results supporting the importance of oxidative stress in the cytotoxicity of silver nanoparticles. Mitochondrial function (MTT reduction) and membrane integrity (assayed by LDH leakage) were decreased and ROS levels (assayed by dichlorofluorescein fluorescence) were increased in concentration-dependent manners in BRL3A rat liver cells exposed for 24 hours to uncoated silver nanoparticles with average diameters of 15 or 100 nm at concentrations ranging from 5 to 50 µg Ag/mL (Hussain et al., 2005). Levels of reduced glutathione were decreased in BRL3A rat liver cells exposed to 25 or 50 µg Ag/mL concentrations of 15- or 100-nm silver nanoparticles (Hussain et al., 2005). In PC-12 rat cells exposed to uncoated 15-nm silver nanoparticles for 24 hours, mitochondrial function was decreased and ROS levels were increased at concentrations ≥10 and 5 µg Ag/mL, respectively (Hussain et al., 2006).

Cytotoxicity in mouse NIH3T3 fibroblast cells exposed for 24 hours to silver nanoparticles at 50 µg Ag/mL was linked to increased ROS species and increased percentage of apoptotic cells (Hsin et al., 2008). The commercial nanoparticles (Ching-Tai Resin, Taichung, Taiwan) were examined by TEM and particles were reported to have diameters ranging from 1 to 100 nm, but no further information on the tested particles was provided. Mitochondrial function (MTT reduction as an assay of cell viability) was not influenced at concentrations of 0.0005, 0.005, 0.05, 0.5, or 5 µg Ag/mL, but was about 35% of control values at 50 µg Ag/mL. At this concentration, the percentage of apoptotic cells (assayed by flow cytometry and annexin V-FITC fluorescence) was about 35–40%, and the percentage of cells producing ROS (detected by dichlorofluorescein fluorescence) and the expression of apoptosis regulators (PARP cleavage, JNK phosphorylation, and p53 phosphorylation detected by Western analyses) were increased. Pretreatment of cells with N-acetylcysteine diminished the apoptotic, JNK phosphorylation, and p53 phosphorylation responses of the cells.

Results from studies of mouse spermatogonia stem cells showed that size and coating can influence the short-term (24-hour) cytotoxicity of silver nanoparticles (Braydich-Stolle et al., 2010). Mitochondria function (reduction of a tetrazolium salt, 3-(4,5, dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphopheyl)-2H-tetrazolium [MTS], taken as a measure of cell viability) was assessed following 24-hour exposure to hydrocarbon-coated silver nanoparticles of 15-, 25-, 80-, or 130-nm average diameters or polysaccharide-coated silver nanoparticles of 10-, 25–30-, 80-, or 130 nm diameters at concentrations of 5, 10, 25, 50, or 100 µg Ag/mL. The 130-nm particles (with either type of coating) were found in large aggregates in the culture medium, and did not influence mitochondria function, compared with non-exposed controls. The polysaccharide coating was reported to lead to better dispersion of the particles, but aggregation "to some extent" was observed with all of the particles after dispersion in deionized water.

Significantly ($p < 0.05$) impaired mitonchondria function occurred at concentrations ≥25 µg Ag/mL with both types of particles (Braydich-Stolle et al., 2010). The degree of impairment was greater with the smaller size classes (15- or 10-nm hydrocarbon-coated and 25- or 25–30-nm polysaccharide-coated), compared with the 80-nm size classes. Within the small class sizes, the degree of impairment after 24 hours of exposure was somewhat greater for the hydrocarbon-coated particles, compared with the polysaccharide-coated particles. For example, at 25 µg Ag/mL, MTS-assessed cell viability was about 70% of controls for 25–30-nm polysaccharide-coated particles, compared with about 40% for 25-nm hydrocarbon-coated particles. At the non-cytotoxic concentration of 10 µg Ag/mL, the percentage of cells producing ROS and percentage of cells in apoptosis were not significantly affected by 24-hour exposure to any the three size classes of the two types of nanoparticles, except for the 10-nm polysaccharide-coated particles, which showed a small increase in these variables, compared with control values. With 6 days of exposure to 15-nm hydrocarbon-coated or 10-nm polysaccharide-coated particles at 10 µg Ag/mL in the presence of glial cell line-derived neurotrophic factor (GDNF), cell proliferation was inhibited equally by either type of coated particle (about 35–40% of control cell proliferation). At this concentration, no effects were observed on the levels of free GDNF in the culture media or on the phophorylation of the Ret receptor, but the activity of Fyn kinase was decreased and the expression of *N-myc* was downregulated. These results suggest that the silver

nanoparticles disrupt GDNF/Fyn kinase signaling in mouse spermatogonia stem cells at concentrations that are noncytotoxic with short-term exposure.

The influence of silver nanoparticle coatings on cytotoxic variables has also been examined in cultured mouse embryonic stem cells and fibroblasts (Ahamed et al., 2008). Polysaccharide-coated silver nanoparticles and hydrocarbon-coated silver nanoparticles with nominal average diameters of 25 nm were tested. The hydrocarbon coating was nonuniform, and the particles tended to agglomerate in the culture media. In contrast, the polysaccharide coating was uniform, and the particles showed less agglomeration. Exposure to either type of particle at 50 µg Ag/mL induced apoptosis (assayed by western blot analysis for annexin V protein) and decreased mitochondrial function (MTT assay). The degree of change in the examined variables was greater for the polysaccharide-coated particles, compared with the hydrocarbon-coated particles. For example, MTT-assessed cell viabilities in embryonic stem cells after 24 hours of exposure were about 40% for the polysaccharide-coated particles, compared with about 60% for the hydrocarbon-coated particles. Ahamed et al. (2008) speculated that the difference in potency may be related to the difference in agglomerization of the two particle types.

In cultured rat coronary endothelial cells exposed to silver nanoparticles, cell proliferation was unaffected at 0.1 and 0.5 µg Ag/mL, inhibited by 1, 5, or 10 µg Ag/mL, and stimulated by 50 or 100 µg Ag/mL (Rosas-Hernandez et al., 2009). TEM analysis of the commercial silver nanoparticles (Novacentrix, Austin, Texas) indicated that the particles were spheres with a mean diameter of 35.75 nm (SD = 13.1 nm, range = 10–90 nm), which showed aggregation in the culture media. Increased cell proliferation at the higher concentrations was associated with increased nitric oxide (NO) production. NO dependence was demonstrated by the partial blocking of proliferation and NO production by N-nitro-L-arginine methyl ester (L-NAME). Activation (via phosphorylation) of endothelial nitric oxide synthase (eNOS, the enzyme that produces NO) was also associated with the increased proliferation at the higher concentrations. The nanoparticles displayed a parallel dual concentration effect on dilation of isolated rat aortic rings: exposure to 5 µg Ag/mL induced vasoconstriction within the aortic rings, whereas 100 µg Ag/mL induced vasodilation. Vasodilation induced by the high concentration was partially blocked by L-NAME treatment. The results suggest that nanoparticles have concentration-dependent effects on cultured vascular endothelium cells, inhibiting cell proliferation at concentrations of 1–10 µg Ag/mL, and stimulating proliferation through a NO-signaling pathway at high concentrations, 50 or 100 µg Ag/mL.

3.6.1.3. *In Vivo* Exposure Studies of Cytotoxicity-related Endpoints

Rahman et al. (2009) evaluated the effects of silver nanoparticles on expression of genes involved in oxidative stress and antioxidant defense pathways in the brain of male C57BL/6N mice. Silver was administered to mice through intraperitoneal injection at doses of 0, 100, 500, or 1,000 mg Ag/kg-bw. The average diameter of the commercial silver nanoparticles (NovaCentrix, Austin, Texas) was 29 nm. Agglomeration occurred whether dispersed in deionized water (average diameter = 118 nm) or phosphate buffer (average diameter = 1,090 nm). Mice were sacrificed 24 hours after injection and brains were removed and regionally dissected. Total ribonucleic acid (RNA) was collected from the caudate, frontal cortex, and hippocampus for use in real-time PCR. SuperArray mouse oxidative stress and antioxidant

defense pathway arrays were used to analyze 84 individual genes following nanoparticle exposure.

Exposure to silver nanoparticles altered the gene expression of 18 genes in the caudate, 14 genes in the frontal cortex, and 29 genes in the hippocampus (Rahman et al., 2009). Most of the alterations in the caudate consisted of gene upregulation, while changes in the hippocampus mostly involved downregulation. In the frontal cortex, there was a nearly even split between up- and downregulated genes (8 and 6, respectively). The Txnip, Gsr, and Fmo2 genes, three genes known to be involved in either oxidative metabolism or protection from oxidative stress, were significantly differentially expressed in all three regions, compared to controls. Txnip, whose induction initiates the intrinsic mitochondrial pathway of apoptosis, was upregulated 2.69-, 3.25-, and 5.79-fold in the hippocampus, frontal cortex, and caudate, respectively. Gsr, a glutathione metabolism gene that is involved in protecting cellular machinery from oxidative stress, was downregulated in all three areas (14-fold in the caudate; changes not specified for frontal cortex or hippocampus). The Fmo2 gene, which is involved in oxidative metabolism, was upregulated 95-fold in the caudate and approximately 4-fold in both the frontal cortex and hippocampus. The results suggest that administration of single high intraperitoneal doses of the tested silver nanoparticles (≥100 mg Ag/kg) to male mice differentially perturbed the expression of genes involved in oxidative stress management pathways in different brain regions. It is unclear from these results if the administered doses actually increased ROS levels or produced cytotoxic effects in the examined brain regions.

3.6.2. Genotoxicity

The frequency of micronucleated erythrocytes in bone marrow was not influenced in Sprague-Dawley rats following 28 days of oral exposure to 30, 300, or 1,000 mg/kg-day of silver nanoparticles, compared with control animals (Kim et al., 2008). Commercial (NAMATECH Co. Ltd. Korea) silver nanoparticles with an average diameter of 60 nm (range = 52.7–70.9 nm), were suspended in 0.05% carboxymethylcellulose. In contrast, a strong induction of micronuclei was observed in cultured human HepG2 cells exposed for 24 hours to a commercial silver nanoparticle (7–10 nm, stabilized with polyethlyenimine) at 1 mg Ag/mL (Kawata et al., 2009). In the same study, no induction of micronuclei was observed following a 24-hour exposure of HepG2 cells to 1 mg/mL of a polystyrene nanoparticle (15 nm) or 1 mg Ag/mL of Ag_2CO_3 (Kawata et al., 2009).

Indirect measures of DNA damage (upregulation and phosphorylation activation of the cell cycle regulator p53 protein and upregulation of the double strand DNA break repair protein Rad 51) were increased in mouse embryonic stem cells and mouse embryonic fibroblasts following 24–72-hour exposures to silver nanoparticles (25 nm) with a uniform polysachharide coating or a nonuniform hydrocarbon coating at 50 µg Ag/mL (Ahamed et al., 2008). The western blot results, shown as photographs in the report, provide semiquantitative indications that the p53 and Rad 51 responses to the polysaccharide-coated particles were greater than those to the hydrocarbon-coated particles.

Increased frequencies of chromosomal aberrations were observed in human IMR-90 lung fibroblasts and human U251 glioblastoma cells exposed to 25 µg Ag/mL silver nanoparticles for

48 hours (AshaRani et al., 2009). At higher concentrations (50 or 100 µg Ag/mL), too few cells were in mitosis to evaluate chromosomal aberrations. The elemental silver particles were stabilized with soluble potato starch, and TEM analysis indicated particles with diameters ranging from 6 to 20 nm. The report did not mention whether agglomeration occurred in the culture media. In both cell types exposed to 25 µg Ag/mL, the frequencies of acentric fragments and centric fragments were increased by at least twofold, compared with control values.

3.7. SUMMARY OF HAZARD AND DOSE-RESPONSE DATA

As discussed in Section 3.6.1, results from comparative studies of in vitro cytotoxicity of elemental silver nanoparticles and ionic silver suggest that cytotoxic responses to silver nanoparticles are dependent on particle physical and chemical characteristics and are similar, although not identical, to responses to ionic silver. Key findings following exposure to either silver nanoparticles or ionic silver include stimulation of apoptosis, increased levels of ROS, and induction of oxidative stress management genes and ROS scavengers. The results from these in vitro studies indicate that cytotoxic potency increases with decreasing particles sizes, and that ionic silver from silver nitrate or silver acetate is more cytotoxic than silver nanoparticles. The study of possible health hazards associated with in vivo exposure to silver nanoparticles is an emerging field with a limited number of animal toxicity studies of repeated exposure via the inhalation, oral, and dermal routes.

The available toxicity database for inhalation exposure to silver nanoparticles contains 28-day (Hyun et al., 2008; Ji et al., 2007a, b) and 90-day (Sung et al., 2009, 2008) inhalation toxicity studies of Sprague-Dawley rats exposed to aerosols of spherical uncoated elemental silver nanoparticles. No chronic inhalation exposure carcinogenicity/toxicity studies or reproductive or developmental toxicity studies are available, although a chronic NTP (2010) bioassay is in the planning stage. Exposure concentrations in the 28-day study were 0, 0.5, 3.5, and 61 µg Ag/cm^3 (Ji et al., 2007a). Geometric mean diameters in the exposed groups were 11.93, 12.40, and 14.77 nm; the higher diameter at the high concentration was explained as being due to some agglomeration of particles. Exposure concentrations in the 90-day study were 0, 49, 133, and 515 µg Ag/cm^3, and geometric mean diameters of the aerosols were 18.12, 18.33, and 18.93 nm (Sung 2009, 2008).

Collective results from the two studies identify the lung and liver as toxicity target organs for inhaled silver nanoparticles. In the 28-day study (Hyun et al., 2008; Ji et al. 2007a), the highest exposure level, 61 µg Ag/m^3, was designated a NOAEL for liver and lung effects; no lung effects were observed in any group of exposed rats, and histological examination, as well as serum chemistry analysis, failed to find clear evidence of biologically significant adverse changes to the liver. In the 90-day study (Sung et al., 2009, 2008), 515 and 133 µg Ag/cm^3 were the LOAEL and NOAEL for increased incidences of male and female rats with lung lesions (chronic alveolar inflammation, alveolar macrophage accumulation, and/or mixed cell perivascular infiltrate) and liver lesions (bile duct hyperplasia). The LOAEL was also associated with increased mean values for markers of inflammation (total protein, albumin, and LDH activity) in BAL fluid from female rats and impaired pulmonary function (decreased tidal volume, minute volume, and peak inspiration flow) throughout the study in male rats.

The available toxicity database for oral exposure to silver nanoparticles contains 28-day (Jeong et al., 2010; Kim et al., 2008) and 90-day (Kim et al., 2010) oral toxicity studies of Sprague-Dawley rats exposed by gavage to aqueous suspensions of commercial silver nanoparticles. No chronic oral exposure carcinogenicity/toxicity studies or reproductive or developmental toxicity studies are available. Reporting and designs of the 28- and 90-day studies by Kim et al. (2010, 2008) are adequate to designate reliable NOAELs and LOAELs useful for dose-response assessment. Both studies utilized silver nanoparticles with pure (99.98%) elemental silver particles (presumably uncoated, as there was no mention of any coating in either study) reported to: (1) range from 52.7 to 70.9 nm (average = 60 nm) for the 28-day study; and (2) have a count median diameter and geometric standard deviation of 56 nm and 1.46 for the 90-day study. Administered daily doses were 0, 30, 300, and 1,000 mg/kg-day for the 28-day study and 0, 30, 125, and 500 mg/kg-day for the 90-day study.

Collected results from the two adequate oral toxicity studies identify the liver as a toxicity target organ for repeated oral exposure to silver nanoparticles. In the 28-day study (Kim et al., 2008), 300 and 30 mg/kg-day were the LOAEL and NOAEL for signs of minimal liver toxicity (increased serum alkaline phosphatase activity and increased serum cholesterol); increased combined incidence of male and female rats with bile duct hyperplasia was observed at the highest dose level, 1,000 mg/kg-day. Results from the 90-day study (Kim et al., 2010) indicate a NOAEL of 125 mg/kg-day and a LOAEL of 500 mg/kg-day based on evidence for minimal liver toxicity (increased serum cholesterol and alkaline phosphatase without clear evidence for increased liver lesions) and yellow pigmentation in intestinal villi.

The available dermal toxicity database contains only a study of weanling pigs given daily topical doses of suspensions of commercial silver nanoparticles (with average diameters of 20 or 50 nm) for 14 days at silver concentrations ranging from 0.34 to 34 µg/mL. Dose-dependent microscopic evidence of inflammation was evident in skin layers below the surface stratum corneum, where most, if not all, of the particles remained, as evidenced by TEM examination, but the study was not designed to look for systemic effects from dermal exposure.

3.8. DERIVATION OF POTENTIAL ACCEPTABLE DAILY INTAKES (ADIs)

For inhalation exposure, a potential ADI is derived using the NOAEL of 133 µg Ag/cm^3 for liver and lung effects in Sprague-Dawley rats exposed for 90 days as the point of departure. An inhalation ADI for silver nanoparticles of 0.2 µg Ag/cm^3 is derived by dividing the duration-adjusted NOAEL (23.45 µg Ag/cm^3 = 133 µg Ag/cm^3 × 6 hours/24 hours × 5 days/7 days) by a total uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for protection of susceptible populations).

For oral exposure, a potential ADI is derived using the NOAEL of 125 mg/kg-day for liver and gastrointestinal effects in Sprague-Dawley rats exposed for 90 days (reported by Kim et al., 2010) as the point of departure. An oral ADI for silver nanoparticles of 1.2 mg/kg-day is derived by dividing the 125-mg/kg-day NOAEL by a total uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for protection of susceptible populations).

3.9. CONCLUSIONS

The available repeated exposure toxicity database for silver nanoparticles identifies mild liver and gastrointestinal effects with oral exposure and pulmonary inflammation and liver effects with inhalation exposure as potential health hazards in rats exposed to uncoated silver nanoparticles with average diameters of about 60 nm (oral) and 10–20 nm (inhalation). The available 28- and 90-day studies adequately describe dose-response relationships for these effects that can be used to derive potential ADIs for uncoated silver nanoparticles via oral and inhalation routes of exposure. No chronic toxicity testing for silver nanoparticles in animals is available, and toxicity from repeated dermal exposure of animals to silver nanoparticles has not been evaluated. There are several areas of uncertainty in extrapolating the observed effects in rats to possible human exposure scenarios.

(1) There is a lack of data to describe exposure-response relationships for pulmonary inflammation and liver effects from chronic inhalation exposure or liver and gastrointestinal effects from chronic oral exposure. The use of the derived ADIs for inhalation or oral exposure for humans with chronic (i.e., lifetime) exposure is based on an assumption that dose-response relationships for these effects from subchronic exposures will be similar to dose-response relationships from chronic exposures. NTP (2010) is currently planning chronic toxicity/carcinogenicity tests for silver nanoparticles.

(2) Results from in vitro toxicity tests suggest that mammalian cytotoxic responses to elemental silver nanoparticles are similar, although not identical, to responses to ionic silver, especially for silver nanoparticles with smaller average diameters (<~30 nm). Across a number of studies, ionic silver from either silver nitrate or silver acetate was consistently more potent than silver nanoparticles in inducing apoptosis and ROS (Foldbjerg et al., 2009) and reducing cell viability (Greulich et al., 2009; Kim et al., 2009b; Miura and Shinohara, 2009; Carlson et al., 2008), but in another comparison, silver carbonate was not as potent as silver nanoparticles (Kawata et al., 2009). In vitro results also indicate that toxic responses are dependent on particle physical and chemical characteristics (such as the particle size and presence of coatings). In cultured rat alveolar macrophages, 55-nm hydrocarbon-coated silver nanoparticles were less cytotoxic than 15- or 30-nm hydrocarbon-coated nanoparticles, which showed similar potency on a mass basis as ionic silver from silver nitrate (Carlson et al., 2008). In mouse spermatogonia cells, smaller size classes of silver nanoparticles (10- or 15-nm hydrocarbon-coated particles or 25- or 25-30 nm polysaccharide-coated particles) were more potent at reducing cell viability than larger size classes (80- or 130-nm particles) (Braydich-Stolle et al., 2010). Within the small size classes, polysaccharide-coated nanoparticles were more potent than hydrocarbon-coated nanoparticles (Braydich-Stoller et al., 2010), and similar results were reported in cultured mouse embryonic stem cells and fibroblasts (Ahamed et al., 2008). In both studies, the difference in potency was associated with a higher degree of agglomeration of the less potent hydrocarbon-coated particles. The in vitro results suggest that in vivo dose-response relationships for silver nanoparticles may be influenced by nanoparticle size and chemical characteristics. Thus, there is inherent uncertainty in the use of the proposed inhalation and oral ADIs, which are based on toxicity data for uncoated silver nanoparticles, to estimate health risks from

exposure to silver nanoparticles with different characteristics (e.g., surface coating). Further in vivo tests with other types of silver nanoparticles would increase confidence in deriving ADIs.

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4.0. TOXICITY DATA ON TITANIUM DIOXIDE NANOPARTICLES

4.1. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Overview: TiO_2 is highly insoluble in water and is generally regarded as an inert substance. As such, metabolic transformation of TiO_2 has not been a focus of research on TiO_2 nanoparticles, and the issue is not further discussed herein. Crystalline morphological forms of TiO_2 nanoparticles have been demonstrated to differ in optical and photocatalytic properties. For example, rutile, which is used in sunscreens, is considered to be a more inert morphological form than anatase, which is used in photocatalytic, catalytic, and sensing applications (Rossi et al., 2010). Whether or not metabolic transformations of TiO_2 morphological forms can occur in mammalian systems is unstudied.

Available results from animals studies indicate that, following inhalation exposure, TiO_2 nanoparticles deposited in the lung can be transported from airspace surfaces into the interstitium and lymph tissue, and cleared from the lung with slower kinetics than microsized TiO_2 particles (Geiser et al., 2008, 2005; Sager et al., 2008a, b; Bermudez et al., 2004, 2002; Oberdörster et al., 1994; Ferin et al., 1992). Mechanisms involved in lung clearance include macrophage engulfment and translocation to the gastrointestinal (GI) tract via mucociliary transport and swallowing, or potentially via lymph or blood circulation. Following inhalation exposure to TiO_2 nanoparticles, rats and mice display similar lung deposition burdens and lung clearance kinetics, whereas hamsters show smaller lung burdens and faster lung clearance kinetics (Bermudez et al., 2004, 2002). Translocation of inhaled and absorbed TiO_2 nanoparticles to nonrespiratory tissues may be limited, based on a report that titanium concentrations were not elevated in the liver, kidney, spleen, or the basal brain with olfactory bulb in rats exposed 6 hours/day for 5 days to aerosols made from TiO_2 nanoparticles at concentrations up to 50 mg/m^3 (Ma-Hock et al., 2009).

A report of elevated titanium tissue concentrations provides qualitative evidence of absorption and tissue distribution in mice exposed to very high (5 g/kg) single oral doses of suspensions of TiO_2 nanoparticles (Wang et al., 2007), but studies providing more quantitative data on the rate and extent of oral absorption or the route, rate, or extent of excretion were not located. Qualitative evidence of skin absorption and tissue distribution (e.g., elevated titanium concentrations in liver) has been reported in hairless mice exposed to suspensions of several types of TiO_2 nanoparticles for 60 days (Wu et al., 2009), but not in minipigs exposed to suspensions of several types of TiO_2 nanoparticles for 22 days (Sadrieh et al., 2010). Examinations of domestic pig or minipig skin samples by transmission electron microscopy (TEM) following in vitro or in vivo exposure of skin to suspensions of TiO_2 particles indicate that most particles were located as agglomerates in the outermost epidermal layer (stratum corneum) and that detection of isolated particles in the underlying dermis was infrequent (Sadrieh et al., 2010; Wu et al., 2009). Evidence of translocation of TiO_2 to brain regions has been reported from studies of mice given intranasal instillations of aqueous suspensions of rutile or anatase nanoparticles (Wang et al., 2008a, b) or abdominal injections of suspensions of anatase nanoparticles (Ma et al., 2010). The relevance of these observations to environmentally pertinent oral or inhalation routes of exposure is uncertain.

The following sections describe in more detail findings from animal studies of the absorption, distribution, and excretion of TiO_2 nanoparticles following inhalation, oral, dermal, and other routes of exposure.

4.1.1. Inhalation: Absorption, Distribution, and Excretion

General Considerations for Inhaled Nanoparticles: In contrast to fine particles with diameters in the 1–2.5 μm range, which are deposited mainly in the peripheral lung, inhaled nanoparticles (with at least one dimension <100 nm) can be deposited in the oral and nasal cavities, the tracheal/bronchiole region of the lung, and the alveolar region of the lung (Kreyling et al., 2002). Nanoparticles deposited in the alveolar region of the respiratory tract may be cleared from the alveolar region by: (1) macrophage phagocytosis and mucociliary transport along the tracheobronchial tree to the GI tract, (2) translocation into interstitial tissue, (3) translocation to the lymphatic system, (4) particle dissolution with subsequent absorption into lung cells and transport into the blood (a very limited process for TiO_2 particles given the insolubility of TiO_2 in aqueous media), and (5) translocation of the particles into lung cells from lung surfaces and possible transport into the blood (Geiser et al., 2008; Kreyling et al., 2002; Oberdörster, 1988). Clearance from tracheal/bronchiole regions may occur by similar pathways. Nanoparticles deposited in the nasal mucosa also may be subject to particle dissolution and absorption into the blood (again, not likely with insoluble TiO_2) or direct translocation into the olfactory bulb of the brain via the olfactory nerve; however, translocation of nanoparticles from the olfactory bulb to other brain regions is poorly studied (Oberdörster et al., 2004).

Absorption Measurements for Inhaled TiO_2 Nanoparticles: Approximately 20% of TiO_2 nanoparticles detected by energy-filtering TEM in alveolar lung regions were located within and beyond the epithelia immediately, or 24 hours, following 1-hour exposure of male WKY/NCRl BR rats to a TiO_2 concentration of 0.11 mg/m^3, corresponding to a mean number concentration of 7.3×10^6 (standard deviation [SD] = 0.5×10^6) particles/cm^3 (Geiser et al., 2005). The remaining percentage of detected particles was located on the luminal side of the epithelium. On average, 79.3 ± 7.6% (mean ± SD) of the particles were found on the luminal side of airways and alveoli, 4.6 ± 2.5% were within epithelial or endothelial cells, 4.8 ± 4.5% were within the connective tissue, and 11.3 ± 3.9% were within the capillaries. TiO_2 nanoparticles (crystalline form not specified) were generated by spark generation and conditioned for inhalation, producing aerosols with a count median diameter (CMD) of 22 nm (geometric standard deviation [GSD] 1.7). Using the conversion equation in U.S. Environmental Protection Agency (U.S. EPA) (1994), this CMD value corresponds to an estimated mass median aerodynamic diameter (MMAD) of about 0.1 μm1. Particle size distribution and number concentration were monitored by a differential electrical mobility particle sizer and a condensation particle counter. The 22-nm aerosol particles were agglomerates of smaller primary particles with estimated diameters of 4 nm. Anesthesized rats were exposed in a plethysmograph box via an endotracheal tube by negative pressure ventilation at a breathing frequency of 45/minute. Particles detected within the alveolar epithelium barrier were located within the epithelial endothelium, connective tissue, and capillary lumen. The methods employed in this study did not allow determination of the percentage of particles that entered general circulation via the lung microvasculature, but the

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1MMAD = $\rho^{0.5}$ CMD exp (3[ln GSD]^2) = $2 \times 22 \times 2.3272 = 102$ nm = 0.1 μm, assuming a particle density (ρ) of 4 g/cm^3; ρ rutile TiO_2 = 4.26 g/cm^3; anatase TiO_2 = 3.84 g/cm^3.

observation of particles in alveolar capillaries provides qualitative evidence that translocation to the blood occurred. A subsequent analysis of the data, which related the number of particles in the various lung compartments with compartment size, indicated that particles were preferentially translocated to connective tissues immediately after exposure and to the capillary lumen at 24 hours after exposure (Muhlfeld et al., 2007).

Particles detected within cells were not membrane bound, suggesting that the particles entered cells via a nonendocytotic mechanism (Geiser et al., 2005). This observation was contrary to other observations of membrane-bound agglomerations of TiO_2 particles within human epithelial A549 cells exposed for 6 or 24 hours to 40 µg/mL TiO_2 nanoparticles in the culture medium (reported average diameter of primary particles ≈50 nm; crystal structure not specified) (Stearns et al., 2001). A possible explanation of this difference is that absorption of highly aggregated, poorly soluble nanoparticles occurs via endocytotic mechanisms at high concentrations in polar liquids such as cell culture medium, whereas nonendocytotic mechanisms of transport across cellular membranes operate at inhalation conditions that limit the extent of agglomeration. The in vivo results suggest that, under the low-concentration, low-agglomeration conditions of the study by Geiser et al. (2005), at least 20% of poorly soluble TiO_2 nanoparticles deposited in the peripheral lung are absorbed into lung tissue via a mechanism that may not involve endocytosis. Other studies of the extent or rate of respiratory tract absorption of inhaled TiO_2 nanoparticles were not located.

Absorption of TiO_2 by pulmonary macrophages was studied by Rossi et al. (2010). Agglomerations (typically >100 nm in size) of TiO_2 particles were observed by light microscopy and TEM in membrane-bound organelles (phagosomes) within pulmonary macrophages of BALB/c/Sca mice after exposure to 10 mg/m^3 aerosolized uncoated TiO_2 nanoparticles for 2 hours/day, 4 days/week for 4 weeks (Rossi et al., 2010). Uncoated TiO_2 test primary nanoparticles ranged in size from 25 to 40 nm (with a general spherical shape) and included anatase, rutile + anatase (9:1) and anatase + brookite (3:1). The MMADs for the aerosols generated in this study were not reported, but the aerosol aerodynamic size distribution range was reported to be 15.9 nm to 10 µm. Most aerosol particles were reported to show mass median diameters in the µm size. Containment of the particles within organelles suggests that macrophages may have absorped the deposited particles via an endocytic mechanism. In contrast, following exposure to aerosols made from silicon dioxide (SiO_2)-coated rutile TiO_2 nanoparticles, most particles were observed in the macrophage cytosol, and phagosomes were absent. This observation was interpreted to suggest that the phagosomes were destroyed and the absorbed nanoparticles were released to the macrophage cytosol. This interpretation is supported by observations that the SiO_2-coated rutile TiO_2 nanoparticles were the only test material in this study that induced obvious pulmonary inflammation.

Lung Deposition and Clearance Kinetics: No studies were located examining lung deposition and clearance kinetics following exposure to nanosized aerosols of TiO_2. Several studies have compared lung deposition and clearance in animals exposed by inhalation to aerosols made from TiO_2 "fine" particles with diameters >100 nm and "ultrafine" (i.e., nano) particles with diameters <100 nm (Bermudez et al., 2004, 2002; Oberdörster et al., 1994; Ferin et al., 1992). However, regardless of particle size of the starting materials, the MMADs of aerosols used in these studies (about 0.7–1.4 µm) were similar and outside of the nanoparticle size range.

Results from these studies indicate that exposure to microsized aerosols made from nanoparticles results in similar lung deposition burdens, but slower lung clearance kinetics, compared with aerosols made from fine TiO_2 particles. The slower lung clearance kinetics were associated with relatively greater translocation to interstitial spaces and lymph nodes. Thus, particle size of the starting material affected lung clearance kinetics, even when the aerosols generated from the different sized starting materials were of similar, non-nanosized. Species comparisons in these studies found that rats and mice have similar lung deposition and lung clearance kinetics (regardless of particle size), whereas hamsters showed smaller lung burdens and faster lung clearance kinetics than rats and mice at equivalent exposure levels.

Bermudez et al. (2004, 2002) exposed female CDF(F344)/CrlBR rats, B3C3F1/CrlBr mice, and Lak:LVG(SYR) BR hamsters (6 hours/day, 5 days/week for 13 weeks) to 0.5, 2.0, or 10 mg/m^3 of aerosols of TiO_2 nanoparticles (average diameter = 21 nm; crystal) with MMADs of 1.29–1.45 µm and GSDs of 2.46–3.65 (Bermudez et al., 2004) or to 10, 50, or 250 mg/m^3 of aerosols of fine ("pigmentary") particles (rutile; primary particle diameter <1 µm) with similar respective MMADs (1.36–1.44 µm) and GSDs (1.50–1.72) (Bermudez et al., 2002). The test material in the Bermudez et al. (2004) studies was "P25" obtained from Degussa-Huls AG, Frankfort, Germany, which is typically 80% anatase: 20% rutile. Ferin et al. (1992; some results from this study were also reported by Oberdörster et al., 1994) exposed male F344 rats (6 hours/day, 5 days/week for 12 weeks) to 23.5 mg/m^3 of aerosols of anatase TiO_2 nanoparticles (average diameter = 21 nm; MMAD = 0.78 µm; GSD = 1.7) or to 23.0 mg/m^3 fine particles (average diameter = 250 nm; MMAD = 0.71 µm; GSD = 1.9). Although MMADs for aerosols generated from nano- and microsized TiO_2 particles were within the same range, key findings from the lung deposition and clearance studies indicate that differential effects were observed for the two particle types:

(1) Immediately following 12–13 weeks of exposure to similarly sized aerosols, rats exposed to aerosols generated from nanoparticles (MMAD = 0.78 µm) at up to 10 mg/m^3 or aerosols generated from fine particles (MMAD = 0.71 µm) at up to 250 mg/m^3 showed similar TiO_2 lung burdens, but total lung clearance during 64 post-exposure weeks was slower for nanoparticles ($t_{1/2}$ = 501 days), compared with fine particles ($t_{1/2}$ = 174 days), and a greater proportion of the TiO_2 mass in the lungs was in the hilar lymph nodes immediately following nanoparticle exposure (6.8%), compared with fine particles (0.4%) (Oberdörster et al., 1994; Ferin et al., 1992). For both particle types, the lavageable TiO_2 content was cleared from the alveolar space with similar kinetics; 93% of the lavageable TiO_2 mass present at the end of exposure was cleared from the alveolar space within 1 year post-exposure. For nanoparticles, a large fraction (44%) of the cleared alveolar mass appeared in the interstitial space within this post-exposure period. A smaller fraction appeared in the interstitial space (13%) following exposure to fine particle aerosols, which were presumably cleared more predominantly via the GI tract.

(2) Immediately following 13 weeks of exposure of rats, mice, or hamsters to aerosols made from nanoparticles (MMAD = 1.29–1.45 µm), exposure-dependent TiO_2 mass lung burdens were similar between rats and mice, but lung burdens were approximately two- to fivefold lower in hamsters (Bermudez et al., 2004). Lung burdens decreased in all three species during 52 weeks without exposure; 1 year following exposure to the highest

concentration (10 mg/m^3), retained TiO_2 mass burdens in the lungs represented about 57, 46, and 3% of the initial burdens in rats, mice, and hamsters, respectively. Respective total lung clearance half-times for the 0.5, 2, and 10 mg/m^3 exposure groups were 63, 132, and 395 days for rats; 48, 40, and 319 days for mice; and 33, 37, and 39 days for hamsters. Similar species differences in lung deposition and lung clearance kinetics were noted with aerosols made from fine TiO_2 particles (Bermudez et al., 2002). For example, following exposure to 10 mg/m^3 of these aerosols for 13 weeks, lung retention half-times during a 52-week post-exposure period were about 50 days for mice and 100 days for rats. Faster lung clearance of TiO_2 from "fine-particle" aerosols is indicated by the shorter lung clearance half-time in rats exposed to "fine-particle" aerosols at 10 mg/m^3 (100 days), compared with rats exposed to "nano-particle" aerosols at 10 mg/m^3 (395 days).

A few observations in rats following inhalation exposure to aerosols of TiO_2 nanoparticles suggest that macrophage-mediated clearance of TiO_2 nanoparticles from the alveolar region and from the lung may be dependent on exposure concentration, the degree to which inhaled aerosols are agglomerated, or lung burden, but current understanding is insufficient to describe the relative importance of these variables on macrophage-mediated clearance of TiO_2 nanoparticles from the lung. Examination of lung surface macrophages by energy-filtering TEM indicated very limited uptake of TiO_2 nanoparticles into macrophages following a 1-hour exposure of male WKY/KyoRj rats to 0.11 mg/m^3 TiO_2 nanoparticle aerosols with a 20-nm CMD (GSD 1.7; estimated approximate MMAD = 0.1 µm) (Geiser et al., 2008). Particles were detected in only 3/1,594 and 27/1,609 examined lung surface macrophages immediately after exposure and 24 hours after exposure, respectively. In contrast, following exposure of female BALB/c/Sca mice to a much higher concentration, 10 mg/m^3, of aerosols generated from TiO_2 nanoparticles of varying sizes (10–40 nm), shapes (spherical or needle-shaped), coatings (uncoated or coated with SiO_2), and crystalline structure (rutile, anatase, and/or brookite) for 2 hours/day, 4 days/week for 4 weeks, light microscopy and TEM showed that particles mainly accumulated in pulmonary macrophages and not in surrounding lung tissue and that the particles were agglomerated within phagosomes (Rossi et al., 2010). The report by Rossi et al. (2010) provided no indication that the different types of TiO_2 nanoparticles showed different degrees of loading of macrophages, but did indicate that following exposure to a SiO_2-coated TiO_2 nanoparticle, which displayed greater toxicity than the other tested TiO_2 nanoparticles (see Section 4.2.1), nanoparticles in macrophages were in the cytosol and phagosomes were disintegrated. The factors contributing to the apparent differences (i.e., minimal [Geiser et al., 2008] versus considerable [Rossi et al., 2010] loading of macrophages with particles) in these observations are unknown, but could include different exposure concentrations and durations, size of the aerosols, or surface characteristics of the particles.

Tissue Distribution Following Inhalation Exposure: No evidence for translocation of TiO_2 to the liver, kidney, spleen, or the basal brain with olfactory bulb was found in a study of male Wistar rats (three animals per group) exposed for 6 hours/day for 5 days to aerosols made from TiO_2 nanoparticles at concentrations of 2, 10, or 50 mg/m^3 (Ma-Hock et al., 2009). Dust aerosols in this study were made by dispersing uncoated TiO_2 primary particles with average diameters of 25.1 ± 8.2 nm; MMADs were between 0.7 and 1.1 µm, and GSDs were between 2.3 and 3.4. Due to agglomerates generated during the aerosolization process, the number

concentration of particles <100 nm in size was only a small fraction (0.1–0.4%) of the total particle mass at each exposure concentration. TiO_2 nanoparticles were characterized as 86% anatase/14% rutile, had an uncoated hydrophobic surface, ranged from 13 to 71 nm in size, and had a surface area of 51.1 ± 0.2 m^2/g. Thus, the aerosols were similar to those used in the studies by Bermudez et al. (2004) and Ferin et al. (1992). Mean TiO_2 mass in lung tissue increased with increasing exposure concentration, and during a 16-day post-exposure period, lung burdens decreased to about 74–82% of values immediately after exposure. TiO_2 was detected in mediastinal lymph nodes of rats exposed to the highest concentration only; the mean amount detected 16 days after exposure was about fivefold higher than the amount immediately after exposure. The increase in lymph node content during the post-exposure period suggests that translocation to the lymph nodes contributed to the lung clearance. In liver, kidney, spleen, and basal brain samples (the only nonrespiratory tissues sampled), TiO_2 could not be detected by inductively coupled plasma-atomic emission spectrometry (ICP-AES; detection limit = 0.5 µg TiO_2 per organ). Within the detection limits of the analytical methods used, the results suggest that clearance of TiO_2 from the lung in the post-exposure period may have occurred predominantly via macrophage-mediated mucociliary transport to the GI or via the lymph system without considerable translocation to the nonrespiratory tract tissues examined. However, fecal samples were not collected and analyzed for TiO_2 contents to examine the mucociliary/ GI transport pathway.

In another study by the same researchers, titanium levels were below the detection limit in liver, kidney, spleen, and basal brain with olfactory bulb from male Wistar rats (three per group) exposed (head/nose) to aerosols of TiO_2 nanoparticles at a concentration of 100 mg/m^3, 6 hours/day for 5 days (van Ravenzwaay et al., 2009). The aerosols were prepared from uncoated nanoparticles (70% anatase: 30% rutile) in the 20–30 nm size range. MMADs were reported to be between 1 and 1.2 µm (GSDs were not reported). TiO_2 content of tissue digest were detected by ICP-AES (detection limit = 0.5 µg TiO_2 per organ). The mean TiO_2 lung concentration was 2,025 µg/lung at the end of exposure and declined to 1,547 µg/lung 14 days later. Concentrations in lymph nodes at these respective timepoints were 2.2 and 8.5 µg/lymph, indicating clearance from the lung to the lymph node. The results indicate that following inhalation exposure to a high concentration of agglomerated aerosols made from uncoated TiO_2 nanoparticles, translocation to nonportal-of-entry tissues is limited. Following intravenous (i.v.) injection of a suspension of the test material (5 mg/kg), TiO_2 was detected mainly in the liver and spleen on days 1, 14, and 28 following injection; mean concentrations ranged across these sampling dates from 99.5 to 133.8 µg/g wet weight in liver and from 33.3 to 78.7 µg/g wet weight in spleen (van Ravenzwaay et al., 2009). Concentrations in lung (2.3–8.8 µg/g wet weight) and kidney (below the level of detection to 0.67 µg/g wet weight) were much lower. The i.v.-administration results suggest that TiO_2 nanoparticles reaching the blood may principally distribute to the liver and spleen.

4.1.2. Oral: Absorption, Distribution, and Excretion

Providing qualitative evidence of oral absorption and distribution to tissues, elevated titanium concentrations in several tissues were reported to have occurred in CD-1 (ICR) mice 2 weeks after administration of single nonlethal 5 g/kg doses of TiO_2 suspensions (Wang et al., 2007). The suspensions contained 3 g of one of several commercial TiO_2 powders (purity >92%;

crystalline form not specified) in 12 mL of 0.5% hydroxypropylmethylcellulose (HPMC) in water: 25- and 80-nm TiO_2 powders (Hangzhou Dayang Nanotechnology Co., China) and fine (155 ± 33 nm) TiO_2 powders (Zhongliam Chemical Medicine Co., China). Titanium concentrations in tissue samples (ng Ti/g tissue) were determined by ICP-mass spectrometry (MS). Mean titanium concentrations in spleen and brain, but not red blood cells (RBCs), were significantly ($p < 0.05$) elevated by approximately two- to threefold in mice exposed to the 25-nm, 80-nm, or fine test materials, compared with mean values in vehicle-exposed control mice 2 weeks post-exposure. Significantly elevated concentrations of a similar magnitude were also found in the kidney of 25- and 80-nm mice and in the lungs of 80-nm mice. In mice exposed to the 25-nm and fine test materials, mean liver concentrations (about 106 ng/g) were not significantly different from control values (about 90 ng/g), but the mean liver concentration in 80-nm mice was about 40-fold higher (3,970 ng/g) than the control value. An explanation for the high hepatic accumulation of titanium following exposure to the 80-nm material (and not after exposure to the other test materials) was not postulated by the authors.

Other studies providing more quantitative data on the rate and extent of oral absorption of TiO_2 nanoparticles or the route, rate, or extent of excretion were not located.

4.1.3. Dermal: Absorption, Distribution, and Excretion

Results from a study of minipigs dermally exposed 5 days/week for about 4 weeks to creams containing TiO_2 indicate that dermal penetration of TiO_2 nanoparticles was very limited (Sadrieh et al., 2010). The creams (formulated as typical sunscreen creams) contained TiO_2 particles of three types at similar concentrations (about 5–6% TiO_2 by weight): sub-µm particles (300–500 nm diameter; 33.7 mg Ti/g cream), uncoated nanoparticles (30–50 nm diameter; 36.6 mg Ti/g), and coated nanoparticles (aluminum hydroxide/dimethicone copolymer coating; 20–30 nm diameter; 28.5 mg/g). Creams were topically applied to female Yucatan minipigs (3/group) at 2 mg cream/cm^2 skin (4 applications/day, 5 days/week, for a total of 22 application days) covering the entire dorsal surface and ventral abdomen ending at the base of the tail. Each pig received about 1.3 L of cream over the 22 application days. Mean titanium concentrations in lymph nodes and liver (determined by ICP-MS) of TiO_2-exposed pigs were not significantly ($p > 0.05$) different from mean concentrations in vehicle control pigs. Regardless of particle type, titanium concentrations in the epidermis were very much higher than concentrations in the underlying dermis (e.g., about 11 and 0.026 mg/g in epidermis and dermis, respectively, in pigs exposed to the sub-µm cream). Significantly ($p < 0.05$) elevated titanium concentrations in dermis samples (compared with control means) were detected in abdominal and neck dermis from pigs exposed to uncoated and coated nanoparticles and in the left inguinal dermis in pigs exposed to sub-µm particles. The authors suspected that minor contamination of dermis samples with epidermis could explain the elevated titanium concentrations in the dermis samples. TEM examination of skin samples provided a qualitative description of the distribution of particles within the skin. All three types of TiO_2 particles were primarily found as highly aggregated particles in the stratum corneum, with only a few isolated particles observed in lower skin layers. No pattern was apparent, indicative of translocation into the lower layers via follicular lumens. In summary, penetration of repeatedly applied TiO_2 sub-µm or nanoparticles in creams was insufficient to detect elevated titanium concentrations in two sentinel organs, the lymph nodes and the liver. TEM examination and examination of titanium concentrations in epidermis and

dermis samples indicated that only a very small fraction of 1% of TiO_2 particles leaves the upper layers of the epidermis.

Corroborative findings for very limited dermal penetration of TiO_2 nanoparticles come from a study of domestic pigs dermally exposed to a test formulation containing 5% (w/w) TiO_2 nanoparticles with average diameters of 4 nm (anatase) or 60 nm (rutile) (Wu et al., 2009). About 24 mg of the test formulation (5% TiO_2 in 2% carbopol 940 and triethanolamine in water or vehicle alone in control pigs) was applied daily to a 3-cm^2 area of the dorsal surface of the right ear of three pigs (3 week old at start) for 30 consecutive days. Exposure to either type of nanoparticle did not cause erythema or edema of the treated area. TEM examination of sections of punch biopsies showed particles only in epidermal layers (the stratum corneum, stratum granulosum, prickle cell layer, and basal cell layers [the latter only with exposure to the 4-nm nanoparticles]), and not in the underlying dermis. In companion in vitro studies with isolated pig ear skin samples mounted in a modified Franz apparatus containing 5% (w/w) suspensions of various TiO_2 nanoparticles in aqueous solutions containing 20% caprylic/capric triglyceride and 1% Tween, no titanium was detected in the receptor fluid by atomic absorption spectrometry following 24 hours of exposure. Tested materials included anatase TiO_2 particles with 4 and 10 nm average diameters; 25, 60, or 90 nm rutile TiO_2; and Degussa P25 (25% rutile/ 75% anatase; 21 nm diameter).

Evidence for dermal penetration and distribution of TiO_2 nanoparticles to nondermal tissues has been presented for hairless BALB/c *nu/nu* mice dermally exposed for 60 days to aqueous suspensions of TiO_2 particles of varying types: 4- and 10-nm anatase; 25- and 60-nm rutile; 21-nm Degussa P25 (75% anatase/25% rutile); and a 300–500-nm TiO_2 powder (Wu et al., 2009). Twenty-four mg of the suspensions (5% TiO_2 in 2% carbopol 940 and triethanolamine in water) were applied daily (n = 3/sex per group) to an uncovered 3-cm^2 area of the dorsal interscapular skin protected with a plastic collar (8 mg suspensions/cm^2 or 400 µg TiO_2/cm^2) for 60 consecutive days. After daily 3-hour exposures, the exposed skin was washed with water and dried. At sacrifice, subcutaneous muscle, liver, heart, spleen, kidneys, brain, and lung were removed, and samples were prepared for histopathology and titanium content by flame atomic absorption spectrometry. In all groups exposed to TiO_2 nanoparticles (but not in the group exposed to 300–500 nm TiO_2), mean titanium concentrations in heart, liver, and spleen, but not in the kidney or brain, were reported to be elevated, compared with control means. Compared with control means, titanium concentrations were elevated by 2.6–3.6-fold in heart, 12.7–20.2-fold in liver, and 1.9–2.2-fold in spleen of mice exposed to TiO_2 nanoparticles (see Table 4.1). The elevated concentrations in these tissues indicate that dermal penetration and distribution to these tissues occurred in hairless mice. This finding is contrasted by failure of particles to penetrate to the dermis of domestic pigs in vivo or in vitro in other experiments by Wu et al. (2009) and the lack of elevated titanium concentrations in nondermal tissues (lymph and liver) in minipigs exposed to several types of TiO_2 for 22 days (Sadrieh et al., 2010). At least some of these differences may be attributed to the models utilized in these studies. The study by Wu et al. (2009) suggests that the porcine in vivo skin model is a more appropriate vehicle for penetration studies than the in vitro model. Furthermore, hairless mice possess a stratum corneum that is less than one-half the thickness of human skin; therefore, lower barrier properties in this animal model may serve to amplify the penetration of nanoparticles.

Table 4.1. Mean titanium concentrations in selected tissues of hairless BALB/c *nu/nu* mice (3/sex/group) dermally exposed to fine TiO_2 and several types of TiO_2 nanoparticles for 60 days (400 µg TiO_2/cm^2 on 3 cm^2 interscapular skin, 3 hours/day)[a]

Tissue	Treatment	Titanium concentration (µg Ti/g)	SD	Fold increase compared with control mean
Heart	Control	3.8	0.9	–
	10 nm	11.7	2.4	3.1
	25 nm	9.8	4.2	2.6
	Degussa P25	13.5	2.8	3.6
	60 nm	10.7	1.4	2.8
Liver	Control	1.2	0.5	–
	10 nm	25.1	8.4	20.2
	25 nm	15.7	5.2	12.7
	Degussa P25	19.4	4.7	15.7
	60 nm	17.5	7.0	14.1
Spleen	Control	5.9	2.7	–
	10 nm	22.6	7.8	3.8
	25 nm	11.0	3.1	1.9
	Degussa P25	12.7	2.5	2.1
	60 nm	13.1	2.7	2.2
Lung	Control	4.0	1.4	–
	10 nm	5.4	1.6	1.4
	25 nm	2.1	0.4	0.5
	Degussa P25	13.6	4.3	3.4
	60 nm	8.3	2.1	2.1

[a]Means and SDs from graphically presented data in Figure 3 were digitized to obtain values in table.

Source: Wu et al. (2009).

In contrast, using nuclear microprobe analysis, titanium signals were only recorded in the outer skin layers, and not in layers >50 µm below the surface, following occluded exposure of human skin transplanted in SCID mice to a commercially available TiO_2-containing sunscreen product for 24 hours (Kiss et al., 2008). This finding indicates very limited penetration of titanium through the skin under the provided conditions (2 mg product/cm^2 skin area; the total treated area of the skin was not reported).

No data were located regarding the route, rate, or extent of excretion of absorbed TiO_2 nanoparticles following dermal exposure.

4.1.4. Other Routes: Absorption, Distribution, and Excretion

Results from intratracheal instillation studies in rats suggest that TiO_2 nanoparticles deposited in the lungs are transported into the interstitium and lymph nodes to a greater degree than larger "fine" TiO_2 particles (Sager, 2008a; Ferin et al., 1992). Following exposure in these studies, excised lungs were lavaged to remove surface fluids and cells, and titanium or TiO_2 concentrations were determined in the collected fluid and lavaged lung tissues. Nonlavagable titanium mass in the lung tissue was interpreted to represent deposited particles translocated to the interstitium. One day following intratracheal instillation of equivalent masses (500 µg) of

TiO_2 nanoparticles (21 nm primary particle diameter) or fine particles (250 nm) in F344 rats, a greater percentage of titanium mass was nonlavagable in nanoparticle-exposed lungs (30%), compared with fine particle-exposed lungs (19%) (Ferin et al., 1992). In another study in which F344/DuCrl rats were instilled with 0.52 mg TiO_2 nanoparticles (primary particle diameter = 21 nm; 80:20 [w/w] anatase:rutile) or 10.7 mg fine particles (primary particle diameter = 1,000 nm; 100% rutile), 81% of TiO_2 mass was nonlavagable in nanoparticle-exposed lungs 7 days after exposure, compared with 9% in fine particle-exposed lungs (Sager et al., 2008a). TiO_2 masses in tracheobronchial and thymic lymph nodes at 1, 7, and 42 days after exposure were also determined in this study. TiO_2 mass in collected lymph nodes increased by 246% from post-exposure days 7 to 42 following exposure to nanoparticles, compared with 134% for the same period following exposure to fine particles (Sager et al., 2008a). Prior to intratracheal instillation, Sager et al. (2008a) suspended the particles in rat bronchoalveolar lavage (BAL) fluid to achieve better dispersion of both types of particles.

Increased titanium concentrations in several brain regions have been measured in CD-1(ICR) female mice following intranasal instillation of aqueous suspensions of two types of TiO_2 nanoparticles: 100% rutile and 100% anatase with average primary particle sizes of about 80 and 155 nm, respectively (Wang et al., 2008a, b). Particles were suspended in water, and 500 µg TiO_2 per rat was intranasally instilled with a syringe every other day for a total of 15 applications. No characterization of the degree of agglomeration in the suspensions was reported. Mice were sacrificed 24 hours after 1, 5, 10, and 15 applications (n = 6 per time point per group, including a vehicle-exposed control group). Brain tissues were dissected (olfactory bulb, cerebral cortex, hippocampus, and cerebellum) and prepared for determination of titanium content by ICP-MS. For each timepoint, mean titanium concentrations were significantly ($p < 0.05$) elevated in most brain compartments in exposed mice, compared with vehicle-control values. At the last timepoint, when maximal concentrations occurred in most compartments, concentrations showed the following order for both types of particles (respective fold increases, compared with control values, are indicated in parentheses for 80- and 155-nm particles): hippocampus (5.3, 4.0) > olfactory bulb (3.8, 3.5) > cerebellum (2.5, 3) > cerebral cortex (3, 4). The data are consistent with the possible transport of particles deposited on the nasal olfactory epithelium to the brain via the olfactory nerve, but the relevance of this pathway to inhalation of aerosols of TiO_2 nanoparticles is uncertain.

Increased brain titanium concentrations have also been observed in CD-1 (ICR) mice following 14 daily abdominal injections of 5, 10, 50, 100, or 150 mg/kg-day TiO_2 nanoparticles (5 nm anatase particles suspended in 0.5% hydroxypropylcellulose K4M in water) (Ma et al., 2010). Agglomeration of the particles in the suspension was not characterized. Mean brain titanium concentrations increased with increasing dose levels as follows (titanium was not detected in brains from vehicle controls): 108, 132, 196, 289, and 500 ng titanium/g brain. The mean brain titanium concentration in mice exposed to 150 mg/kg-day bulk TiO_2 (average grain size = 10–15 µm) for 14 days was 349 ng titanium/g brain. The results suggest that abdominally injected TiO_2 particles are transported by some unknown mechanism to the brain. The relevance of this observation to oral exposures to TiO_2 nanoparticles is uncertain.

4.2. ACUTE TOXICITY

4.2.1. Acute/Short-Term Inhalation Toxicity

Overview: Signs of pulmonary inflammation have been observed in several studies of animals exposed for short-term durations to aerosols made from different crystalline forms of TiO_2 nanoparticles (Rossi et al., 2010; Ma-Hock et al., 2009; van Ravenzwaay et al., 2009; Grassian et al., 2007a, b). Aerosols were nanoparticle agglomerations in the 100–200 nm size range in studies by Rossi et al. (2010) and Grassian et al. (2007a, b) and in the μm size range in other studies (Ma-Hock et al., 2009; van Ravenzwaay et al., 2009). Signs of pulmonary inflammation were observed in C57B1/6 mice exposed for 4 hours/day for 10 days to 8.9 mg/m^3 or for 4 hours to 7.2 mg/m^3, but not 0.7 mg/m^3, uncoated anatase TiO_2 nanoparticles (Grassian et al., 2007a); in C57B1/6 mice exposed for 4 hours to 0.6 or 7.16 mg/m^3 uncoated anatase/rutile TiO_2 nanoparticles (Grassian et al., 2007b); in male Wistar rats exposed to 88 mg/m^3 uncoated 70% anatase:30% rutile TiO_2 nanoparticles for 6 hours/day for 5 days (van Ravenzwaay et al., 2009); in male Wistar rats exposed to concentrations as low as 2 mg/m^3 uncoated 86% anatase:14% rutile TiO_2 nanoparticles for 6 hours/day for 5 days (Ma-Hock et al., 2009); and in BALB/c/Sca mice exposed to 10 mg/m^3 SiO_2-coated rutile TiO_2 nanoparticles 2 hours/day for 4 days or 4 days/week for 4 weeks (Rossi et al., 2010). The last of these studies provides evidence that pulmonary inflammation can be influenced by coatings applied to nanoparticles. In contrast to positive findings from repeated exposure to aerosols of SiO_2-coated rutile TiO_2 nanoparticles, Rossi et al. (2010) found no evidence for pulmonary inflammation in BALB/c/Sca mice following single or repeated exposure to 10 mg/m^3 concentrations of aerosols made from uncoated anatase, uncoated rutile/anatase (9:1), or uncoated anatase/brookite (3:1) nanoparticles. Impairment of endothelium-dependent dilation in systemic arterioles has been reported in male Sprague-Dawley rats exposed for 4–12 hours to aerosols made from fine rutile TiO_2 particles or uncoated nanosized 80% anatase:20% rutile particles at concentrations ranging from about 1.5 to 20 mg/m^3 (Nurkiewicz et al., 2009; 2008). Exposure conditions in these studies did not produce signs of pulmonary inflammation, and TiO_2 nanoparticles, at equivalent lung burdens, produced more severe microvascular dysfunction than TiO_2 fine particles (Nurkiewicz et al., 2008). In summary, the results from short-term inhalation exposure studies in rodents identify pulmonary inflammation and microvascular dysfunction in systemic arterioles as effects of concern from TiO_2 nanoparticles.

C57B1/6 mice were whole-body exposed to anatase TiO_2 nanoparticles as an aerosol at 0.8 or 7.2 mg/m^3 for 4 hours (6 males/group) or 8.9 mg/m^3 for 4 hours/day for 10 days (24 males/group) (Grassian et al., 2007a). Sentinel mice and mice exposed to nebulized water (6 males/group) served as controls. The average measured size and surface area of the TiO_2 particles used as the starting material were 3.5 ± 1.0 nm (range 2–5 nm) and 219 ± 3 m^2/g, respectively. Surface analyses indicated the presence of oxygen atoms and OH groups on the surface of the TiO_2 particles. To produce the aerosols, the TiO_2 powder was suspended in water and passed through a six-jet Collision nebulizer. Water was removed (by drying) from the nebulized droplets to produce a dried powder aerosol. Analyses of resulting TiO_2 aerosols showed marked aggregation, with geometric means ranging from 120 to 128 nm (GSDs ranged from 1.6 to 1.7). These analyses also detected the presence of small amounts of impurities in the water used to generate TiO_2 nanoparticle aerosols (not quantified). Body weights were recorded

pre-exposure and at necropsy; groups of mice were subjected to necropsy immediately after exposure (acute and subacute studies) and on weeks 1, 2, and 3 post-exposure (10-day exposures). BAL fluid from sacrificed animals was collected and used to assess total and differential cell counts, total protein content, and the levels of cytokines (including IFN-γ, IL-6, and IL-1β after 10-day exposures only) in the supernatant. Lung tissues were examined histopathologically using light microscopy.

No adverse effects associated with single exposures to the low concentration of TiO_2 aerosols were reported (Grassian et al., 2007a). In mice exposed to the high concentration for 4 hours, numbers of total cells and macrophages in the BAL fluid immediately after exposure were significantly increased with respect to controls ($p < 0.05$); other BAL parameters (number of neutrophils, total protein content, and lactate dehydrogenase [LDH] activity) and histopathological findings were similar to controls. Mice exposed for 10 days showed no clinical signs of toxicity and exhibited weight gains within the normal range. The number of alveolar macrophages (AMs) in the BAL fluid of mice exposed for 10 days increased significantly at weeks 1 and 2 post-exposure with respect to sentinels, but showed recovery by week 3 post-exposure (data not shown). No other BAL parameters (including total cell counts, neutrophil or lymphocyte counts, total protein content, LDH activity, or cytokine levels) varied significantly from control values in the mice exposed for 10 days. Alveolar macrophages in the lung tissue of treated mice were laden with particles (in the absence of other pathologic abnormalities). A decrease in the particle content of AMs isolated from BAL fluid from week 0 to 3 post-exposure was noted. The authors stated that increased numbers of particle-laden AMs in the lungs of treated rats were indicative of a modest but significant inflammatory response. The results indicate that the 0.8 and 7.2 mg/m^3 concentrations of anatase TiO_2 nanoparticles were the no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) values, respectively, for pulmonary inflammation (increased number of AMs in BAL fluid) for single 4-hour exposures. With 10 4-hour exposures, the only tested concentration, 8.9 mg/m^3, was a LOAEL for pulmonary inflammation (increased number of AMs without changes in other BAL fluid variables such as LDH activity or cytokine levels).

In a companion study, Grassian et al. (2007b) reported that significantly ($p < 0.05$) increased numbers of macrophages in BAL fluid were found immediately following whole-body exposure of male C57B1/6 mice for 4 hours to 0.612 or 7.16 mg/m^3 concentrations of aerosols made from uncoated anatase/rutile TiO_2 nanoparticles with a mean primary particle size of 17.8 nm. Geometric means for aerosol sizes were 138.8 nm (GSD = 1.44) for the high concentration and 152.9 nm (GSD = 1.38) for the low concentration. Total protein and LDH activities in BAL fluid were not significantly elevated in BAL fluid from exposed mice, compared with controls.

In another study, BALB/c/Sca mice (8 females/group) were whole-body exposed to TiO_2 particles as an aerosol at 0 or 10 mg/m^3 as either a single 2 hour-exposure, 2 hours/day for 4 days, or 2 hours/day, 4 days/week for 4 weeks (Rossi et al., 2010). Three commercially available and one in situ-produced form of TiO_2 particles were utilized; the particle types were differentiated throughout the study by their size and crystalline morphology. The size and surface area of the particles used as starting materials were measured experimentally: anatase nanoparticles were <25 nm and had a surface area of 222 m^2/g; rutile/anatase nanoparticles (9:1)

were 30–40 nm and had a surface area of 23 m^2/g; SiO_2-coated rutile nanoparticles were 10 × 40 nm needle-shaped particles coated in SiO_2 and had a surface area of 132 m^2/g; anatase/brookite nanoparticles (3:1; and in situ-produced by gas-to-particle conversion) were ~21 nm in size and had a surface area of 61 m^2/g; and coarse rutile particles were <5 µm in size and had a surface area of 2 m^2/g. Aerosols of anatase/brookite TiO_2 were produced in situ by thermal decomposition of titanium tetraisopropoxide in a particle generator; aerosols of all other particle types were generated using a solid particle dispenser. Upon dispersion, measurements of aerodynamic size distributions showed that particles formed agglomerates (about 100 nm and larger in size for solid particles and about 80 nm or smaller for generated particles). Groups of mice were sacrificed 4 and/or 24 hours after the last exposure. Lungs were lavaged; differential cell counts and cytokine levels were evaluated in the BAL fluid.

The percentage of neutrophils making up the BAL fluid was significantly ($p < 0.001$) increased in mice treated repeatedly (2 hours/day for 4 days or 2 hours/day, 4 days/week for 4 weeks) with SiO_2-coated TiO_2 nanoparticles only; neutrophils made up ≥9% of the total BAL cells after treatment with SiO_2-coated TiO_2 nanoparticles for 4 days or 4 weeks (data for controls not shown) (Rossi et al., 2010). No signs of inflammation (i.e., changes in BAL fluid parameters) were apparent in the lungs of mice from other treatment groups. Similarly, treatment with SiO_2-coated TiO_2 nanoparticles elicited dramatic expression of CXCL1 messenger ribonucleic acid (mRNA) (a neutrophil-attracting chemokine) in the lungs (about threefold higher than controls), whereas treatment with other particle types did not. Tumor necrosis factor-alpha (TNF-α) mRNA expression in the lungs was also increased approximately two- to threefold fold in mice treated with SiO_2-coated TiO_2 nanoparticles, compared with untreated controls. Neutrophil levels in the BAL fluid and cytokine expression in the lungs were not increased in mice treated with nanosized SiO_2 particles. Based on unpublished data indicating that needle-like rutile nanosized TiO_2 particles did not elicit inflammatory effects in vitro, the authors concluded that surface coating (rather than surface area, primary or aggregate particle size or shape, or radical formation capacity) was the best correlate for lung inflammation parameters. The results indicate that exposure to aerosols of SiO_2-coated rutile TiO_2 nanoparticles at a concentration of 10 mg/m^3 2 hours/day for 4 days or 4 days/week for 4 weeks was a LOAEL for pulmonary inflammation in BALB/c/Sca mice (increased numbers of neurtrophils in BAL fluid and increased expression of TNF-α and a neutrophil-attracting chemokine, CXCL1 in lung tissue). Single 2-hour exposures to this material at 10 mg/m^3 were without effect on these endpoints. Other test materials (including aerosols made from uncoated anatase, uncoated rutile/anatase (9:1), and uncoated anatase/brookite (3:1) nanoparticles) did not elicit changes in the examined pulmonary inflammation endpoints following single or repeated exposure to 10 mg/m^3.

Groups of male Wistar rats were head- and nose-exposed to aerosols generated from TiO_2 nanoparticles at a target exposure concentration of 100 mg/m^3 or aerosols generated from pigmentary TiO_2 at a target exposure concentration of 250 mg/m^3 for 6 hours/day for 5 consecutive days (van Ravenzwaay et al., 2009). The measured exposure concentrations to aerosols generated from nano-TiO_2 and pigmentary TiO_2 were 88 ± 6.4 and 274.0 ± 30.5 mg/m^3, respectively; rats exposed to conditioned air served as controls. The TiO_2 nanoparticles used as the starting material were 70% anatase/30% rutile, had no surface coating, ranged from 20 to 30 nm in size (measured by TEM) with a density of 4.2 g/cm^3, and had a surface area of

48.6 m^2/g. Pigmentary TiO_2 was 99.4% pure rutile, had a median particle size of 200 nm in ethanol, and an average surface area of 6 m^2/g. TiO_2 nanoparticles were dispersed in de-ionized water at 0.5% by weight; the resulting suspension was nebulized by a two-component atomizer. In contrast, dust aerosols of pigmentary TiO_2 were produced by dry dispersion of powder pellets using brush generators. For both particle types, aerosols were generated using compressed air in a mixing stage, mixed with conditioned dilution air, and passed via a cyclone (to separate particles >3 µm) into the head-nose inhalation system. During this process, water used to aerosolize nano-TiO_2 evaporated to generate dry dust aerosols in the test atmosphere. The aerosols generated from nano-TiO_2 particles had MMAD and GSD values of 1.0 µm and 2.2, respectively; the number concentration of TiO_2 particles remaining <100 nm in size after aerosolization was a small fraction (0.5%) of the total particle mass. For pigmentary TiO_2, MMAD and GSD values were not different than those of the nanoparticles (1.1 µm and 2.2, respectively); the calculated mass fraction of particles <100 nm was 0.05%. Three and 14 days after exposure, groups of five rats were sacrificed; BAL fluid was collected from the lungs and analyzed for total and differential cell counts, total protein content, and the activities of LDH, alkaline phosphatase (ALP), γ-glutamyltransferase (γ-GGT), and N-acetyl-β-glucosaminidase (NAG). Additional animals (6/group) were sacrificed immediately after exposure or 14 days post-exposure; organ weights (of the lung, mediastinal lymph nodes, liver, kidneys, adrenals, thymus, spleen, and brain) were recorded and respiratory tract tissues (including the lungs, mediastinal lymph nodes, and nasal cavity) were examined microscopically.

No mortality or clinical signs of toxicity were reported (van Ravenzwaay et al., 2009). Increases in the numbers of total cells and polymorphonuclear neutrophils (PMNs) in the BAL fluid of nanoTiO_2-treated rats were significant (*p*-value not reported) compared with controls, particularly on post-exposure day 3 (~10- and 1,000-fold changes, respectively, based on data presented graphically). Lymphocyte levels were also increased 10–100-fold over control levels on day 3 post-exposure. By 14 days post-exposure, total and differential cell counts in the BAL fluid of treated rats varied <10-fold from control levels (indicative of recovery); however, the number of PMNs in the lavage fluid was still significantly increased (almost 100-fold) relative to controls. Total protein content and the activities of LDH, ALP, GGT and NAG in the BAL fluid were moderately but significantly (*p*-value not reported) increased with respect to controls on day 3 post-exposure, but returned to near-normal levels by day 14. With respect to BAL fluid parameters, effects from exposure to pigmentary TiO_2 were completely comparable (in direction and magnitude) to effects elicited by aerosols generated from nano-TiO_2. A decrease in absolute lung weight of >30% was reported in TiO_2-treated rats (both treatment groups) immediately following exposure; lung weight was similar to controls by day 14 post-exposure (organ weight data not shown).

After exposure for 5 days, histopathological changes in the lungs (diffuse histiocytosis and mild neutrophilic inflammation), mediastinal lymph nodes (lymphoreticulocellular hyperplasia), and nasal cavity (detection of free particles in the olfactory epithelium) of nano-TiO_2 treated rats were noted; the inflammatory response was reportedly reduced by day 14 post-exposure (van Ravenzwaay et al., 2009). Mediastinal lymph node activation and diffuse histiocytosis in the absence of neutrophilic inflammation was observed in the lungs of animals treated with pigmentary TiO_2. Based on changes in BAL fluid parameters (including changes in differential cell counts, total protein levels, and enzyme activities of LDH, ALP, GGT, and

NAG) and histopathological findings in the lungs (including histiocytosis and mild neutrophilic inflammation and hyperplasia in the mediastinal lymph nodes), the tested concentration of 88 mg/m^3 (6 hours/day for 5 days) is identified as a LOAEL for pulmonary inflammation in male Wistar rats exposed to aerosols made from uncoated 70% anatase:30% rutile TiO_2 nanoparticles. Similar effects were observed in rats exposed to aerosols made from pigmentary rutile TiO_2 particles at a concentration of 274 mg/m^3.

In another short-term study by the same research group, male Wistar rats were head- and nose-exposed to TiO_2 nanoparticles as an aerosol at targeted exposure concentrations of 2, 10, or 50 mg/m^3 for 6 hours/day for 5 days (Ma-Hock et al., 2009). Measured exposure concentrations were within ~2 mg/m^3 of the targeted exposure concentrations; rats exposed to conditioned air served as controls. TiO_2 nanoparticles used as the starting material were characterized as 86% anatase/14% rutile, had an uncoated hydrophobic surface, ranged from 13 to 71 nm in size (with an average primary particle size of 25.1 ± 8.2 nm), and had a surface area of 51.1 ± 0.2 m^2/g. Dust aerosols of TiO_2 nanoparticles were produced by dry dispersion of powder pellets using brush generators. Aerosols were generated using compressed air in a mixing stage, mixed with conditioned dilution air, and passed via a cyclone (to separate particles >3 µm) into the head-nose inhalation system. The aerosols generated from nano-TiO_2 particles had MMAD and GSD values of 0.8–1.1 µm and 2.3–3.4, respectively. Due to agglomerates generated during the aerosolization process, the number concentration of TiO_2 particles <100 nm in size was only a small fraction (0.1–0.4%) of the total particle mass. Body weights were recorded (time points not specified). Groups of three to six rats were sacrificed immediately after exposure, 3 days post-exposure, and 16 days post-exposure. BAL fluid was collected from the lungs of animals sacrificed at each time point and analyzed for total and differential cell counts, total protein content, activities of BAL enzymes (LDH, ALP, GGT, and NAG) and levels of 60 cell mediators (including cytokines, chemokines, and signaling proteins). Rates of cell proliferation and apoptosis were also measured for three lung compartments (large/medium bronchi, terminal bronchioli, and alveoli). The levels of cell mediators, serum troponin I, as well as additional hematology parameters (not specified) were evaluated in high-dose rats on days 0 and 3 post-exposure. For rats sacrificed immediately after exposure and 16 days post-exposure, organ weights were recorded (organs not specified) and respiratory tissues (including the nasal cavity, larynx, lungs, and mediastinal lymph nodes) were examined histopathologically.

No mortality or clinical signs of toxicity were reported (Ma-Hock et al., 2009). Body weights of treated rats were reportedly similar to controls (data not shown). The BAL fluid of high-dose rats showed significant increases in the numbers of total cells on days 0 and 3 post-exposure (three- and sevenfold changes, respectively) and neutrophils at all post-exposure time points (with a maximal 400-fold change over control levels on post-exposure day 3; see Table 4.2. Neutrophil counts were also significantly increased compared to controls in the BAL fluid of mid-dose rats immediately following exposure and 3 days post-exposure. A transient increase in the number of atypical cells in the mid-dose group (on day 3 post-exposure) was observed. No treatment-related effects on eosinophil, lymphocyte, or macrophage counts in the BAL fluid were detected. In contrast, total protein content of the BAL fluid was moderately but significantly increased (approximately two- to threefold over controls) in all treatment groups on day 3 post-exposure (Table 4.2). Significant, concentration-dependent changes in the activity of BAL enzymes were also observed in all TiO_2 treatment groups; the activities of LDH and ALP in

the low-dose group and all enzymes in the high-dose group were significantly increased ($p < 0.01$) with respect to controls on day 3 post-exposure. The most dramatic effects on BAL fluid cytology and clinical chemistry were observed 3 days after exposure; by day 16 post-exposure, only neutrophil counts (33-fold change) and LDH activity (2-fold change) in the high-dose group were significantly different from control values (Table 4.2).

Table 4.2. Significant changes in male Wistar rats exposed to TiO_2 nanoparticles (86% anatase:14% rutile; average primary particle size 25 nm) via inhalation for 5 days

	Exposure concentration (mg/m^3)			
	0	**2**	**10**	**50**
BAL fluid clinical pathology				
Total cells (counts/µL)				
D 5	48.1[a]	35.0	49.4	170.9[b]
D 8	30.5	31.4	42.6	208.7[b]
Neutrophils (counts/µL)				
D 5	0.62	0.91	16.0[b]	133.2[b]
D 8	0.38	0.74	20.5[c]	152.1[c]
D 21	0.74	0.64	0.77	24.61[b]
Atypical cells (counts/µL)				
D 8	0	0.01	0.27[c]	2.36
Total protein (mg/L)				
D 8	33	51[c]	59[c]	108[b]
GGT activity (nkat/L)				
D 5	8	12	55[b]	117[b]
D 8	3	26	29[c]	93[b]
LDH activity (µkat/L)				
D 5	0.31	0.25	0.37	1.07[b]
D 8	0.18	0.3[c]	0.37	1.04[b]
D 21	0.24	0.22	0.18	0.45[b]
ALP activity (µkat/L)				
D 5	0.60	0.62	1.19	1.75[b]
D 8	0.56	0.65[c]	0.91[b]	1.36[b]
NAG activity (nkat/L)				
D 5	20	20	21	49[b]
D 8	20	24	28	48[b]
Lung cell proliferation[d]				
Large/medium bronchi				
D 5	0.55	2.22[b]	2.93[b]	3.80[b]
D 8	0.88	2.09	3.18[b]	3.37[b]
Terminal bronchioli				
D 5	0.50	6.07[b]	8.55[b]	22.50[b]
D 8	0.72	2.56[c]	8.60[b]	11.91[b]
Alveoli				
D 5	4.27	4.99	5.27	7.28[b]
Absolute lung weight (g)				
D 0	0.82	0.77	0.83	0.92[c]

Table 4.2. Significant changes in male Wistar rats exposed to TiO_2 nanoparticles (86% anatase:14% rutile; average primary particle size 25 nm) via inhalation for 5 days

	Exposure concentration (mg/m^3)			
	0	**2**	**10**	**50**
Histopathology				
Minimal focal alteration of the epithelium (epiglottis)	0/6[e]	0/6	0/6	2/6
Diffuse alveolar infiltration with histiocytes	0/6	0/6	6/6 (minimal)	6/6 (minimal to mild)
Increased epithelial thickness (bronchi and bronchioli)	0/6	0/6	0/6	6/6
Pigment-loaded macrophages (mediastinal lymph nodes)	0/6	0/6	4/6	6/6

[a]Median values.
[b]Significantly different from control at $p < 0.01$ (two-sided Wilcoxon test).
[c]$p < 0.05$.
[d]Expressed as mean labeling indices (percentage of nuclei counted undergoing replicative deoxyribonucleic acid [DNA] synthesis).
[e]Number affected/number animals examined.
Source: Ma-Hock et al. (2009).

Treatment-related increases in the levels of nine cell mediators in the BAL fluid were observed; the levels of mediators were significantly higher than controls in mid- and high-dose rats immediately after exposure, in all TiO_2-treated rats 3 days post-exposure, and in high-dose rats 16 days post-exposure (Ma-Hock et al., 2009). Although apoptosis was not induced by TiO_2 treatment, levels of cell replication in the various compartments of the lungs were dose-dependently increased in treated rats; the most marked effects were observed in the terminal bronchioli. Replication in the terminal bronchioli was increased in all treated rats immediately after treatment (12–45-fold control values) and on day 3 post-exposure (3–16-fold control values). In the large/medium bronchi, significant (four- to sevenfold) increases in cell proliferation were seen in all treated rats on day 0 post-exposure and at the two highest doses on day 3 post-exposure. Replication was increased significantly in the alveoli only in the high-dose group immediately after exposure (to 170% of the control value). Lung cell replication was similar among treated and control rats in all compartments by day 16 post-exposure. No significant changes in hematological parameters were reported for any exposure group (data not shown); effects on cell mediator levels in the serum were minor. Levels of serum troponin I (considered a biomarker for myocardial damage) were similar in control and high-dose rats on days 0 and 3 post-exposure.

The high-dose group showed a significant (12%) increase in absolute lung weights immediately following exposure; lung weights were similar to controls in all treatment groups on day 16 post-exposure (Ma-Hock et al., 2009). Histopathological analyses of respiratory tissues revealed only minimal lesions immediately following TiO_2 treatment. The nasal cavities of all high-dose rats showed TiO_2 particles on the surface of epithelial cells (mainly in posterior areas); larynx sections indicated that a fraction of high-dose animals also had minimal focal alteration of the epithelium at the base of the epiglottis. Histiocytes (presumed by the authors to be AMs) were observed in lung sections from all treated rats. Diffuse alveolar infiltration of macrophages in the lungs was noted at $\geq$10 mg/m^3; these effects ranged from minimal (mid-dose rats) to mild

(high-dose rats). A minimal to mild increase of epithelium thickness (interpreted by the authors as hypertrophy/hyperplasia) was observed in rats treated with 50 mg/m^3; a single high-dose rat also showed minimal multifocal infiltration with neutrophils. At ≥10 mg/m^3, pigment-laden macrophages (presumably containing TiO_2 particles) were detected in the mediastinal lymph nodes of most animals. On day 16 post-exposure, the lungs of all treated rats still contained pigment-laden macrophages; for the majority of mid- and high-dose animals, the distribution of histiocytes was changed from a diffuse to a multifocal pattern.

The results from this study (Ma-Hock et al., 2009) indicate that the lowest concentration, 2 mg/m^3, was a LOAEL for changes in BAL fluid indicative of minimal pulmonary inflammation (increased total protein, LDH, and ALP activities in BAL fluid) and increased cell proliferation indices in the large/medium and terminal bronchioli in male Wistar rats exposed for 5 days to aerosols made from 86% anatase:14% rutile TiO_2 particles. No NOAEL was identified. At the higher tested concentrations, 10 and 50 mg/m^3, pulmonary effects were progressively more pronounced. At 50 mg/m^3, a greater number BAL fluid markers of inflammation were markedly increased (e.g., total cell and neutrophil counts, total protein, activities of LDH, GGT, ALP, and NAG), cell proliferation indices were increased in bronchial regions, as well as in alveolar regions, and histological changes in the lung of all exposed rats included diffuse alveolar infiltration with histiocytes, increased epithelial thickness in bronchiolar regions, and pigment loaded macrophages in mediastinal lymph nodes. Extra respiratory tissues were not examined histologically and standard clinical chemistry variables were not examined in blood samples, but serum troponin I levels (a marker for myocardial damage in rodents) were not different in rats in the control and high-exposure groups.

In a study by Nurkiewicz et al. (2008), Sprague-Dawley rats (6–7 weeks old) were whole-body exposed to filtered air (control), fine TiO_2, or ultrafine TiO_2 via the inhalation route to evaluate effects on systemic microvascular function. Primary particle sizes for fine (rutile) and ultrafine (80% anatase:20% rutile) TiO_2 were 1 µm and 21 nm, respectively. TiO_2 powders were sieved, dried, and stored under conditions designed to limit agglomeration. Animals (three to five males/group) were exposed to aerosols at exposure concentrations ranging from 1.5 to 20 mg/m^3 for 4–12 hours; these exposures were selected by the authors because they were not anticipated to significantly affect BAL markers of pulmonary inflammation or elicit overt lung damage. Particle size distributions (or geometric diameters) of the resulting aerosols indicated that the primary and secondary modes for aerosols generated from ultrafine particles were 100 and 400 nm, respectively; primary and secondary modes for aerosols generated from fine TiO_2 were 710 and 120 nm, respectively. Count mean diameters (GSDs) for fine and ultrafine particles were 402 nm (2.4) and 138 nm (2.2), respectively. These count mean diameter values correspond to estimated MMADs of about 16.0 µm for fine TiO_2 and 3.6 µm for ultrafine TiO_2. Exposure groups were identified by the authors by total target exposure (expressed in µg); based on this classification, 8, 20, 36, 67, and 90 µg groups were identified for fine TiO_2, and 4, 6, 10 (three groups), 19, and 38 µg groups were identified for ultrafine TiO_2. At 24 hours post-exposure, intraluminal infusion of A2187 (a Ca^2+ ionophore) was utilized to evaluate endothelium-dependent arteriolar dilation in the spinotrapezius muscle by in vivo microscopy. Resting variables (including resting diameter, passive tone, and resting tone) for arterioles used in the intravital studies were recorded. Histopathological examinations of the left lung of sacrificed rats were performed 24 hours post-exposure.

Resting variables were similar among all experimental groups regardless of aerosol dose, particle size, or exposure duration (Nurkiewicz et al., 2008). However, exposure to fine or ultrafine TiO_2 particles impaired arteriolar dilation in a dose-dependent manner. Arteriolar dilations were significantly ($p < 0.05$) impaired in 67 and 90 µg groups (fine TiO_2 particles) and 10, 19, and 38 µg groups (ultrafine TiO_2 particles). Arteriolar constriction was also observed in rats exposed to 38 µg ultrafine TiO_2. Based on results from an additional experiment, in which groups of rats were exposed to ultrafine TiO_2 at a total dose of 30 µg by means of various exposure conditions, the authors concluded that microvascular responses were dictated by total (mass) exposure rather than specific exposure times or aerosol concentrations. Importantly, pair-wise comparisons of arteriolar responsiveness in rats exposed to fine TiO_2 (at 8, 20, or 36 µg) or ultrafine TiO_2 particles (at 10, 19, or 38 µg, respectively) showed that, at similar pulmonary burdens, ultrafine TiO_2 elicited significantly ($p < 0.05$) more pronounced microvascular dysfunction than fine TiO_2 particles. Histopathological examination of the lungs showed no evidence of inflammation in any treatment group. Lung changes observed in TiO_2-treated rats included particle accumulation inside AMs, the presence of anuclear macrophages (indicative of apoptosis; in 16/29 fine- [all exposure groups] and 1/20 ultrafine-treated rats [at 38 µg], versus 0/21 controls) and close approximation between particle-laden macrophages and the alveolar wall (indicative of macrophage activation; data not quantified). Ultrafine TiO_2 particles were visualized inside macrophages (only agglomerations visible by light microscopy); fine TiO_2 particles were localized both intra- and extracellularly. Taken together, the data indicate that at exposure levels that do not cause lung inflammation, treatment with TiO_2 elicited significant microvascular dysfunction; although aerosols generated from either fine TiO_2 or ultrafine TiO_2 were in the µmeter range, microvascular dysfunction was more pronounced after treatment with ultrafine particles than fine TiO_2 particles of the same chemical composition at the same mass.

In additional studies performed by the same research group, treatment with aerosols generated from TiO_2 nanoparticles (21 nm in size) was also shown to impair endothelium-dependent vascular responses in coronary arterioles (at a deposition of 9.5 µg; LeBlanc et al., 2009a, b); in systemic arterioles, microvascular dysfunction was associated with increased local oxidative stress and decreased nitric oxide (NO) bioavailability (at depositions of 4–90 µg/rat) (Nurkiewicz et al., 2009). The effects of treatment with aerosols generated from fine TiO_2 particles on these endpoints were not assessed.

4.2.2. Acute Oral Toxicity

In an acute oral toxicity study, a single dose of ultrafine TiO_2 particles (79% rutile/ 21% anatase (90% wt titania/7% alumina/1% amorphous silica; median particle size 140 nm in water) suspended in deionized water was administered via gavage to one fasted female rat per dose (strain not specified) at 0, 175, 550, or 1,750 mg/kg; three additional females were dosed at 5,000 mg/kg (Warheit et al., 2007). Rats were observed for mortality, body weights, and clinical signs for 14 days after dosing. All rats were necropsied to detect gross lesions. No mortality occurred. The rat dosed at 1,750 mg/kg and all rats dosed at 5,000 mg/kg exhibited gray-colored feces; no other clinical signs of toxicity were reported. The authors reported no biologically significant changes in body weights and no gross lesions at necropsy. Based on these findings, the authors concluded that the oral LD_{50} is >5,000 mg/kg for female rats.

CD-1 ICR mice (10/sex/group) were administered a suspension of TiO_2 particles (>99% pure) at 5,000 mg/kg-day via gavage as a single oral dose and survivors were sacrificed 2 weeks after dosing (Wang et al., 2007). A vehicle-only control group was administered 0.5% HPMC. Nanosized (25 and 80 nm) and fine TiO_2 particles (crystalline forms were not specified) were used; TEM analyses of the particles reportedly showed agreement with the nominal sizes. The size of fine TiO_2 particles (measured by TEM) was 155 ± 33 nm. Mortality and clinical signs were monitored regularly, particularly during the first 24 hours after dosing. Body weights were recorded prior to treatment and at sacrifice. Clinical chemistry parameters, including serum levels of total bilirubin, ALP, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (to assess liver function), uric acid, blood urea nitrogen (BUN), and creatinine (to assess kidney function), and creatine phosphokinase (CK), LDH, and α-hydroxybutyrate dehydrogenase (α-HBDH; to assess heart function) were evaluated. The heart, liver, spleen, kidneys, lung, brain, testicle, and ovary were weighed and subjected to histopathological examination.

Mortality was reported in all TiO_2 treatment groups (Wang et al., 2007). Two females in the 25 nm group, one female in the 80 nm group, and two females in the fine TiO_2-treated group were spiritless, anorectic, and inactive and died within 2 days. Two additional mice (a male in the fine group and a female in the 25 nm group) died on the third day. These deaths were attributed by the researchers to the administration of TiO_2 particles (i.e., ruptured esophagus) rather than the test material. No clinical signs of toxicity were observed in surviving animals. Body weights were similar among control and treated mice 2 weeks following treatment. Statistically significant increases in ALT (25%) and the ALT/AST ratio (33%), as well as a significant increase in BUN concentration (21%), were observed in female mice exposed to 25 nm TiO_2. Levels of LDH and α-HBDH were likewise increased in females treated with 25 (32–35% for both enzymes) or 80 nm particles (81–83%). In males, only slightly elevated ALT/AST ratios (all treatment groups) and nonsignificant increases in serum BUN and creatinine levels (in nanosized TiO_2 groups) were reported (data not shown). No significant changes in relative spleen or kidney weights were observed; however, relative liver weight was significantly higher in females (but not males) exposed to nanosized TiO_2 particles (25 or 80 nm) compared to controls. Relative liver weight was not increased in mice exposed to fine TiO_2 particles. No histopathological changes were observed in mice exposed to 25 nm nanoparticles. Mice (male and female) exposed to 80 nm and fine TiO_2 particles showed evidence of injury in the brain (slight lesions characterized by vacuoles in the neurons of the hippocampus, indicative of fatty degeneration), kidney (renal tubules filled with proteinic liquids in the 80 nm group and swelling in the glomerulus in the fine-treated group), and liver (hydropic degeneration around the central vein and spotty necrosis of hepatocytes); incidence data were not shown. No abnormal findings were reported in the heart, lung, spleen, testicle, or ovary. These data suggest that a single 5,000 mg/kg oral dose of TiO_2 (as 25 nm, 80 nm, or fine particles) was toxic to mice, producing evidence of effects on the heart, liver, kidney, and brain. The researchers concluded that there was no clear relationship between particle size and toxicity in this study.

4.2.3. Acute Dermal Toxicity

No studies regarding the acute dermal toxicity of TiO_2 nanoparticles were identified.

4.3 REPEATED DOSE TOXICITY (INCLUDING INHALATION/ RESPIRATORY EFFECTS)

4.3.1 Inhalation Exposure

Overview: The best available data to assess the "repeated-dose" toxicity of TiO_2 nanoparticles via the inhalation route (discussed below) are limited in that, although the TiO_2 particles used as the starting materials for these experiments were in the nano-range (i.e., at least one dimension was <100 nm), aerosolization of the nanoparticles typically induced agglomeration and resulted in exposures to TiO_2 aerosols with MMAD values well into the µm range. Although there is inherent uncertainty associated with applying data from µm-sized TiO_2 aerosols to evaluate the toxicity of nanosized TiO_2, data from several experiments suggest that TiO_2 nanoparticles are more potent at producing pulmonary inflammation, and lead to slower lung particle clearance, than "fine" TiO_2 particles following exposure to equivalent airborne mass concentrations. Results from several subchronic-duration inhalation studies of µm-sized aerosols made from anatase or anatase:rutile TiO_2 nanoparticles identify pulmonary inflammation as the principal effect of concern in F344 rats exposed to concentrations of about 23 mg/m^3 (Baggs et al., 1997; Janssen et al., 1994; Oberdörster et al., 1994; Ferin et al., 1992); in CDF (F344)/CrlBr rats exposed to 2 or 10 mg/m^3, but not 0.5 mg/m^3 (Bermudez et al., 2004), and in B3C3F1/CrlBR mice and Lak:LVG (SYR) BR hamsters exposed to 10 mg/m^3, but not 0.5 or 2 mg/m^3 (Bermudez et al., 2004).

The results of subchronic-duration studies performed by a single research group according to the same basic exposure protocol were presented in several published reports (Baggs et al., 1997; Janssen et al., 1994; Oberdörster et al., 1994; Ferin et al., 1992). In this study, F344 rats (4 males/group) were exposed whole-body to fine (230 or 250 nm) or ultrafine TiO_2 (anatase; 21 nm primary particle size) particles as aerosols at a concentration of about 23 mg/m^3 for 6 hours/day, 5 days/week for up to 12 weeks, and observed up to 52 weeks post-exposure (Ferin et al., 1992). A group of rats breathing clean air served as controls. The TiO_2 nanoparticles used as a starting material reportedly had a surface area of 50 m^2/g and a density of 4.28; the surface area of fine TiO_2 particles was 6.4 m^2/g. TiO_2 aerosols were generated using a Wright dust feed; MMAD (σ_g) values of the resulting aerosols were 0.78 µm (1.7) and 0.71 µm (1.9) for ultrafine and fine particles, respectively (according to Ferin et al., 1992), indicating that aerosolization of ultrafine and fine TiO_2 samples generated particle aggregates that were nearly identical in size. The measured average aerosol concentration during the experiment was 23.5 ± 3.2 mg/m^3 for ultrafine particles and 23.0 ± 4.0 mg/m^3 for fine particles. Each of the reports (described below) presented data for similar and overlapping endpoints (namely BAL fluid parameters and microscopic examinations of the lungs) to assess the occurrence and degree of lung inflammation associated with exposure to TiO_2 particles.

According to Ferin et al. (1992), levels of PMNs in the BAL fluid were measured at weeks 4, 8, 12, 41, and 64 after the start of exposure; additional groups of rats were sacrificed and the lungs (not lavaged) were examined microscopically at the same time points. Compared to control rats, rats treated with ultrafine TiO_2 showed significantly increased numbers of PMNs (indicative of inflammation) at weeks 4, 8, 12, and 41; PMN levels were increased maximally at the end of exposure (reaching 129 times the control value), but decreased, almost reaching

control levels, by 52 weeks post-exposure. A modest (but significant) increase in PMN levels was also observed in rats treated with fine TiO_2 particles. In rats exposed to ultrafine (or fine) TiO_2 for 12 weeks, large volumes of particle-laden AMs were observed. In some cases, cell debris (indicative of AM death and release of previously phagocytized particles) was detected in alveoli. TiO_2 particles were seen in type I pneumocytes, agglomerated in endosomes, and within the pulmonary interstitium. Cell-associated and free particles were also found in bronchus-associated lymphoid tissue (BALT) and peribronchial and perivascular connective tissue after 12 weeks exposure (incidence data not quantified).

Based on the data presented in Oberdörster et al. (1994), the lungs of animals (6/group) sacrificed at 4, 8, and 12 weeks were subjected to BAL; lavage fluid was analyzed for differential cell counts, total protein, and lysosomal and cytosolic enzyme activities. Additional animals were sacrificed 41 and 64 weeks after the start of exposure; histopathological analyses of the lung tissues were performed. Consistent with data presented in the earlier publication, PMN levels in the BAL fluid of ultrafine TiO_2-treated rats increased substantially with respect to controls by the end of the exposure period (estimated 10-fold induction at 12 weeks based on data presented graphically); the number of PMNs remained elevated 41 weeks after the initiation of exposure. Total cells and the number of AMs in the BAL fluid were approximately 3- and 2.5-fold higher, respectively (estimated from data presented graphically), in nano-TiO_2-treated rats than controls at the end of exposure period; total and differential cells counts were similar to controls by week 64. Total protein content and the levels of LDH and β-glucuronidase in the BAL fluid (data not shown) were similarly elevated in rats treated with nano-TiO_2 at week 12 and persisted until week 41. These effects were not observed (or only modestly observed) in fine TiO_2-treated rats. Histopathological analyses showed signs of early fibrotic reactions (marked by the beginning of interstitial fibrotic foci), type II cell hyperplasia, and occlusion of the pores of Kohn in nano-TiO_2-treated rats (and to a lesser extent, in fine TiO_2-treated rats) at 41 weeks; these changes regressed 1 year post-exposure. Based on inflammatory effects (including effects on total and differential cell counts in the BAL fluid and histopathological changes), the test concentration of 23.5 mg/m^3 is identified as a LOAEL in F344 rats exposed for up to 12 weeks to aerosols made from anatase TiO_2 nanoparticles. No NOAEL was identified.

In another publication, data were presented for additional parameters associated with the inflammatory response induced by TiO_2 exposure (Janssen et al., 1994). In this study, F344 rats were again exposed to filtered air, 23.5 mg/m^3 ultrafine (20 nm) anatase TiO_2 or 22.5 mg/m^3 fine (250 nm) anatase TiO_2 according to the same exposure protocol. Aerosols of nano-TiO_2 were generated using a Jet-o-Mizer; aerosols of fine TiO_2 were produced with a Wright dust feeder. Characteristics of the fine and ultrafine TiO_2 particles used were reportedly similar to those described in the earlier studies. At 4, 8, 12, and 29 weeks after the start of exposure, groups of rats (n = 4) were sacrificed; lungs were subjected to BAL. Total and differential cell counts in the BAL fluid were performed; additionally, gene and protein expression of antioxidant enzymes (including glyceraldehyde-3-phosphate dehydrogenase [GAPDH], catalase [CAT], manganese-containing superoxide dismutase [SOD] or MnSOD, glutathione peroxidase [GPX], and copper-zinc-containing SOD [CuZnSOD]) were analyzed in the lungs. The lungs of additional groups of rats, sacrificed at 29 and 52 weeks post-exposure, were evaluated histopathologically for signs of inflammation and/or fibrosis.

Analyses of BAL fluid showed that, in comparison to controls, total cell counts and the percentage of PMNs (indicative of an inflammatory response) were significantly elevated ($p < 0.05$) in rats treated with nano-TiO_2 particles throughout the exposure and observation periods; these values were highest (increases of approximately twofold and 18%, respectively, based on data presented graphically) at the end of the exposure period (Janssen et al., 1994). The percentage of AMs in the BAL was significantly reduced compared to controls in ultrafine TiO_2-treated rats at all time points (dropping to ~80% of control at 12 weeks), whereas the percentage of lymphocytes significantly increased only immediately after exposure (at 12 weeks). These changes were not seen (or only modestly seen) in rats treated with fine TiO_2. Compared to controls and fine TiO_2-treated rats, rats treated with ultrafine TiO_2 had significantly increased mRNA expression of MnSOD ($p < 0.05$) at all time points; based on data presented graphically, expression ranged from approximately 200 to 400% of control levels (Janssen et al., 1994). Pearson correlation analysis indicated that parameters of inflammation (namely PMN and total cell counts) correlated with levels of MnSOD mRNA expression ($p < 0.01$). Similarly, significant increases in the steady-state levels of CAT, GPX, and GAPDH mRNAs ($p < 0.05$), but not CuZnSOD mRNA, were observed in ultrafine TiO_2-treated rats compared to controls at 8 and 12 weeks after initiation of exposure. Western analyses indicated that, at 8 weeks exposure, MnSOD protein expression was markedly increased compared to the control (data not quantified); however, increases in CAT and GPX mRNA levels were not paralleled by increases in their respective protein levels. Histopathological examination at 29 weeks post-exposure revealed some inflammation and fibrosis in the lungs of nanoTiO_2-treated rats (and to a lesser extent, in fine TiO_2-treated rats; data not quantified); however recovery was apparent at 52 weeks post-exposure. Based on inflammatory effects (increased total cell counts and percentage of PMNs in the BAL fluid and histopathological changes), the test concentration of 23.5 mg/m^3 is identified as a LOAEL in F344 rats exposed for up to 12 weeks to aerosols made from anatase TiO_2 nanoparticles; no NOAEL was identified.

Another study by the same authors focused mainly on the histopathological changes associated with exposure to nanosized TiO_2 particles (Baggs et al., 1997). In this study, male F344 rats (number not specified) were exposed whole-body to filtered air or fine (250 nm) or ultrafine (20 nm) TiO_2 particles at the same exposure concentrations and exposure times. Particle characterization data (including surface area, crystalline form, method of aerosolization, and MMAD and σ_g values for generated aerosols) were not reported. Six and 12 months post-exposure, 3–4 animals were sacrificed; cell replication in the lungs was measured histologically and lung tissues were subjected to histopathological analyses. Measurements of cell proliferation in macrophages, alveolar epithelial cells, and fibroblasts in the lungs of ultrafine (or fine) TiO_2-treated rats were not significantly different from control levels at 6 or 12 months post-exposure. Histopathological analyses of lung tissues from nanoTiO_2-treated rats (and, to a lesser extent, rats treated with fine TiO_2) showed increased alveolar epithelial thickness, mild septal fibrosis (accompanied by significant increases in septal collagen levels; $p < 0.05$), and an abundance of particle-laden macrophages within the alveoli 6 months post-exposure (data not quantified). By 12 months post-exposure, numbers of AMs (most containing particles) were still elevated in the lungs of TiO_2-treated rats compared to controls; however, other signs of lung injury (including the amount of interstitial fibrosis and septal collagen levels) had returned to control levels. Based on histopathological changes in the lung (including alveolar thickness, septal fibrosis, and increased numbers of AMs), the test concentration of 23.5 mg/m^3 is identified

as a LOAEL in F344 rats exposed to aerosols made from uncoated anatase TiO_2 nanoparticles for up to 12 weeks; no NOAEL was identified.

In a comparative study, Bermudez et al. (2004) exposed female CDF (F344)/CrlBR rats, B3C3F1/CrlBR mice, and Lak:LVG (SYR) BR hamsters (25/group/time point) to nanosized TiO_2 (average primary particle size of 21 nm, P25 obtained from Degussa-Huls AG, Frankfort, Germany, typically 80% anatase: 20% rutile) as an aerosol at targeted concentrations of 0 (filtered air control), 0.5, 2.0, or 10 mg/m^3 for 6 hours/day, 5 days/week for 13 weeks; animals were observed for up to 1 year post-exposure. Aerosols were generated using a brush generator; during this process, particles were dispersed in jet streams of air and passaged through a mixing chamber before being delivered to the exposure chambers. Aerosolized particles showed evidence of agglomeration; measured MMADs and GSDs for these experiments were 1.29–1.44 µm and 2.46–3.65, respectively. In each experiment, actual (measured) exposure concentrations varied <1 mg/m^3 from target exposure concentrations. Clinical signs of toxicity were regularly monitored. Body weights were recorded prior to exposure, weekly for 17 weeks, and biweekly thereafter. Groups of animals were sacrificed immediately after exposure, and at weeks 4, 13, 26, and 52 (49 for hamsters) post-exposure. The BAL fluid of some sacrificed animals was assessed for cytological and biochemical markers of toxicity (including total and differential cell counts, total protein level, and LDH activity). The lungs of other sacrificed animals were used to measure cell proliferation and subjected to histopathological examination.

Seven rats died in the observation period following exposure (treatment groups not specified); these deaths did not appear to be related to treatment (Bermudez et al., 2004). A depression in body weight gain (magnitude not specified) at the end of exposure, followed by recovery in the next 3–4 weeks, was reported for all treatment groups (data not shown). Immediately following exposure, BAL fluid from rats treated with TiO_2 at the high concentration showed significantly increased total cell counts; the total cell count was ~3 times higher than the mean number of lavaged cells recovered from control animals. In addition, the numbers of neutrophils and lymphocytes (weeks 0, 4, and 13 post-exposure) and the numbers of macrophages (weeks 0 and 4 post-exposure) were significantly higher in high-concentration rats compared to controls ($p < 0.05$). Total cell counts and the numbers of macrophages, neutrophils, and lymphocytes were similar to controls by 26 weeks post-exposure. However, the proportion of the population of cells that was made up of macrophages and neutrophils was significantly different from controls at 52 weeks post-exposure ($p < 0.05$; magnitude and direction of responses not specified). Levels of LDH activity (at weeks 0, 4, and 13 post-exposure) and total protein (at weeks 0 and 4 post-exposure) in the BAL of high concentration rats were significantly higher (by approximately three- to fivefold) than concurrent controls (see Table 4.3). Rates of terminal bronchiolar cell replication were significantly elevated in mid- and high-concentration rats (in a concentration-dependent manner) immediately following exposure, but were not different from controls by week 4 post-exposure. Alveolar cell replication was also significantly increased (by 1.7- and 2.7-fold) in the same treatment groups immediately after exposure, but returned to normal levels by weeks 4 and 26 post-exposure for mid- and high-concentration groups, respectively.

Table 4.3. Significant changes in female CDF (F344)/CrlBr rats exposed to TiO_2 nanoparticles (21 nm) via inhalation for 13 weeks

	Exposure concentration (mg/m^3)			
	0	**0.5**	**2**	**10**
Number of animals examined	25	25	25	25
LDH level (U/I) in BAL fluid				
Wk 0 post-exposure	24 ± 2[a]	26 ± 2	29 ± 7	122 ± 18[b]
Wk 4 post-exposure	29 ± 1	32 ± 11	36 ± 17	112 ± 8[b]
Wk 13 post-exposure	29 ± 3	29 ± 16	26 ± 6	83 ± 14[b]
Protein in BAL fluid (µg/mL)				
Wk 0 post-exposure	83 ± 15	111 ± 25	104 ± 91	236 ± 28[b]
Wk 4 post-exposure	79 ± 5	80 ± 7	102 ± 15	223 ± 12[b]
Terminal bronchiolar cell replication (labeling index)[c]				
Wk 0 post-exposure	2.09 ± 1.15	2.32 ± 1.80	3.79 ± 1.52[b]	5.45 ± 0.95[b]
Alveolar cell replication (labeling index)[c]				
Wk 0 post-exposure	4.53 ± 1.78	6.23 ± 2.42	7.81 ± 1.22[b]	12.18 ± 2.53[b]
Wk 4 post-exposure	4.59 ± 1.05	6.30 ± 1.18	7.15 ± 1.44	10.06 ± 0.96[b]
Wk 13 post-exposure	5.09 ± 1.83	5.48 ± 2.06	7.17 ± 4.37	9.40 ± 2.66[b]

[a]Mean ± SD.
[b]Significantly different from control at $p < 0.05$.
[c]Labeling indices are reported as the percentage of BrdU-labeled cells of the cells counted (minimum of 400 cells counted).

Source: Bermudez et al. (2004).

Histopathological examination confirmed that minimal lung damage was induced in rats by the low concentration of TiO_2; changes were restricted to the appearance of particle-laden AMs, and slight changes in patterns of macrophage accumulation (Bermudez et al., 2004). The lung tissues of mid-level rats showed minimal-to-mild lesions, consisting of particle-laden macrophage accumulation and aggregation in subpleural regions and centriacinar zones (accompanied by minimal hypertrophy and hyperplasia of type II alveolar epithelial cells).

In the post-exposure period, aggregations became more focally concentrated and were detected in interstitial regions. More dramatic changes in epithelial proliferation, including metaplastic effects in the centriacinar region (bronchiolization of the alveolar epithelium), associated with the accumulation of particles and particle-containing macrophages, were observed in high-concentration rats; these effects were most pronounced 13 weeks post-exposure. Although these effects diminished with increasing post-exposure time, occasional foci of epithelial hyperplasia and hypertrophy, minimal hyperplasia, and a minimal fibrotic response (characterized by thickening of the alveolar septae in centriacinar regions associated with particle aggregation) were noted at 52 weeks post-exposure. Based on increased terminal bronchiolar and alveolar cell replication and histopathological changes in the lungs (including accumulation of particle-laden macrophages, their migration to interstitial regions, and aggregation in subpleural and centriacinar zones with minimal hypertrophy and hyperplasia), NOAEL and LOAEL values of 0.5 and 2.0 mg/m^3, respectively, are identified for CDF (F344)/CrlBR rats exposed to aerosols of uncoated anatase:rutile TiO_2 nanoparticles for 13 weeks.

In mice, four deaths during exposure were reported (treatment groups not specified); these deaths were considered to be treatment-related by the researchers. Four additional mice

(distributed over the various treatment groups) died during the post-exposure period (Bermudez et al., 2004). A depression in body weight gain (magnitude not specified) at the end of exposure, followed by recovery in the next 3–4 weeks, was reported for all treatment groups (data not shown). The BAL fluid from mice treated at the high concentration showed significantly increased total cell counts throughout the post-exposure period; total cell counts were approximately three- and twofold higher than the mean number of cells lavaged from control animals at weeks 0 and 52 post-exposure, respectively. Similarly, the numbers of macrophages, neutrophils, and lymphocytes were significantly elevated in high-concentration mice compared to concurrent controls at all time points following exposure. Changes in LDH activity and total protein were also observed in the high-concentration mice (see Table 4.4); LDH activity was significantly increased at 4 and 13 weeks post-exposure (2.7- and 3.2-fold, respectively), and total protein content was increased at all post-exposure time points (approximately ~2–4-fold changes). Compared to concurrent controls, mice exposed to the high concentration of TiO_2 showed increased terminal bronchiolar cell replication (at weeks 0 and 26 post-exposure) and alveolar cell replication (at 13 and 26 weeks post-exposure).

Table 4.4. Significant changes in female B3C3F1/CrlBR mice exposed to TiO_2 nanoparticles (21 nm) via inhalation for 13 weeks

	Exposure concentration (mg/m^3)			
	0	**0.5**	**2**	**10**
Number of animals examined	25	25	25	25
LDH level (U/I) in BAL fluid				
Wk 4 post-exposure	38 ± 10[a]	46 ± 18	48 ± 17	103 ± 24[b]
Wk 13 post-exposure	37 ± 16	41 ± 14	45 ± 17	120 ± 81[b]
Protein in BAL fluid (µg/mL)				
Wk 0 post-exposure	92 ± 19	92 ± 9	67 ± 25	257 ± 31[b]
Wk 4 post-exposure	91 ± 12	82 ± 5	85 ± 17	256 ± 42[b]
Wk 13 post-exposure	69 ± 15	80 ± 12	89 ± 5	274 ± 126[b]
Wk 26 post-exposure	68 ± 27	97 ± 27	92 ± 26	169 ± 31[b]
Wk 52 post-exposure	115 ± 15	129 ± 24	98 ± 23	206 ± 44[b]
Terminal bronchiolar cell replication (labeling index)[c]				
Wk 0 post-exposure	2.16 ± 0.69	3.47 ± 1.58	3.93 ± 1.74	5.27 ± 1.55[b]
Wk 26 post-exposure	1.31 ± 0.47	2.17 ± 0.50	2.20 ± 1.82	3.44 ± 2.33[b]
Alveolar cell replication (labeling index)[c]				
Wk 13 post-exposure	5.64 ± 1.12	7.06 ± 2.36	6.09 ± 1.88	10.21 ± 1.99[b]
Wk 26 post-exposure	4.91 ± 1.17	6.53 ± 1.91	5.92 ± 1.81	9.46 ± 2.45[b]

[a]Mean ± SD.
[b]Significantly different from control at $p < 0.05$.
[c]Labeling indices are reported as the percentage of BrdU-labeled cells of the cells counted (minimum of 400 cells counted).

Source: Bermudez et al. (2004).

In low- and mid-concentration mice, particles were observed free, within macrophages, and in alveolar septal regions, but no particle-induced lesions were observed (Bermudez et al., 2004). Histopathological findings in high-concentration mice showed minimal epithelial changes; lesions were typically characterized by aggregations of particle-laden macrophages concentrated in the central lobar centriacinar sites. With increasing post-exposure time up to 52 weeks, macrophage aggregates became more concentrated and migrated to interstitial areas;

they were primarily found around blood vessels and peribronchial interstitium. Significant perivascular lymphoid proliferation was also noted in high-concentration mice. Based on changes in increased total and differential cell counts (including increased numbers of macrophages, neutrophils, and lymphocytes), changes in BAL fluid parameters (increased total protein and LDH activity), and histopathological changes in the lungs (including aggregations of particle-laden macrophages in centriacinar sites and their migration to interstitial areas, and perivascular lymphoid proliferation), NOAEL and LOAEL values of 2.0 and 10 mg/m^3, respectively, are identified for B3C3F1/CrlBR mice exposed for 13 weeks to aerosols made from uncoated anatase:rutile TiO_2 nanoparticles.

During the post-exposure period, 35 hamsters (distributed over the various treatment groups) died; the authors attributed these deaths to the occurrence of age-related conditions including chronic renal disease (Bermudez et al., 2004). Frank body weight loss (9–15%) at the end of exposure, followed by a slow recovery during the post-exposure period (up to 49 weeks) was reported for all hamsters (data not shown). While the number of neutrophils was significantly elevated in high-concentration hamsters immediately following exposure, no other BAL parameters (total or differential cell counts, LDH activity, or total protein) differed significantly from concurrent controls in any treatment group at any post-exposure time point. Levels of terminal bronchiolar cell replication, but not alveolar cell replication, were increased significantly relative to controls in high-concentration animals immediately following exposure; recovery was apparent 4 weeks after termination of exposure. A similar increase in terminal bronchiolar cell replication in low-concentration hamsters 4 weeks post-exposure was not considered by the authors to be biologically significant. The lung tissues of high-concentration hamsters showed the presence of alveolar and interstitial macrophages laden with particles, occasional aggregation of particle-laden macrophages, and no other histopathological changes. Few free particles or particle-laden macrophages were detected by week 26 post-exposure. Based on significant increases in neutrophil counts in the BAL fluid and the level of bronchiolar cell replication, NOAEL and LOAEL values of 2.0 and 10 mg/m^3, respectively, are identified in Lak:LVG (SYR) BR hamsters exposed for 13 weeks to aerosols made from uncoated anatase:rutile TiO_2 nanoparticles.

4.3.2. Oral Exposure

Groups of male and female Wistar rats (8/sex/group) were administered TiO_2 nanoparticles via gavage at 0, 160, 400, or 1,000 mg/kg-day once per day for 14 days (Bu et al., 2010). The TiO_2 nanoparticles were a mixture of anatase and rutile phases (composition not specified), 10% weight in water, and measured <50 nm in size; no further details regarding the test material were provided. Animals were sacrificed on day 15. Body weights were recorded every 3 days. Clinical chemistry (activities of ALT, AST, LDH, and CK and levels of creatinine, cholesterol, total bilirubin, and triglyceride) and hematological parameters (counts of white blood cells [WBCs], neutrophils, lymphocytes, monocytes, erythrocytes, and platelets, and hemoglobin concentration) were evaluated. Metabonomic analyses of the urine (from samples collected from male rats on the day prior to dosing, and days 1, 3, 7, and 14 of treatment) and the serum (collected from sacrificed rats) were performed. The liver, kidney, brain, adrenal gland, spleen, lung, heart, testis, ovary, and uterus were weighed and subjected to histopathological analysis. Samples of the heart, brain, kidney, and liver were prepared for TEM analysis.

Body weights and relative organ weights were not affected in any treatment group (data not shown; Bu et al., 2010). Statistically significant dose-related increases in mean serum activities of CK, AST, and LDH were observed in all exposure groups (see Table 4.5). Increases in WBC, lymphocyte, and monocyte counts were also reported at the highest tested dose (Table 5). Metabonomic analyses showed that TiO_2 induced variations in the levels of many metabolites typically found in the serum, including increased trimethylamine-N-oxide (TMAO), choline, creatine, phosphocholine, and 3-D-hydroxybutyrate (3-D-HB), and decreased glutamine, pyruvate, glutamate, acetoacetate, glutathione, and methionine. Changes in the metabonomic profile of the urine at various times after the initiation of treatment were also detected, including (from summarized findings) increased levels of hippurate, histidine, taurine, TMAO, citrulline, citrate, α-ketoglutarate, acetate, and phenylacetylglycine; and decreased levels of lactate, betaine, choline, methionine, 3-D-HB, and pyruvate. It was not specified at which dose levels the metabonomic changes were found.

Table 4.5. Significant changes in male and female Wistar rats administered TiO_2 nanoparticles via gavage for 14 days

Endpoint	Dose (mg/kg-d)			
	0	160	400	1,000
Serum Enzyme Activities				
AST (U/L)	137.49 ± 20.45[a]	160.00 ± 24.34[b]	186.98 ± 27.22[b]	179.71 ± 19.82[b]
LDH (U/L)	1,756.25 ± 512.11	2,176.56 ± 522.06[c]	2,898.50 ± 511.62[b]	3,010.14 ± 432.55[b]
CK (U/L)	874.4 ± 271.2	905.4 ± 242.5	1,245.4 ± 214.6[b]	1,395.9 ± 212.9[b]
Hematology				
WBC (10^9/L)	3.63 ± 1.11	3.27 ± 0.51	3.52 ± 1.08	4.55 ± 0.97[b]
Lymphocytes (10^9/L)	2.86 ± 0.99	2.47 ± 0.44	2.69 ± 0.83	3.65 ± 0.79[b]
Monocytes (10^9/L)	0.14 ± 0.06	0.15 ± 0.06	0.17 ± 0.08	0.21 ± 0.07[b]

[a]Mean ± SD (n = 16 animals/group).
[b]Statistically significant at $p < 0.01$ compared with controls.
[c]$p < 0.05$.

Source: Bu et al. (2010).

No histopathological changes in the liver, kidney, brain, adrenal gland, spleen, lung, heart, testis, ovary, or uterus were observed in TiO_2-treated rats (Bu et al., 2010). TEM examination showed mitochondrial swelling in the heart tissues of TiO_2-treated rats (incidence and dosage groups affected not reported), and no changes to other tissues. Bu et al. (2010) concluded that the observed exposure-related changes in metabonomic profiles were indicative of disturbances of energy metabolism and amino acid metabolism that may have been responsible for possible toxicological effects on the liver and heart. The low dose level in this study, 160 mg/kg-day for 14 days, is considered to be a LOAEL in Wistar rats for changes in serum enzyme activities and hematological endpoints.

In another study, CD-1 (ICR) female mice (20/group) were administered TiO_2 nanoparticles (anatase; 5 nm in size; containing 58% titanium, 41% oxygen, 0.2% carbon and 0.1% hydrogen) suspended in 0.5% HPMC K4 M 4 M) via gavage at 0, 62.5, 125, or 250 mg/kg-day every other day for 30 days (Duan et al., 2010). Clinical signs of toxicity and mortality were

monitored daily. Body weights were recorded at the start of treatment and prior to sacrifice (after treatment for 30 days). Clinical chemistry (including serum levels of ALT, ALP, AST, LDH, cholinesterase [ChE], total protein, albumin, globulin, total bilirubin, triglycerides, and total cholesterol) and hematological parameters (including counts of reticulocytes, platelets, WBCs and RBCs, levels of hemoglobin, and measurements of platelet distribution width [PDW], red [cell] distribution width [RDW], thrombocytocrit, hematocrit, mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], mean corpuscular volume [MCV], and mean platelet volume [MPV]) were evaluated in blood samples collected at the end of treatment. Differential lymphocyte counts (of B, CD3, CD4, CD8, B, and NK cells) were performed, and concentrations of interleukin-2 (IL-2) and NO in the plasma were measured. Organ weights (of the kidney, liver, spleen, and thymus) were recorded, and the kidney and liver were subjected to histopathological analyses.

Significant effects elicited by treatment with nano-TiO_2 are shown in Table 4.6 (Duan et al., 2010). No mortality or clinical signs of toxicity were observed. In comparison with controls, mice treated with TiO_2 nanoparticles at 125 or 250 mg/kg-day showed reductions in body weight gain of 15 and 22%, respectively, over the 30-day treatment period. In general, dose-dependent changes in clinical chemistry and hematological parameters were observed; statistically significant changes were reported for most endpoints for mid- and high-dose mice. Decreased thrombocytocrit (56–72%), and decreased numbers of WBCs (37–54%), RBCs (19–44%), and reticulocytes (69–82%) were observed in mid- and high-dose mice, whereas the number of platelets was increased (by 43 and 66% respectively) in the same treatment groups. Significant decreases in the number of WBCs (32%) and reticulocytes (27%) were also observed in low-dose mice. Hematocrit was significantly increased with respect to controls in the high-dose group only (37%). At 125 mg/kg-day, mean platelet and corpuscular volumes were significantly increased compared to the control group (by 18 and 12%, respectively); in addition to these parameters, MCH and RDW values were significantly increased (by 8 and 25%, respectively) at 250 mg/kg-day. In contrast, MCHC and hemoglobin levels were significantly decreased in the two highest dose groups (by 8–15 and 26–38%, respectively). Counts of immunologically competent cells (including CD3, CD4, and CD8 cells) were significantly decreased by 39–42% in mice treated with TiO_2 nanoparticles at the high-dose; counts of B cells and NK cells were also significantly decreased in all treated mice.

Table 4.6. Significant effects in female CD-1 (ICR) mice administered TiO_2 nanoparticles (anatase, 5 nm) via gavage for 30 days

Endpoint	Dose (mg/kg-d)			
	0	62.5	125	250
Body weight gain (g)	7.12 ± 0.36[a]	6.91 ± 0.35	6.06 ± 0.30[b] (-15)	5.57 ± 0.28[c] (-22)
Relative organ weight				
Liver (mg/g)	40.56 ± 2.03	44.92 ± 2.25	46.77 ± 2.34[b] (+15)	51.52 ± 2.58[c] (+27)
Kidney (mg/g)	13.56 ± 0.68	14.52 ± 0.73	15.23 ± 0.77[b] (+12)	17.38 ± 0.87[c] (+28)
Spleen (mg/g)	3.69 ± 0.18	4.05 ± 0.20	4.45 ± 0.22[b] (+21)	4.97 ± 0.25[c] (+35)
Thymus (mg/g)	2.22 ± 0.11	2.79 ± 0.14	3.08 ± 0.15[b] (+39)	3.26 ± 0.16[c] (+47)

Table 4.6. Significant effects in female CD-1 (ICR) mice administered TiO_2 nanoparticles (anatase, 5 nm) via gavage for 30 days

Endpoint	Dose (mg/kg-d)			
	0	62.5	125	250
Clinical chemistry				
ALT (U/L)	29 ± 1.45	36 ± 1.80	44 ± 2.22[b] (+52)	59 ± 2.95[c] (+103)
AST (U/L)	168 ± 40	149 ± 7.45	201 ± 10.05[b] (+20)	245 ± 12.25[c] (+46)
ALP (U/L)	90 ± 4.50	94 ± 4.70	114 ± 5.70[b] (+27)	133 ± 6.65[c] (+48)
LDH (U/L)	1,035 ± 51.75	1,182 ± 59.10	1230 ± 61.50[b] (+19)	1,795 ± 89.75[c] (+73)
ChE (U/L)	1,179 ± 58.95	1,223 ± 61.15	1,447 ± 72.35[b] (+23)	1,681 ± 84.05[b] (+43)
Total protein (g/L)	51.0 ± 2.55	56.90 ± 2.85	61.32 ± 3.07[b] (+20)	65.91 ± 3.30[b] (+29)
Albumin/globulin ratio	1.40	1.47	1.22[b] (-13)	1.13[b] (-19)
Total bilirubin (mmol/L)	1.4 ± 0.07	1.1 ± 0.06	0.8 ± 0.04[b] (-43)	0.5 ± 0.03[c] (-64)
Total cholesterol (mmol/L)	2.33 ± 0.12	2.59 ± 0.13	2.95 ± 0.15[b] (+27)	3.13 ± 0.16[c] (+34)
Triglycerides (mmol/L)	1.55 ± 0.08	1.99 ± 0.10	2.3 ± 0.12[b] (+48)	2.48 ± 0.12[c] (+60)
IL-2 (pg/mL)	1.25 ± 6.25	1.08 ± 5.40[d] (-14)	8.3 ± 4.15[d] (+564)	5.7 ± 2.85[d] (+356)
NO (µmol/L)	3.34 ± 0.17	5.58 ± 0.28[c] (+40)	7.21 ± 0.36[c] (+116)	9.66 ± 0.48[c] (+189)
Hematology				
WBC (10^9/L)	4.92 ± 0.25	3.33 ± 0.17[b] (-32)	3.08 ± 0.15[b] (-37)	2.25 ± 0.11[c] (-54)
RBC (10^{12}/L)	9.26 ± 0.46	8.21 ± 0.41	7.53 ± 0.38[b] (-19)	5.15 ± 0.26[c] (-44)
Hemoglobin (g/L)	168 ± 8	148 ± 7	125 ± 6[b] (-26)	105 ± 5[c] (-38)
MCV (fL)	53.15 ± 2.66	55.59 ± 2.78	59.28 ± 2.96[b] (+12)	66.47 ± 3.32[c] (+25)
MCH (pg)	17.6 ± 0.9	18.2 ± 0.9	18.5 ± 0.9	19.0 ± 1.0[b] (+8)
MCHC (g/L)	358 ± 18	346 ± 17	329 ± 16[b] (-8)	306 ± 15[c] (-15)
RDW (%)	15.78 ± 0.79	15.14 ± 0.76	16.46 ± 0.82	19.72 ± 0.99[b] (+25)
Platelets (10^9/L)	423 ± 21	482 ± 24	605 ± 30[c] (+43)	701 ± 35[c] (+66)
Thrombocytocrit (%)	39.05 ± 1.95	29.11 ± 1.45	17.08 ± 0.85[b] (-56)	11.01 ± 0.55[c] (-72)
Reticulocytes (%)	6.56 ± 0.33	4.79 ± 0.24[b] (-27)	2.06 ± 0.10[c] (-69)	1.18 ± 0.06[c] (-82)
Hematocrit (%)	40.11 ± 2.00	42.05 ± 2.10	45.06 ± 2.25	55 ± 2.75[b] (+37)
MPV (fL)	6.2 ± 0.3	6.6 ± 0.3	7.3 ± 0.4[b] (+18)	8.1 ± 0.4[c] (+60)
CD3 (%)	54.02 ± 2.70	72.80 ± 3.64	56.80 ± 2.84	31.62 ± 1.58[c] (-42)
CD4 (%)	75.30 ± 3.77	74.16 ± 3.21	72.36 ± 3.62	40.54 ± 2.03[c] (-46)
CD8 (%)	59.40 ± 2.97	61.05 ± 3.05	59.25 ± 2.96	36.5 ± 1.83[c] (-39)
CD4/CD8	1.27 ± 0.06	1.21 ± 0.06	1.22 ± 0.06	1.11 ± 0.06[b] (-13)
B cell (%)	18.00 ± 0.90	12.20 ± 0.61[b] (-32)	8.60 ± 0.43[c] (-52)	5.30 ± 0.27[c] (-71)
NK cell (%)	80.72 ± 4.04	70.80 ± 3.54[b] (-12)	65.50 ± 3.28[c] (-19)	56.10 ± 2.81[c] (-31)

[a]Mean ± standard error (n = 10 animals/group).
[b]Statistically significant at $p < 0.05$.
[c]$p < 0.01$.
[d]Reported to be statistically significant by the researchers, but this could not be verified using the data from the original report.

Note: numbers in parentheses represent the percent change in compared to the control group and were calculated for this review.

Source: Duan et al. (2010).

Biochemical parameters of liver function were also affected by treatment; the activities of all serum enzymes evaluated were significantly increased in mid- and high-dose mice (Duan et al., 2010). Similarly, significant elevations in total protein (20–29%), total cholesterol (27–34%), and triglycerides (48–60%) were observed in the same treatment groups; whereas total bilirubin was significantly decreased (by 43–64%) relative to controls. Levels of NO were significantly increased (by 40–189%) in treated mice (all dosage groups) compared to controls. Although the authors also reported statistically significant changes in IL-2 levels in all treated mice, these results could not be verified by independent statistical analyses (unpaired *t*-tests performed for this review). Relative weights of the liver, kidney, spleen, and thymus were 12–47% higher in mid- and high-dose mice than controls. Histopathological examination of the liver showed no abnormalities at 125 mg/kg-day; however, mice administered 250 mg/kg-day showed histopathological changes including congestion of interstitial vessels and altered hepatocyte structure (incidence data not reported). The results are consistent with designating 62.5 mg/kg-day as a NOAEL and 125 mg/kg-day as a LOAEL for decreased body weight gain, increased relative organ weights (liver, kidney, spleen, and thymus), increased serum enzyme markers for hepatotoxicity (e.g., ALT, AST, ALP, LDH), marked increases in serum levels of NO, and marked decreases in several hematological endpoints including WBCs, RBCs, hemoglobin, and reticulocytes in CD-1 (ICR) female mice exposed by gavage for 30 days to suspensions of anatase TiO_2 nanoparticles. At the highest dose level in this study, 250 mg/kg-day, exposed mice also showed histological signs of interstitial congestion and altered hepatocyte structure in the liver. Statistical changes in some hematological and immune endpoints at the low dose of 62.5 mg/kg-day were not considered to be biologically significant by the researchers.

4.3.3. Dermal Exposure

BALB/c *nu/nu* hairless mice (6/group; sex not specified) were administered suspensions of 5% TiO_2 nanoparticles (in carbopol 940, triethanolamine, and demineralized water) to a 3-cm^2 patch of the dorsal skin (in the interscapular region) for 60 days (Wu et al., 2009). A vehicle-only control group was used. The physical properties of the particles utilized were measured experimentally: 10 nm anatase TiO_2 particles (99.5% pure) had a hydrophobic surface with an area of 160 m^2/g; 25 nm rutile TiO_2 particles (99.6% pure) had a hydrophilic surface with an area of 80 m^2/g; Degussa P25 particles (99.5% pure) were ~21 nm in size, 25% rutile/75% anatase, and had a hydrophilic surface with an area of 50 m^2/g; and 60 nm rutile TiO_2 particles (99.6% pure) had a hydrophobic surface with an area of 40 m^2/g. An additional group of mice was exposed to coarse TiO_2 particles (300–500 nm in size). Each day, 24 mg of suspension (or 400 µg TiO_2/cm^2, as estimated by the authors) was applied as uniformly as possible to the treatment area and left uncovered for 3 hours; residual TiO_2 was removed after treatment by washing. The estimated daily dose was 6.7 mg/kg-day ([1.2 mg TiO_2/day] × [1/0.18 kg body weight]). Animals were observed daily for mortality and clinical signs of toxicity. Body weights were recorded at the start of the experiment and every 7 days thereafter. Mice were sacrificed 24 hours following the last exposure; levels of malondialdehyde (MDA) and SOD activity (biomarkers of antioxidant response) were measured in homogenized skin and liver samples. Skin samples were also evaluated for hydroxyproline (HYP) content. Organ weights (of the skin, subcutaneous muscle, liver, heart, spleen, kidneys, brain, and lung) were recorded; these tissues were also subjected to histopathological analyses.

Significant findings from this experiment are shown in Table 4.7 (Wu et al., 2009). No mortality or clinical signs of toxicity were reported for any treatment group. Mice treated with the three smallest-sized (10 nm, 25 nm, or P25) TiO_2 nanoparticles showed decreased body weight gains of >10%, compared with controls. Similarly, relative organ weights (expressed as percent body weight) for the liver were 8–12% higher in the same treatment groups compared with controls; relative spleen weights were 13 and 24% higher in P25- and 10-nm treatment groups, respectively. The relative heart weights of treated mice were similar to controls. With respect to the control group, the activity of SOD in the liver and skin was significantly decreased (27–43%) in mice treated with 10 nm or P25 particles. MDA content was increased in the skin of all nanosized TiO_2-treated mice; treatment groups affected were 10 nm (fourfold change) > P25 > 25 nm > 60 nm (1.4-fold change). In the liver, MDA levels were elevated significantly by treatment with the three smallest TiO_2 nanoparticles only; levels were 85, 34, and 109% higher than controls in 10 nm, 25 nm, and P25 treatment groups, respectively. Significant reductions in HYP content of the skin were also observed in all nano-TiO_2 treated mice; the most dramatic effects (approximately twofold changes) were seen in the 10 nm and P25 treatment groups.

Table 4.7. Significant changes in BALB/c *nu/nu* hairless mice treated with a 5% formulation of TiO_2 nanoparticles via the dermal route for 60 days

	Particle size (nm)					
Endpoint	**Control**	**10**	**25**	**21**	**60**	**300–500 (coarse)**
Number of animals examined	6	6	6	6	6	6
Initial body weight (g)	18.34 ± 1.91[a]	18.15 ± 2.10	17.81 ± 2.12	18.56 ± 1.72	17.93 ± 2.01	18.22 ± 1.73
Terminal body weight (g)	21.82 ± 1.31	17.46 ± 2.32[b]	18.51 ± 2.64[b]	17.73 ± 2.66[b]	21.43 ± 1.92	22.47 ± 2.52
Organ coefficients (percent body weight)						
Liver	6.36 ± 0.51	6.91 ± 0.72[b]	6.88 ± 0.41[b]	7.13 ± 0.6[b]	6.52 ± 0.55	6.28 ± 1.09
Spleen	0.46 ± 0.13	0.57 ± 0.09[b]	0.49 ± 0.10	0.52 ± 0.12[b]	0.47 ± 0.06	0.46 ± 0.08
SOD activity (U/mg protein)						
Skin	27.02 ± 8.31	16.76 ± 4.31[b]	24.54 ± 3.67	17.76 ± 4.61[b]	25.46 ± 6.07	26.87 ± 5.51
Liver	45.61 ± 10.31	25.86 ± 9.27[c]	43.61 ± 10.31	33.18 ± 12.23[b]	42.98 ± 15.65	46.15 ± 15.52
MDA level (nmol/mg protein)						
Skin	2.79 ± 1.09	11.15 ± 2.12[c]	5.32 ± 1.17[b]	8.12 ± 2.72[c]	3.91 ± 1.54[b]	3.22 ± 1.15
Liver	4.89 ± 1.42	8.99 ± 2.15[c]	6.54 ± 1.86[b]	10.21 ± 3.41[c]	5.62 ± 1.93	5.16 ± 2.10
HYP level (μg/mg protein)						
Skin	2.36 ± 0.67	1.21 ± 0.32[c]	1.88 ± 0.51[b]	1.13 ± 0.25[c]	1.92 ± 0.59[b]	2.28 ± 0.69

[a]Mean ± SD.
[b]Significantly different from control at $p < 0.05$.
[c]$p < 0.01$.

Source: Wu et al. (2009).

Histopathological changes were apparent in nano-TiO_2-treated mice (Wu et al., 2009). Compared to controls, the skin of all treated mice showed excessive keratinization, and a thinner and more wrinkled epidermis (particularly evident in the 10 nm and P25 treatment groups). In the liver, focal necrosis (25 nm, P25, and 60 nm treatment groups) and liquefaction necrosis (10 nm treatment group) were apparent (incidence data not reported). With the exception of small traces of WBC in heart tissue sections of mice treated with 10 nm particles, no signs of heart damage were observed in any treatment group. Minor lesions in the spleen (increased proliferation of local macrophages), kidney (subcutaneous saccus lymphaticus), and lung (slight alveolar thickening) were noted in nano TiO_2-treated mice (incidence data not reported). No histopathological lesions were detected in the brain. Based on decreased body weight gain, changes in relative weights of the liver and spleen, changes in the activities of SOD and HYP in the liver and skin, and histopathological changes to the skin (keratinization and wrinkling) and liver (focal necrosis), which occurred mainly in mice treated with TiO_2 with primary particle sizes of 10, 21, and/or 25 nm, the test concentration of 5% TiO_2 (about 6.7 mg/kg-day) is identified as a LOAEL in BALB/c *nu/nu* hairless mice exposed dermally for 60 days; no NOAEL was identified.

Also as part of the Wu et al. (2009) study, domestic pigs were dermally exposed to test formulations containing 5% (w/w) TiO_2 nanoparticles with average diameters of 4 nm (anatase) or 60 nm (rutile) (Wu et al., 2009). About 24 mg of the test formulation (5% TiO_2 in 2% carbopol 940 and triethanolamine in water or vehicle alone in control pigs) was applied daily to a 3-cm^2 area (clipped 24 hours before treatment) of the dorsal surface of the right ear of three pigs (3 week old at start) for 30 consecutive days. Skins of untreated ears served as controls. No clinical signs of toxicity were reported. Skin irritation was evaluated by visual scoring using a modified Draize method. Exposure to either type of nanoparticle did not cause erythema or edema of the treated area. However, TEM analyses of the epidermis showed pathological changes in pigs treated with nano-TiO_2 (4 nm in size), including extended intracellular space, impairment of the desmosome, and vacuoles around the nuclei in basal cells. Based on these histopathological changes in the skin, a free-standing LOAEL of 5% nano-TiO_2 (the only concentration tested) is identified for this study.

4.4. CHRONIC TOXICITY/CARCINOGENICITY

In several epidemiological studies, there was no clear evidence of increased respiratory disease or lung cancer mortality ratios in workers exposed occupationally to TiO_2 dusts (Boffetta et al., 2004; Fryzek at al., 2003, as cited in Boffetta et al., 2004; Chen and Fayerweather, 1988). Although workers were presumably exposed to both fine and ultrafine TiO_2 particles, these studies were not designed to investigate the relationship between TiO_2 particle size and mortality risk; specific exposures to nanosized particles were not estimated.

Chronic-duration cancer/toxicity bioassays, conducted by Heinrich et al. (1995), are available for rats and mice exposed to aerosols of TiO_2 nanoparticles, but these studies included only one exposure level and evaluated endpoints were limited to mortality and clinical signs, body and lung weights, BAL fluid variables, and respiratory tract histology.

Female Wistar rats were whole-body exposed to TiO_2 nanoparticles as an aerosol at an average exposure concentration of 0 (clean air control) or 10 mg/m^3 for 18 hours/day, 5 days/week for up to 2 years (Heinrich et al., 1995). Actual exposure concentrations were 7.2 mg/m^3 for the first 4 months, 14.8 mg/m^3 for 4 additional months, and 9.4 mg/m^3 for the remaining exposure period (16 months). Several groups of rats were exposed to TiO_2 using this exposure protocol to evaluate different endpoints; 80, 66, and 28 rats/group were used to evaluate histology, particle mass/lung, and lung clearance, respectively. Additional groups of animals (100 TiO_2-treated rats and 220 controls) were exposed to TiO_2 for 24 months and observed for 6 months post-exposure to assess carcinogenicity. TiO_2 particles used as a starting material were 80% anatase/20% rutile, measured 15–40 nm in size, and had a surface area of 48 m^2/g. Aerosols of TiO_2 were generated by dry dispersion using a screw feeder and a pressurized air dispersion nozzle. The aerosols generated from TiO_2 nanoparticles had a MMAD of ~1.5 µm. To increase deposition efficiency, particle size distribution was reduced via filtering of coarse particles to produce MMAD and GSDs of 0.80 µm and 1.80, respectively. The concentrations of various gaseous exhaust components in the exposure chamber were monitored continuously; none were detected at mean exposure concentrations >2 ppm. Animals were monitored regularly for mortality and clinical signs of toxicity. Body weights were recorded every 4 weeks. Animals in the histology group were sacrificed at 6, 12, 18, and 24 months (20 per time point). Lungs of sacrificed animals were lavaged; biochemical and cytological parameters (including LDH and β-glucoronidase activities, total protein, HYP content, total number of leukocytes, and differential cell counts) were evaluated in the BAL fluid. Lung weights (wet) were recorded 3, 6, 12, 18, 22, and 24 months after the start of exposure (particle mass/lung group). Histopathological analyses of the nasal and paranasal cavities (four sections), larynx, trachea, and lung (five sections) were performed on all animals. Examination of animals for gross or histological changes in other tissues is not mentioned in the published report (Heinrich et al., 1995).

Mortality in TiO_2-treated rats reached 60% by 24 months, compared to 42% in control rats (Heinrich et al., 1995). The researchers did not provide an explanation for the high mortality rates and did not attempt to correlate mortality with lung tumor incidence. By the end of the observation period (carcinogenicity group), mortality was 90 and 85% in treated and control groups, respectively. Compared with control rats, the mean lifespan of rats treated with TiO_2 was significantly shortened ($p < 0.05$; Kaplan-Meier method). Body weights of exposed rats were also significantly lower than controls at ≥400 days exposure (data not shown); by the end of the exposure, the mean body weight of treated rats was 12.5% less than control rats. Exposure-related effects on BAL parameters (differential cell counts and the concentrations of LDH, β-glucoronidase, HYP, and total protein; direction and magnitude of changes not reported) were observed throughout the entire exposure period (24 months; data not shown). Wet lung weights increased with respect to controls in a duration-dependent manner in TiO_2-treated rats (peaking to ~6 times higher than controls at 18 months exposure, based on data presented graphically). After exposure to TiO_2 for 6 months, rats (histology group) showed slight bronchioalveolar hyperplasia (20/20; incidence in control group not specified) and very slight to slight interstitial fibrosis (incidence not reported). Exposure for 24 months resulted in moderate-to-severe bronchiolar hyperplasia (99/100 in the carcinogenicity group) and slight to moderate interstitial fibrosis and the detection of free particles and particle-laden macrophages in the alveolar region of the lungs (all treated animals; incidence data for control group not reported). Based on

shortened life span, decreased body weight, changes in BAL fluid parameters (including differential cell counts and the concentrations of LDH, β-glucoronidase, HYP, and total protein), and histopathological changes in the lung (bronchioalveolar hyperplasia, interstitial fibrosis, and particle-laden macrophages in the alveolar region), 10 mg/m^3 (the only exposure concentration tested) is identified as a LOAEL in Wistar rats exposed for up to 24 months to uncoated TiO_2 nanoparticles (80% anatase: 20% rutile); no NOAEL was identified.

Lung tumors were observed in groups of treated rats (serial histology group) sacrificed after ≥18 months of exposure; at 18 months, the incidence of lung tumors (benign squamous-cell tumors, squamous cell-carcinomas, and adenocarcinomas) was significantly higher ($p < 0.05$) in TiO_2-treated rats (5/20) than control rats (0/18). Similarly, at 24 months, the incidence of lung tumors was 4/9 in TiO_2 treated rats and 0/10 in controls. In rats sacrificed at 30 months (carcinogenicity group), tumor incidence was 32% in rats exposed to TiO_2 compared to ~0% (1/217) in control rats (see Table 4.8); 8% of treated rats had two lung tumors. Despite few findings of lung tumors in control rats, high mortality within this group may have contributed to a lower rate of lung tumor incidence than might be expected given a lower mortality rate. The published report did not mention finding tumors in other tissues examined histologically (i.e., nasal and paranasal cavities, larynx, and trachea).

Table 4.8. Incidence of lung tumors in female Wistar rats after exposure to TiO_2 nanoparticles (15–40 nm primary particle size) via inhalation for 24 months and 6 months recovery (carcinogenicity group).

Exposure concentration (mg/m^3)	Incidence of lung tumors
0	1/217 (~0%)
10	32/100 (32%)

Source: Heinrich et al. (1995).

As part of the same experiment, NMRI mice (80, 40, and 40 females in the carcinogenicity, serial histology, and serial particle mass/lung groups, respectively) were whole-body exposed to TiO_2 nanoparticles (exhibiting the same particle characteristics as reported above for rats) at an average exposure concentration of 10 mg/m^3 for 18 hours/day, 5 days/week, for 13.5 months (Heinrich et al., 1995). With the exception of BAL fluid parameters, which were not evaluated in mice, the same endpoints were assessed in rats and mice. Actual exposure concentrations were 7.2 mg/m^3 for the first 4 months, 14.8 mg/m^3 for 4 additional months, and 9.4 mg/m^3 for the remaining exposure period (5.5 months). Animals were exposed to clean air and observed for up to 9.5 months post-exposure. Although it was not explicitly stated by the researchers that ~15 mg/m^3 was intended to be the exposure concentration for the duration of the study, the researchers did state that the exposure concentration was reduced to ~10 mg/m^3 8 months after the initiation of exposure because mice exhibited increased mortality and showed clinical signs of toxicity (individual body weight loss and bad general condition). By the end of the exposure period (13.5 months), mortality reached 33% in the TiO_2 treatment group, compared with 10% in the control group; the researchers did not indicate the cause of death in untreated or treated mice. Compared with control mice, the mean lifespan of mice treated with TiO_2 was significantly shortened ($p < 0.05$). A statistically significant decrease in the body weight of treated rats (5–7%) compared with controls was reported 8 months after the start of

exposure; however, the body weights of treated and control rats were similar by the end of the exposure period (data not shown). Lung weights (measured at 3, 6, 12, 18, and 21 months) were significantly increased in TiO_2-treated mice compared with control mice during the exposure period; wet lung weights were 1.5- and 4.5-fold higher in treated mice 3 and 12 months after the initiation of exposure, respectively (see Table 4.9; data for other time points not provided). By the end of exposure period, the wet lung weight of treated mice decreased slightly relative to the 12-month time point. Nonneoplastic lesions in the histologically examined tissues (nasal and paranasal cavities, larynx, trachea, and lung) were not mentioned in the published report. Based on clinical signs of toxicity, shortened life span, decreased body weight, and increased wet lung weights, 10 mg/m^3 (the only exposure concentration tested) is identified as a LOAEL in NMRI mice exposed for up to 13.5 months to uncoated TiO_2 nanoparticles (80% anatase: 20% rutile); no NOAEL was identified.

Table 4.9. Wet lung weights in NMRI mice treated with TiO_2 nanoparticles (15–40 nm primary particle size) via inhalation for 13.5 months

	Average exposure concentration (mg/m^3)	
	0	10
Wet lung weight (g)		
3 mo after initiation of exposure	0.2	0.3
12 mo after initiation of exposure	0.2	0.9
13.5 mo after initiation of exposure	Not reported	0.7

Source: Heinrich et al. (1995).

Although lung tumors (adenomas and adenocarcinomas) were observed in TiO_2-treated mice, the incidence in treated mice (11.3 and 2.5% for the aforementioned tumor types, respectively) were not significantly different from control animals, which actually had higher incidences of these tumor types than treated mice (25 and 15.4%, respectively). Similarly, the combined tumor incidence was not significantly different among treated (13.8%) and control (30%) mice. The use of the NMRI mouse, which has a very high spontaneous lung tumor rate (historical control of ~30%), and the shortened life-span of exposed mice may have masked detection of treatment-related lung tumors.

4.5. REPRODUCTIVE/DEVELOPMENTAL TOXICITY

Time-mated C57BL/6BomTac mice (22 or 23/group) were whole-body exposed to TiO_2 particles as an aerosol at targeted concentrations of 0 (filtered air control) or 40 mg/m^3 for 1 hour/day on gestation days (GDs) 8–18 (Hougaard et al., 2010). The mean particle mass concentration (measured) was 42 ± 2.9 mg/m^3. A commercially available form of TiO_2 particles (chemically modified with zirconium, silicon, and aluminum, and coated with polyalcohols) was used as a starting material; particle properties (determined experimentally) showed that they were composed of ~71% TiO_2 by weight, elongated and rutile, and had an average size and surface area of 21 nm and 108 m^2/g, respectively. TiO_2 nanoparticles were aerosolized with a microfeeder through a dispersion nozzle. Based on filter measurements, the geometric mean number diameter of aerosols generated from TiO_2 nanoparticles was 97 nm; the fraction of nanosized (<100 nm) particles was <1% the total particle mass. A cumulative exposure

equivalent to 840 µg TiO_2/animal was estimated by the authors. Exposed females were observed for mortality and clinical signs of toxicity. Body weights of dams were recorded prior to exposure, on GDs 9, 11, 14, and 18; and on postnatal days (PNDs) 1, 8, 11, 16, 19, and 22. Nonpregnant time-mated females (sacrificed 5 days post-exposure) as well as dams that produced litters (sacrificed on PNDs 24–25) were subjected to BAL; numbers of total and dead cells and differential cell counts were evaluated in the BAL fluid. Sacrificed dams were examined for determination of uterine implantation sites; organs (including the lungs, liver, heart, brain) were weighed and dissected. Individual pups were counted and sexed on PND 1; they were weighed on the same days as dams. On PND 22, one male and one female pup/litter were selected for a battery of behavioral testing, including assessments of learning and memory (Morris water maze; evaluated at ages 11 and 15 weeks for males and 12 and 16 weeks for females), activity (dry water maze pool, tested in open field at age 14 weeks), and acoustic startle reaction and prepulse inhibition (tested at age 4 months). The organs of pups, sacrificed on PNDs 2 (one pup from litters with five or more pups) and 23–24 (one male and one female pup/litter), were weighed and dissected (same organs as dams). At 19 weeks of age, surviving control and exposed offspring were cross-mated to naïve CBA/J mice; time-to-first-delivery of F2 litter, litter size, and gender were recorded. The litter was considered the statistical unit for all analyses.

No mortality, clinical signs of toxicity, or variations in body weight were reported in females exposed to TiO_2 (Hougaard et al., 2010). Similar numbers of control and TiO_2-exposed F0 mice produced litters; a slight decrease in the viability of pups from TiO_2-exposed dams was not statistically significant ($p = 0.08$). However, the number of F1 pups that died during lactation was significantly higher in the TiO_2-treated group than the control group (Table 4.10). Neutrophil counts in the BAL fluid of exposed mice were significantly higher than control values (numerical data not reported); this effect was more marked in exposed nonpregnant females (19-fold higher than controls; n = 9; $p < 0.01$) than exposed pregnant females (threefold higher than controls; n = 14; $p < 0.05$). Lymphocytes were also significantly increased (approximately fourfold) and macrophages were significantly decreased (by approximately 35%) in the BAL fluid of exposed mice (nonpregnant) compared to concurrent controls ($p < 0.05$; based on data presented graphically). All other BAL parameters (total cell counts and numbers of eosinophils and epithelial cells) were similar for unexposed and exposed F0 mice. No implantation sites were detected in any of the timed-pregnant females that failed to produce litters. Absolute and relative lung weights, but no other organ weights, were significantly increased in pregnant exposed mice with respect to controls ($p \leq 0.05$; data not shown).

Table 4.10. Significant changes in C57BL/6BomTac mice exposed to TiO_2 nanoparticles via inhalation for 1 hour/day on GDs 8–18

Endpoint	Exposure concentration (mg/m^3)	
	0	40
Number of time-mated females	22	23
Pups dead during lactation	0.31 ± 0.24[a]	0.71 ± 0.30[b]
Total dead cell count (BAL fluid)		
D 26–27 post-exposure	13,600 ± 3,748 (9)	25,857 ± 3,141[c](15)

[a]Data expressed as mean ± standard error of the mean.
[b]Significantly different from control at $0.05 < p < 0.01$.
[c]$p < 0.01$.

Note: value in parenthesis indicates percent of total cell count.

Source: Hougaard et al. (2010).

No significant changes in the number, sex distribution, or body weights of pups in TiO_2-exposed litters were reported (Hougaard et al., 2010). Exposed and unexposed pups performed similarly in the Morris water maze (data not shown). In comparison to unexposed controls, exposed pups paid shorter and fewer visits to the central zone in open field tests; this indicated to the researchers that exposed pups exhibited altered neurological behavior. Exposed males visited ~45% less frequently than unexposed males ($p < 0.05$), and exposed females spent ~35% less time per visit than unexposed females ($p < 0.01$) in the central zone (percentages estimated based on data presented graphically). A significantly stronger prepulse inhibition response was also observed in female TiO_2-exposed offspring compared to concurrent controls at the highest prepulse level (86 dB). Altered performance in a battery of neurobehavioral tests suggested to the researchers that the fetal nervous system is a sensitive target after prenatal inhalation exposure. No exposure-related effects on organ weights in the F1 offspring were reported. Time-to-first-delivery of F2 litters was similar in control and exposed female offspring, but was extended by 30% in females mated to exposed males compared to females mated to unexposed males (difference not statistically significant). Litter sizes were similar for control and exposed F2 litters. Based on evidence of lung inflammation (including increased numbers of neutrophils and lymphocytes in the BAL fluid) and increased absolute and relative lung weights, 40 mg/m^3 (the only concentration tested) is identified as a maternal LOAEL; no NOAEL was identified. Based on increased pup mortality during lactation and neurobehavioral effects in pups (altered performance in neurobehavioral tests), 40 mg/m^3 (1 hour/day on GDs 8–18) is also identified as a free-standing LOAEL for developmental toxicity. No NOAEL for developmental toxicity was identified. The results of the cross-mating trials for the F1 offspring indicated that the exposure conditions did not adversely affect the ability of the F1 offspring to produce F2 litters.

4.6. MECHANISTIC DATA ON TITANIUM DIOXIDE NANOPARTICLES

4.6.1. Genotoxicity

Results from genotoxicity studies are summarized in Table 4.11.

Table 4.11. Summary of genotoxicity data for TiO_2 nanoparticles

Endpoint	Assay	Test system	Exposure	Particle data	Result	Comments	Reference
Mutation	Reverse mutation assay	*Salmonella typhimurium* strains TA97a and TA100 and *Escherichia coli* WP2*uvr*A	0, 10, 100, or 1,000 µg/plate for 72 hrs	<100 nm in size	Negative, with or without metabolic activation (*S. typhimurium*); positive in WP2 with activation		Pan et al., 2010
	Reverse mutation assay	*S. typhimurium* strains TA 98, TA100, TA1535, and TA1537 and *E. coli* WP2*uvr*A	0, 100, 333, 1,000, or 5,000 µg/plate	Median primary particle size of 140 nm (in water); 79% rutile/21% anatase; surface area = 38.5 m^2/g	Negative, with or without metabolic activation		Warheit et al., 2007
	Gpt-delta assay	MEF cells	0, 0.1, 1, 10, or 30 µg/mL for 24 hrs	Anatase particles with average primary particle diameters of 5 or 40 nm and surface areas of 114 and 38 m^2/g, respectively; and -325 mesh TiO_2 (fine) with a surface area of 9 m^2/g	Positive for 5 and 40 nm particles (all doses); negative for -325 mesh TiO_2		Xu et al., 2009
	HPRT assay	WIL2-NS (human B-cell lymphoblastoid) cells	0, 26, 65, or 130 µg/mL for 6, 24, or 48 hrs	Particles 99% pure	Positive results reported at 130 µg/mL for 24 hrs (data not shown)	Particle size distribution: by volume 6.57 nm (100%); by intensity 8.2 nm (80%) and 196 nm (19%)	Wang et al., 2007
	HPRT assay	Alveolar type II cells (rats)	0, 10, or 100 mg/kg via intratracheal instillation	Anatase; median diameter 180 nm; surface area of 8.8 m^2/g	Positive at 100 mg/kg		Driscoll et al., 1997

Table 4.11. Summary of genotoxicity data for TiO_2 nanoparticles

Endpoint	Assay	Test system	Exposure	Particle data	Result	Comments	Reference
Clastogenicity	SCE	CHO-K1 cells	0, 5,10, or 25 µg/mL for 24 hrs	Average particle size 20 ± 7 nm; surface area of 142 m^2/g	Positive at 1–5 µg/mL	Cytotoxicity was apparent at 10 and 25 µg/mL	Di Virgilio et al., 2010
	SCE	CHO-K1 cells	1–20 µM (0.08–1.6 µg/mL) for 24 hrs	No data available	Positive	Phase and particle size not reported	Lu et al., 1998, as cited in Di Virgilio et al., 2010
	Chromosomal aberrations	CHO-WBL cells	Concentrations up to 5,000 µg/mL or that resulted in ≥50% toxicity (whichever was lowest)	Eight test particles (coated, doped, and uncoated), various crystal types, particle sizes ranging from 14 to 60 nm	Negative (in the presence or absence of UV light)		Theogaraj et al., 2007
	DNA deletions	C57B1/6Jp^{un}/p^{un} mice	500 mg/kg in drinking water during pregnancy	75% anatase, 25% rutile particles; purity 99.5%, primary particle size 21 nm	Positive	Offspring were sacrificed at PND 20 and assessed for deletions	Trouiller et al., 2009
	MN formation	V79 cells (hamster lung fibroblasts)	0, 1, 5, 10, 15, or 25 µg/cm^3 for 24, 48, or 72 hrs	Anatase with a diameter of 30–50 nm, uncoated or coated with V_2O_5	Negative (uncoated particles); positive (coated particles) at 2 µg/cm^3 for 24 or 48 hrs	Pure V_2O_5 alone did not induce genotoxic effects in V79 cells at the applied concentrations	Bhattacharya et al., 2008
	MN formation	CHO cells	0, 750, 1,250, or 2,500 µg/mL without activation (4 hrs), 0, 62.5, 125, or 250 µg/mL with activation (4 hrs), 0, 25, 50, or 100 µg/mL without activation (20 hrs)	Median primary particle size of 140 nm (in water); 79% rutile/21% anatase; surface area = 38.5 m^2/g	Negative		Warheit et al., 2007
	MN formation	CHO-K1 cells	0, 0.6, 1, 6, or 10 µg/mL for 24 hrs	Average particle size 20 ± 7 nm; surface area of 142 m^2/g	Positive at 0.5 and 1 µg/mL	Cytotoxicity was apparent at 10 µg/mL	Di Virgilio et al., 2010
	MN formation	CHO-K1 cells	1–20 µM (0.08–1.6 µg/mL) for 24 hrs		Positive	Phase and particle size not reported	Lu et al., 1998, as cited in Di Virgilio et al., 2010

Table 4.11. Summary of genotoxicity data for TiO_2 nanoparticles

Endpoint	Assay	Test system	Exposure	Particle data	Result	Comments	Reference
	MN formation	SHE cells	0, 0.5, 1.0, 5, or 10 μg/cm^2 for 12, 24, 48, 66, or 72 hrs	Particle size ≤20 nm	Positive at 1.0 μg/cm^2 for 24, 48, 66, or 72 hrs; at 5.0 μg/cm^2 for 24 hrs		Rahman et al., 2002
	MN formation	RLE cells	0, 5, 10, or 20 μg/cm^2 for 21 hrs	Anatase particles, 20 nm in size	Negative		Linnainmaa et al., 1997
	MN formation	BEAS-2B (human bronchial) cells	0, 1, 5, 10, 20, 40, 60, 80, or 100 μg/cm^2 (or 0, 3.8, 19, 38, 76, 114, 228, 304, or 380 μg/mL) for 24, 48 or 72 hrs	Uncoated anatase (<25 nm); rutile (10 × 40 nm) coated in SiO_2	Positive for anatase particles at 10 or 60 μg/cm^2 for 72 hrs Negative for coated rutile particles	Average agglomerate sizes were 5.5 and 4.5 μm for anatase and coated rutile particles, respectively	Falck et al., 2009
	MN formation	BEAS-2B (human bronchial) cells	10 μg/mL for 24 hrs	Anatase particles, 10 nm in diameter	Positive		Gurr et al., 2005
	MN formation	Human peripheral blood lymphocytes	0, 20, 50, or 100 μg/mL for 48 hrs	70–85% anatase, 15–30% rutile particles, ~30 nm in size	Positive at ≥50 μg/mL		Kang et al., 2008
	MN formation	L-02 (human embryo) hepatocytes	0, 0.01, 0.1, or 1 μg/L for 24 hrs	The anatase: rutile ratio of particles was 8:2; primary particle diameter ~25 nm	Negative		Shi et al., 2010
	MN formation	WIL2-NS (human B-cell lymphoblastoid) cells	0, 26, 65, or 130 μg/mL for 6, 24, or 48 hrs	Particles 99% pure	Positive at 26 μg/mL (48 hrs), 65 μg/mL (all time points), and 130 μg/mL (6 hrs)	Particle size distribution: by volume 6.57 nm (100%); by intensity 8.2 nm (80%) and 196 nm (19%) Cytotoxicity (>60%) was observed at 130 μg/mL in cells treated ≥24 hrs	Wang et al., 2007
	MN formation	C57B1/6Jp^{un}/p^{un} mice (erythrocytes from peripheral blood)	50, 100, 250, or 500 mg/kg in drinking water	75% anatase, 25% rutile particles; purity 99.5%, primary particle size 21 nm	Positive at 500 mg/kg		Trouiller et al., 2009

Table 4.11. Summary of genotoxicity data for TiO_2 nanoparticles

Endpoint	Assay	Test system	Exposure	Particle data	Result	Comments	Reference
DNA damage	Comet assay	Bottlenose dolphin leukocytes	0, 20, 50, or 100 µg/mL for 4, 24, or 48 hrs	Anatase (<25 nm) or rutile (<5,000 nm) particles	Positive for anatase particles at 50 µg/mL for 24 or 48 hrs; positive for rutile particles at ≥50 µg/mL for 48 hrs	In solution, both particles types showed aggregation. The size frequency distribution was similar for both particles; limited numbers of single particles or small aggregates (<100 nm in size) and a large number of aggregates in the µm range were observed	Bernardeschi et al., 2010
	Comet assay	BEAS-2B or IMR-90 (human bronchial) cells	0, 2, 5, 10, or 50 µg/cm^2 for 24 hrs	Particles with an average diameter of 91 nm; composed of 56% titanium, 41% oxygen, and 3% carbon	Negative		Bhattacharya et al., 2009
	Comet assay	BEAS-2B (human bronchial) cells	0, 1, 5, 10, 20, 40, 60, 80, or 100 µg/cm^2 (or 0, 3.8, 19, 38, 76, 114, 228, 304, or 380 µg/mL) for 24, 48, or 72 hrs	Uncoated anatase (<25 nm); rutile (10 × 40 nm) coated in SiO_2	Positive for anatase particles at 10–80 µg/cm^2 for 24 hrs, 60–100 µg/cm^2 for 48 hrs, or 40, 80, or 100 µg/cm^2 for 72 hrs Positive for coated rutile particles at 80 µg/cm^2 for 24 hrs or ≥80 µg/cm^2 for 72 hrs	Average agglomerate sizes were 5.5 and 4.5 µm for anatase and coated rutile particles, respectively	Falck et al., 2009
	Comet assay	BEAS-2B (human bronchial) cells	10 µg/mL for 1 hr	Anatase particles, 10, 20, or ≥200 nm in size; rutile TiO_2 (200 nm in size)	Positive for 10 or 20 nm anatase and 200 nm rutile particles; negative for ≥200 nm anatase particles		Gurr et al., 2005
	Comet assay	Human nasal epithelial cells	0, 10, 25, 50, or 100 µg/mL for 24 hrs	Anatase, <25 nm in size	Negative	Mean size of aggregated particles was 285 ± 52 nm	Hackenberg et al., 2010

Table 4.11. Summary of genotoxicity data for TiO_2 nanoparticles

Endpoint	Assay	Test system	Exposure	Particle data	Result	Comments	Reference
	Comet assay	Human peripheral blood lymphocytes	20, 50, or 100 µg/mL for 0, 6, 12, or 24 hrs	70–85% anatase, 15–30% rutile particles, ~30 nm in size	Positive (all concentrations and time points)		Kang et al., 2008
	Comet assay	A549 (human lung epithelial) cells	1, 20, or 40 µg/cm^2 (2, 40, or 80 µg/mL) for 4 hrs	Mixture of anatase and rutile, average particle size 63 nm	Positive at ≥20 µg/cm^2	Aggregations (typically 10 larger than the primary particle size) were noted	Karlsson et al., 2008
	Comet assay	L-02 (human embryo) hepatocytes	0, 0.01, 0.1, or 1 µg/L for 24 hrs	The anatase: rutile ratio of particles was 8:2; primary particle diameter ~25 nm	Negative		Shi et al., 2010
	Comet assay	WIL2-NS (human B-cell lymphoblastoid) cells	0, 26, 65, or 130 µg/mL for 6, 24, or 48 hrs	Particles 99% pure	Positive results reported at 65 µg/mL for 24 hrs (data not shown)	Particle size distribution: by volume 6.57 nm (100%); by intensity 8.2 nm (80%) and 196 nm (19%)	Wang et al., 2007
	Comet assay	C57B1/6Jp^{un}/p^{un} mice (peripheral blood)	500 mg/kg in drinking water	75% anatase 25% rutile particles; purity 99.5%, primary particle size 21 nm	Positive		Trouiller et al., 2009
	Double-stranded breaks	C57B1/6Jp^{un}/p^{un} mice (bone marrow)	50, 100, 250, or 500 mg/kg in drinking water	75% anatase 25% rutile particles; purity 99.5%, primary particle size 21 nm	Positive (all doses)		Trouiller et al., 2009
	Damage to DNA fragments in test tube	DNA fragments incubated with TiO_2 nanoparticles in presence of UVA light	0, 4, 8, or 16 µg/mL, ± Cu II	Photo-irradiated anatase or rutile nanoparticles (50–300 nm; commercial products)	Positive	Anatase was more potent than rutile; DNA damage was dependent on UVA irradiation and stimulated by Cu II	Hirakawa et al., 2004

Table 4.11. Summary of genotoxicity data for TiO_2 nanoparticles

Endpoint	Assay	Test system	Exposure	Particle data	Result	Comments	Reference
	DNA adduct formation	IMR-90 (human bronchial) cells	5 or 10 µg/cm^3 for 24 hrs	Particles with an average diameter of 91 nm; composed of 56% titanium, 41% oxygen, and 3% carbon	Positive		Bhattacharya et al., 2009
	DNA adduct formation	L-02 (human embryo) hepatocytes	0, 0.01, 0.1, or 1 µg/L for 24 hrs	The anatase rutile ratio of particles was 8:2; primary particle diameter ~25 nm	Positive at 1 µg/L		Shi et al., 2010

CHO = Chinese hamster ovary; MEF = mouse embryo fibroblast; MN = micronucleus; RLE = rat liver epithelial; SCE = sister chromatid exchange; SHE = Syrian hamster embryo

In general, short-term tests to assess the mutagenicity of TiO_2 nanoparticles found mixed results in bacteria, but were primarily positive in mammalian tissues (Table 11). In two assays, nanoscale TiO_2 particles (characterized by size distributions that include particles <100 nm) tested negative for reverse mutation at concentrations up to 5,000 µg/plate in several *Salmonella typhimurium* strains in the presence or absence of metabolic activation (Pan et al., 2010; Warheit et al., 2007). However, positive results for mutation were obtained by Pan et al. (but not Warheit et al.) using the *Escherichia coli* WP2*uvr*A (oxidative-stress sensitive) strain in the presence of metabolic activation. Both Xu et al. (2009; using 5 and 40 nm anatase particles) and Wang et al. (2007; primary particle size and phase not specified) obtained positive results for mutation in gpt delta (mouse fibroblast or mouse embryo fibroblast [MEF] cells) and *HPRT* (human B-cells) assays, respectively; positive results were also obtained in alveolar type II cells isolated from rats 15 months after intratracheal instillation with TiO_2 (anatase; median diameter 180 nm; surface area 8.8 m^2/g) at 100 mg/kg (Driscoll et al., 1997). Based on additional data, indicating that TiO_2 at -325 mesh in diameter (i.e., fine TiO_2) failed to induce mutation in MEF cells at the same concentrations in which nanoscale TiO_2 tested positive, Xu et al. (2009) suggest that as the size of particles change from µm -scale to nanoscale, particle diameter is an important factor in the toxicity response.

In assays performed to evaluate clastogenic effects, TiO_2 nanoparticles tested positive for sister chromatid exchange (SCE) in Chinese hamster ovary (CHO)-K1 cells (Di Virgilio et al., 2010; Lu et al., 1998, as cited in Di Virgilio et al., 2010). In one of these assays, SCE frequencies were significantly increased ($p < 0.01$) in CHO-K1 cells treated with nano-TiO_2 (average particle size 20 ± 7 nm; surface area 142 m^2/g) at 1 or 5 µg/mL for 24 hours (Di Virgilio et al., 2010). Treatment of the same cell line with eight different types of nanoscale TiO_2 (comprising various surface coatings [di-iron trioxide, alumina and/or silica], crystal phases [anatase or rutile or a combination of both] and particle sizes [14–60 nm]) provided no evidence of chromosomal aberrations at concentrations up to 5,000 µg/mL (Theogaraj et al., 2007). Deoxyribonucleic acid (DNA) deletions were reported in the offspring of C57B1/6Jp^{un}/p^{un} mice treated in vivo with nano-TiO_2 (75% anatase/25% rutile; primary particle size 21 nm) at 500 mg/kg in the drinking water during pregnancy (Trouiller et al., 2009).

In 9 of 11 assays, TiO_2 nanoparticles tested positive for micronuclei formation (Table 11). In CHO cells, Warheit et al. (2007) found that TiO_2 nanoparticles (median particle size 140 nm, but including a distribution of particles <100 nm in size) did not induce micronuclei at concentrations up to 250 (with metabolic activation) or 2,500 µg/mL (without metabolic activation). Using 20 nm TiO_2 nanoparticles, Linnainmaa et al. (1997) also reported negative results for micronuclei induction. A study by Bhattacharya et al. (2008) compared directly responses in V79 cells to treatment with uncoated particles (anatase; 30–50 nm in size) and particles of the same size and composition coated with vanadium pentoxide. The researchers found that coated particles, but not uncoated particles, induced micronuclei formation, suggesting that the coating of nanoparticles can influence the genotoxic response. Additional studies in CHO cells (and one study in Syrian hamster embryo [SHE] cells; Rahman et al., 2002) showed that TiO_2 nanoparticles (but not fine TiO_2; Rahman et al., 2002) induced micronuclei formation at concentrations as low as 0.5 µg/mL (Di Virgilio et al., 2010; Lu et al., 1998). TiO_2 particles (anatase:rutile 8:2; approximately 25 nm in size) did not induce micronuclei formation in embryo hepatocytes (Shi et al., 2010), but micronuclei were significantly induced by TiO_2 nanoparticles (10–30 nm in size) in several other human cell lines (including bronchial cells, blood lymphocytes, and B-cells) at concentrations in the range of 10–130 µg/mL (Falck et al., 2009; Kang et al., 2008; Wang et al., 2007; Gurr et al., 2005). Notably, Falck et al. (2009)

showed that anatase nano-TiO_2 (<25 nm in size), but not rutile nano-TiO_2 (10 × 40 nm in size, coated in SiO_2) induced micronuclei formation, suggesting that the shape, phase composition, and surface coatings of nanoscale particles likely factor into their genotoxicity. Micronuclei formation (erythrocytes) was also reported in C57B1/6Jp^{un}/p^{un} mice treated in vivo with nano-TiO_2 (75% anatase/25% rutile; primary particle size 21 nm) at 500 mg/kg in the drinking water during pregnancy (Trouiller et al., 2009).

With respect to DNA damage, the same in vivo study (Trouiller et al., 2009) showed that TiO_2 nanoparticles (75% anatase/25% rutile; 21 nm in size), administered in drinking water during pregnancy, also induced DNA damage in the peripheral blood (comet assay) and double-stranded DNA breaks in the bone marrow of treated mice. Nano-TiO_2 tested positive in six of nine additional comet assays; all but one of these assays were performed in human cell lines (see Table 11). Negative results were obtained in human bronchial cells treated with 91 nm TiO_2 particles at 0–50 µg/cm^2 (Bhattacharya et al., 2008), nasal epithelial cells treated with 25 nm particles at up to 100 µg/mL (Hackenberg et al., 2010), and embryo hepatocytes treated with ~25 nm TiO_2 particles at up to 1 µg/L (Shi et al., 2010). In contrast, TiO_2 (10–40 nm in size) induced DNA damage in two assays in human bronchial cells (Falck et al., 2009; Gurr et al., 2005), and was positive as well in human blood lymphocytes (primary size of particles ~30 nm; Kang et al., 2008), human lung epithelial cells (average particle size 63 nm; Karlsson et al., 2008), and human B-cells (Wang et al., 2007). Damage to DNA fragments in test tube was induced by UVA-irradiated commercial anatase or rutile nanoparticles; anatase was more active than rutile in this system (Hirakawa et al., 2004).

In a few studies, effects from several different types of TiO_2 particles were evaluated. In an assay testing responses in human bronchial cells to anatase (10, 20, or ≥200 nm in size) or rutile TiO_2 particles (200 nm in size), oxidative DNA damage was observed in response to rutile TiO_2 and nano-, but not µm-scale, anatase TiO_2 (Gurr et al., 2005), leading the authors to conclude that the capacity to induce oxidative DNA damage may be influenced by particle size and/or crystal form. While these data suggest that reducing the particle sizes from the µm-range to the nano- range heightens the capacity of TiO_2 to induce DNA damage, this may not always be the case. For instance, Karlsson et al. (2009) reported that following short-term (4–16 hours) exposure of human A549 cells in culture to a concentration of 40 µg/cm^2, µm-sized (rutile, average primary particle size of 1 µm) TiO_2 particles or nanosized (unspecified mixture of rutile and anatase, average primary particle size of 63 nm) TiO_2 particles induced no cytotoxic effects, mitochondrial damage, or oxidative DNA damage, but induced DNA damage (as assayed by the Comet assay), compared with control conditions; the DNA damage induced by the µm-sized particles was larger than that produced by the nanosized particles (24 and 20% of DNA in Comet tail, respectively, compared with about 8% in controls). Additionally, Falck et al. (2009) showed that silica-coated TiO_2 particles (10 × 40 nm; rutile) were less effective at inducing DNA damage than uncoated TiO_2 particles (anatase, <25 nm in size); however, a number of other particle variables (size, phase, shape) confounded any clear conclusions.

In a few additional assays, DNA adducts were formed in cells treated with TiO_2 nanoparticles (see Table 11). In two of these assays, Bhattacharya et al. (2009; 91 nm particles, phase not specified) and Shi et al. (2010; anatase:rutile 8:2; 25 nm in size) showed that significant levels of DNA adducts were observed in TiO_2-treated cells.

In only a few studies have nanoparticle characteristics (such as primary particle size, degree of agglomeration, shape and/or structural isomer of particles) been evaluated for their role

in eliciting a genotoxic response. Taken together, the data suggest that all characteristics of TiO_2 nanoparticles (including size, coating, and phase) can influence genotoxicity depending on the test system and exposure conditions, but no broad conclusions based on these characteristics can be made at this time.

4.6.2. Cytotoxicity

4.6.2.1. Overview

A broad database aimed at determining the mechanism by which TiO_2 nanoparticles induce toxicity is available. The majority of these studies suggest that TiO_2 nanoparticles induce inflammatory and oxidative effects on cells; these responses can lead to DNA damage and cytotoxicity.

Various studies indicate that exposure to TiO_2 nanoparticles triggers an oxidative response. For instance, in mice treated subchronically (13 weeks) with TiO_2 nanoparticles via the inhalation route (Janssen et al., 1994), increased expression of MnSOD (an antioxidant enzyme) in the lung, at both the mRNA and protein levels, was observed. Similarly, splenic congestion and proliferation in mice treated with nano-TiO_2 via gavage was associated with oxidative stress, and was evidenced by the accumulation of reactive oxygen species (ROS), increased lipid peroxidation, and increased expression of heme-oxygenase-1 (HO-1; Wang et al., 2010). By other routes of administration (intratracheal instillation or intraperitoneal [i.p.] injection), nano-TiO_2 treatment was shown to induce NO release, increase H_2O_2 production and lipid peroxidation, increase the activities of antioxidant enzymes (including GPX, glutathione reductase, and CAT), and decrease levels of glutathione and ascorbic acid (Ma et al., 2010; Afaq et al., 1998a, b).

Numerous in vitro studies corroborate these in vivo data. In studies performed using various cell lines, treatment with TiO_2 nanoparticles elicited an oxidative response characterized by increased ROS generation, lipid peroxidation, upregulation of antioxidant activities and/or decreased antioxidant capacity, and/or perturbations in mitochondrial function (Helfenstein et al., 2008; Kang et al., 2008; Rogers et al., 2008; Long et al., 2007, 2006a, b; Gurr et al., 2005); concentration- and/or time-dependent effects, in the absence of cytotoxicity, were observed in some studies (Kim et al., 2010; Jin et al., 2008). Furthermore, studies by Bhattacharya et al. (2009, 2008) indicated that, in V79 and human lung cells, treatment with TiO_2 nanoparticles led to the production of cellular and acellular free radicals; DNA damage (in the form of DNA adduct or micronuclei formation) was identified as a downstream effect. Additional studies have shown that ROS production associated with nano-TiO_2 treatment activates the p53-mediated DNA damage checkpoint (in peripheral blood lymphocytes; Kang et al., 2008) and transforms benign mouse fibrosarcoma cells into tumor cells (Onuma et al., 2009).

Exposure to TiO_2 nanoparticles also induces a characteristic inflammatory response. This response is well-documented in animal studies conducted via the inhalation route; changes in differential cell counts in the BAL fluid (including neutrophil recruitment) and histopathological changes in the lung were consistently observed (see Section 4.3.1). Moreover, studies conducted via other routes of administration (intratracheal instillation or i.p. injection) also showed that TiO_2 treatment induced oxidant-dependent inflammatory signaling and initiated the production and/or release of numerous inflammatory mediators (including IL-1, IL-6, IL-13, and TNF-α) (Park et al., 2009; Kang et al., 2008; Ahn et al., 2005).

Genomics-based data indicate that TiO_2 exposure induces gene expression changes consistent with oxidative stress and inflammation; additional genes modulated by treatment were associated with processes such as apoptosis, cell cycle regulation, DNA damage/repair, cellular transport, and energy metabolism (Huang et al., 2009; Shimizu et al., 2009; Long et al., 2007; Chen et al., 2006; Gwinn et al., 2006). In human bronchial epithelial (BEAS-2B) cells, treatment with TiO_2 nanoparticles (21 nm in size) upregulated the expression of oxidative-stress related genes (including HO-1, thioredoxin reductase, glutathione-S-transferase, and CAT) and inflammation-related genes (including IL-1, IL-6, IL-8, TNF-α, and CXCL2) (Park et al., 2008). Similarly, in an additional microarray analysis performed by Park et al. (2009) on the lung tissues of ICR mice treated with nano-TiO_2 (at 5–50 mg/kg-day) via a single intratracheal instillation, upregulation of genes associated with the induction of pro-inflammatory cytokines, antigen presentation, the induction of chemotaxis of immune cells, and inflammatory proteins was observed.

As a consequence of inflammatory and oxidative-stress responses to nano-TiO_2 treatment (and other effects induced by nano-TiO_2 treatment), TiO_2 nanoparticles have predominantly tested positive in assays designed to assess cytotoxicity. In a subset of these assays, the influence of particle characteristics (including size, shape, crystalline form, and surface modifications) on nano-TiO_2-induced cytotoxicity was evaluated. With regard to particle size, some in vivo data (Renwick et al., 2004) suggest that the cytoxic potential of TiO_2 increases with decreasing particle size (from fine- to nanoscale), but other in vivo (Warheit et al., 2006) and in vitro data in human cell lines indicate that fine and nano-TiO_2 elicit cytotoxicity to comparable degrees (Lai et al., 2008; Park et al., 2007). Other in vitro data (L'Azou et al., 2008; Simon-Deckers et al., 2008) suggest that particle size, with other factors, influences the cytotoxicity response. Indeed, studies by Aisaka et al. (2008), Simon-Deckers et al. (2008), and Sayes et al. (2006), provided data suggesting that the crystalline morphological form of TiO_2 nanoparticles can influence their cytotoxicity in vitro. Additional studies indicate that the primary shape of TiO_2 nanoparticles might also influence cytotoxicity responses in vitro (Chen et al., 2010; Hamilton et al., 2009). Evidence that surface modifications can also modulate the cytotoxic potency of TiO_2 nanoparticles in vivo and in vitro comes from studies conducted by Tiano et al. (2010), Onuma et al. (2009), Pan et al. (2009), Bhattacharya et al. (2008), and Warheit et al. (2007). Results from cytotoxicity assays for TiO_2 nanoparticles are summarized in Table 4.12.

Table 4.12. Summary of cytotoxicity data for TiO_2 nanoparticles

Author	Experimental design	Results	Conclusions
Animal in vivo cytotoxicity			
Renwick et al., 2004	Male Wistar rats intratracheally instilled a single time with TiO_2 nanoparticles (29 nm in size) or fine TiO_2 (250 nm in size) at an equal mass (0, 125, or 500 µg) and sacrificed 24 hrs post-exposure	Positive; nano-TiO_2 more cytotoxic than fine TiO_2	Nano-TiO_2 induced greater cytotoxicity than fine TiO_2 via a heightened inflammatory response.
Warheit et al., 2006	Crl:CD(SD)IGS BR rats intracheally instilled with TiO_2 nanoparticles (anatase nanoscale rods [92–233 nm × 20–35 nm] or anatase nanoscale dots [5.8–6.1 nm]) or fine TiO_2 particles (rutile; 300 nm) at 5 mg/kg	Positive; nano-TiO_2 not more cytotoxic than fine TiO_2	Nanoscale TiO_2 was not more cytotoxic than fine TiO_2; however multiple variables (i.e., size and phase composition) were evaluated.

Table 4.12. Summary of cytotoxicity data for TiO_2 nanoparticles

Author	Experimental design	Results	Conclusions
Warheit et al., 2007	Crl:CD(SD)IGS BR rats intracheally instilled with TiO_2 nanoparticles (80/20 anatase/rutile P25 [129 nm in water], or two types rutile [136 or 149 nm in water]) or fine TiO_2 particles (382 nm in water) at 1 or 5 mg/kg	Positive; ranking: anatase/rutile P25 TiO_2 > fine TiO_2 > ultrafine rutile TiO_2	Crystal structure, pH, and/or surface reactivity influence the cytotoxic potency of TiO_2.
Human cell in vitro cytoxicity			
Bregoli et al., 2009	Human hematopoietic progenitor cells were treated with TiO_2 particles (20–160 nm in size, aggregates >2,300 nm) at 0, 5, 25, or 100 µg/mL and incubated for 14 d	Negative	
Hackenberg et al., 2010	Human nasal mucosa cells treated with TiO_2 (15–30 nm in size; sphere-shaped; mean aggregate size 285 nm) at 0, 10, 25, 50, or 100 µg/mL for 24 hrs	Negative	
Berntsen et al., 2010	Human airway smooth muscle cells treated with TiO_2 (25 nm in size)	Negative	
Kim et al., 2010	Human lung cell lines (lung epithelial cells, A549 carcinoma cells, and L-132 cells) treated with TiO_2	Negative	
Park et al., 2007	Human alveolar epithelial (A549) cells treated with TiO_2 nanoparticles (30 nm in size) or fine TiO_2 (1 µm in size) at up to 200 µg/mL for 24 hrs	Negative; no significant differences in response between nano- and µm-sized TiO_2	No difference in cytotoxicity was observed between fine- and nano-TiO_2 particles.
Sayes et al., 2006	Human dermal fibroblasts and lung epithelial cells treated with nano-TiO_2 anatase (10 nm in size), nano-TiO_2 rutile (5 nm in size), or nano-TiO_2 anatase-rutile (3 nm in size) at 3 µg/mL to 3 mg/mL for up to 48 hrs	Positive, only at ≥100 µg/mL	Anatase particles were 100-fold more cytotoxic than an equivalent sample of rutile particles. Phase a better correlate for cytotoxicity than the surface area.
Chen et al., 2010	HeLa cells treated with 0-D (monodisperse particles <100 nm in size), 1-D (anisotropic; two dimensions, <100 nm in size) or 3-D (bulky aggregates of particles) TiO_2 at up 140 µg/mL for 24 hrs	Positive for 0-D particles at ≥125 µg/mL; negative for other particle types	The shape of TiO_2 nanoparticles is a determinant for cytotoxicity.
Wang et al., 2007	WIL2-NS cells treated with TiO_2 nanoparticles (particle characteristics not specified) at up to 130 µg/mL for up to 48 hrs	Positive (concentration-dependent)	
Lai et al., 2008	Human astrocytoma U87 cells or normal fibroblasts treated with TiO_2 nanoparticles (anatase, <25 nm in size) or fine TiO_2 (1–1.3 µm in size) at up to 100 µg/mL for 48 hrs	Positive at ≥1 µg/mL (both particle types)	Nano-TiO_2 and fine TiO_2 were about equally effective in decreasing cell survival at ≥1 µg/mL.
Park et al., 2008	Human bronchial epithelial (BEAS-2B) cells treated with TiO_2 particles (21 nm in size) at 0, 5, 10, 20, or 40 µg/mL for 24, 48, 72, or 96 hrs	Positive at 5–40 µg/mL; time- and concentration-dependent)	

Table 4.12. Summary of cytotoxicity data for TiO_2 nanoparticles

Author	Experimental design	Results	Conclusions
L'Azou et al., 2008	Human glomerular mesangial and epithelial tubular cells treated with various TiO_2 nanoparticles (13–75 nm in size, mix of structural isomers) at up to 160 µg/cm^2 for 24 hrs	Positive; smallest particles showed greatest cytotoxicity	Cytotoxicity was particle type-, particle size-, and cell type-dependent.
Aisaka et al., 2008	Human erythrocytes treated with anatase (<25 or <5,000 nm in size), rutile (<5,000 nm in size), or amorphous TiO_2 (<50 nm in size) at up to 20 mg/mL for 60 mins	Positive; effect on hemolysis varied by particle type	Hemolytic effects of TiO_2 vary depending on the polymorph but not on the primary size (nano- or µm-scale) of particles.
Pan et al., 2009	Human dermal fibroblasts treated with rutile TiO_2 (oblate, with an average width of 15 nm; aggregated into complexes several µm in diameter), coated rutile (coated with polymer brush), or anatase TiO_2 (aggregates with an average diameter of 200 nm) at up to 0.8 mg/mL for up to 2 d	Positive for uncoated particles; anatase more cytotoxic than rutile particles; negative for coated rutile particles	Functionalization can alter the toxicity of TiO_2 nanoparticles.
Onuma et al., 2009	QR-32 fibrosarcoma cells treated with TiO_2-1 (coated with $ZnO_2Al[OH]_3$) or TiO_2-2 (coated with $ZnO_2Al[OH]_3$ and stearic acid) for up to 48 hrs	Positive; TiO_2-2 more cytotoxic than TiO_2-1	Surface coating of TiO_2 nanoparticles is a determinant for cytotoxicity.
Tiano et al., 2010	Human skin fibroblasts treated with TiO_2 nanoparticles (various surface modifications and structural isomers) for 16 hrs and exposed to UVA for 0, 30, or 60 mins	Positive	Surface coatings and phase composition of TiO_2 nanoparticles are determinants for cytotoxicity.
Simon-Deckers et al., 2008	Human (A549) lung epithelial cells treated with TiO_2-Degussa 25 (21 nm in size; 75% anatase), TiO_2-CEA (12 nm in size; 95% anatase), or TiO_2-Sigma-rutile (elongated; 68 × 9 nm in size; rutile) for up to 72 hrs	Positive	In this study, cytoxicity responses (measured using the MTT assay) were a function of the size and crystalline phase of the TiO_2 particles.
Animal cell in vitro cytotoxicity			
Jeng and Swanson, 2006	Neuro-2A (mouse neuroblastoma) cells treated with TiO_2 nanoparticles (spherical and needle-shaped; <40 nm in size) at up to 200 µg/mL for up to 48 hrs	Negative	
Linnainmaa et al., 1997	RLE cells treated with anatase TiO_2 (20 nm in size) or rutile TiO_2 (20 nm in size; coated with aluminum hydroxide and stearic acid) at up to 200 µg/cm^2 for 20 hrs	Negative	
Bhattacharya et al., 2008	V79 (hamster lung fibroblast) cells treated with TiO_2 nanoparticles (anatase; 30–50 nm in size, with or without vanadium pentoxide coating) at 0, 1, 2, or 5 µg/cm^2 for 24, 48, or 72 hrs	Positive (both particle types); V_2O_5-treated particles more cytotoxic than untreated particles	Surface coating of TiO_2 nanoparticles is a determinant for cytotoxicity; V_2O_5-treated particles showed increased cytotoxicity; possibly via ↑ surface reactivity and consequently ↑ free radical generation.
Di Virgilio et al., 2010	CHO-K1 cells treated with TiO_2 nanoparticles for 24 hrs	Positive	

Table 4.12. Summary of cytotoxicity data for TiO_2 nanoparticles

Author	Experimental design	Results	Conclusions
Jin et al., 2008	Mouse fibroblast (L929) cells treated with homogenous TiO_2 (5 nm in size) or weakly aggregated TiO_2 (20–30 nm in size) at up to 600 µg/mL for 24 or 48 hrs	Positive	Dose-dependent effects were observed for both particle types.
Hamilton et al., 2009	Murine AMs treated with TiO_2 nanoparticles (60–200 nm in size); short TiO_2 nanobelts (width 60–300 nm; 0.8–4 µm long), or long TiO_2 nanobelts (width 60–300 nm; 15–30 µm long) at up to 200 µg/mL for up to 4 hrs	Positive; for long TiO_2 nanobelts at ≥100 µg/mL	Shape of TiO_2 nanoparticles is a determinant for cytotoxicity.
Hussain et al., 2005	Rat liver-derived cell line (BRL 3A) treated with TiO_2 nanoparticles (40 nm in size) at up to 250 µg/mL for 24 hrs	Positive (marginally) at 100–250 µg/mL	

4.6.2.2. Animal in Vivo Cytotoxicity

In a study by Renwick et al. (2004; Table 12), male Wistar rats were treated with nano-TiO_2 (29 nm in size) or fine TiO_2 (250 nm in size) via a single intratracheal instillation at 125 or 500 µg to assess the ability of TiO_2 particles to induce inflammation, cause epithelial cell injury, and impair AM function. TiO_2 nanoparticles induced cytotoxicity (evaluated by measuring LDH activity in the BAL fluid); nanoscale particles induced more cytotoxicity (as well as greater inflammation and epithelial damage) than its fine counterpart administered at an equal mass. The researchers attributed these effects to an increased inflammatory response associated with the decreased particle size of TiO_2 nanoparticles. However, a later study by Warheit et al. (2006) showed that pulmonary cytotoxicity elicited by treatment with TiO_2 nanoparticles (anatase nanoscale rods [92–233 × 20–35 nm] and anatase nanoscale dots [5.8–6.1 nm]) in Crl:CD(SD)IGS BR rats via intratracheal instillation was not different from that induced by larger rutile (300 nm) particles. Further, in groups of rats intratracheally instilled with TiO_2 particles of various sizes and composition at two doses (1 or 5 mg/kg), Warheit et al. (2007) showed that the ranking of cytotoxicity responses was anatase/rutile P25 TiO_2 > fine rutile TiO_2 > ultrafine rutile TiO_2, suggesting to the authors that crystal structure, pH, and/or surface reactivity contribute to particle-specific responses to TiO_2.

4.6.2.3. Human Cell in Vitro Cytotoxicity

TiO_2 nanoparticles were negative in tests for cytotoxicity in human cells in only a few assays (see Table 12). In human hematopoietic progenitor cells treated with TiO_2 nanoparticles (20–160 nm in size; with aggregates >2,300 nm in size), no cytotoxicity was observed at concentrations up to 100 µg/mL (Bregoli et al., 2009). Nano-TiO_2 (sphere-shaped; 15–30 nm in size, mean aggregate size 285 nm) also failed to induce cytotoxicity in human nasal mucosa cells at the same concentration (up to 100 µg/mL; Hackenberg et al., 2010). Berntsen et al. (2010) indicated that after treatment with TiO_2 nanoparticles (25 nm in size), there was no indication of altered cell contractility or decreased cell viability in human airway smooth muscle cells. An additional study by Kim et al. (2010) showed that, while TiO_2 treatment induced oxidative stress in a time- and concentration-dependent manner, treatment did not induce adverse effects on the proliferation or cell viability of lung epithelial cells, A549 carcinoma cells, or L-132 cells. A study by Park et al. (2007) showed that neither TiO_2 nanoparticles (30 nm in size) nor fine TiO_2

particles (1 µm in size) significantly decreased cell viability (evaluated by annexin V/PI staining) in human alveolar epithelial (A549) cells.

The majority of studies evaluating the cytotoxicity of TiO_2 nanoparticles in human cell lines have produced positive results (Table 12). Nano-TiO_2 decreased the viability of WIL2-NS (human B lymphoblastoid) cells in a dose-dependent manner at concentrations up to 130 µg/mL (Wang et al., 2007). Using human bronchial epithelial (BEAS-2B) cells, Park et al. (2008) also observed time- and concentration-dependent cytotoxicity after treatment of cells with TiO_2 nanoparticles (21 nm in size) at concentrations ranging from 0 to 40 µg/mL. The cytotoxic response was associated with increased ROS production, decreased levels of reduced glutathione, and the upregulation of genes involved in oxidative stress and inflammatory responses. Based on additional observations that generated ROS activated caspase-3 and elicited chromatin condensation, the researchers concluded that TiO_2 nanoparticles exert cytotoxicity via apoptosis.

In several studies, multiple types of TiO_2 particles have been used to assess cytotoxicity. Comparing TiO_2 nanoparticles (anatase; <25 nm in size) to fine TiO_2 (1–1.3 µm in size), Lai et al. (2008) found that both particle types induced cytotoxicity (evaluated by MTT assay) in human astrocytoma U87 and normal fibroblasts in a time- and concentration-dependent manner; nano-TiO_2 and fine TiO_2 were about equally effective in decreasing cell survival at ≥1 µg/mL. Based on additional assays, the researchers implicated necrosis and apoptosis as mechanisms for TiO_2-induced cytotoxicity. In human glomerular mesangial and epithelial cells, L'Azou et al. (2008) evaluated the cytotoxic potential of TiO_2 particles comprising different structural isomers (amorphous or anatase/rutile) and of various sizes (range: 13–75 nm) at concentrations reaching 160 µg/cm^2. Cytotoxicity was induced by the different particle types to varying degrees, and was associated with particle internalization and activation of intracellular mechanisms (including ROS production). Although the smallest particle was most effective at reducing cell viability (in both cell lines), a direct association between particle size and cytotoxicity was not observed for all particle types, leading the researchers to conclude that cytotoxicity is both particle-size- and particle-type-dependent. In agreement, treatment of human lung epithelial cells with three types of TiO_2 (TiO_2-Degussa 25 [75% anatase/25% rutile, 21 nm in size); TiO_2-CEA [95% anatase/5% rutile; 12 nm in size], or TiO_2-Sigma-rutile (elongated; 100% rutile; 9 × 68 nm in size]) induced cytotoxicity to varying degrees; these responses were dependent on the assay performed (LDH, XTT, or MTT; Simon-Deckers et al., 2008). In cytotoxicity tests based on measurements of cell membrane integrity or mitochondrial impairment, all particle types induced small but significant decreases in cell viability (LDH assay) or decreased viability by about 15% after treatment for 48 hours at 100 µg/mL (XTT assay; statistically significance not reported). In contrast, varying degrees of cytotoxicity were observed in the MTT assay based on particle type; TiO_2-CEA, TiO_2-Degussa 25, and TiO_2-Sigma-rutile decreased cell viability by 25, 24, and 9%, respectively. Based on these data, the researchers concluded that cytoxicity responses (MTT assay) were a function of the size and crystalline form of the TiO_2 particles.

Additional studies indicate that other particle characteristics influence the cytotoxic potential of TiO_2 nanoparticles. In a study measuring hemolysis (evaluated by measuring percentage hemoglobin release) induced by TiO_2 particles (in a plasma-free system), Aisaka et al. (2008) showed that the effectiveness of various TiO_2 particle types to induce hemolysis was anatase TiO_2 (<5,000 nm in size) > amorphous TiO_2 (<50 nm in size) > rutile (<5,000 nm in size) > nanoscale anatase TiO_2 (<25 nm in size). Based on these data, the researchers concluded that hemolytic effects were dependent on the crystalline morphological form, but not the primary

particle size, of the TiO_2 particles utilized. A study by Sayes et al. (2006) also found differences in the cytotoxic responses elicited by different crystalline morphological forms of nano-TiO_2. In human dermal fibroblasts and lung epithelial cells treated with nano-TiO_2 anatase (10 nm in size), nano-TiO_2-rutile (5 nm in size), or nano-TiO_2 anatase-rutile (3 nm in size), cytotoxicity was induced in a dose- and time-dependent manner at ≥100 µg/mL. However, nano-TiO_2 anatase particles were 100-fold more cytotoxic than an equivalent sample of nano-TiO_2 rutile particles; this led the researchers to suggest that crystalline form serves as a better correlate for cytotoxicity than surface area. Further, this study indicated that the most cytotoxic TiO_2 particles were also the most effective at generating ROS (in an ex vivo assay).

In a study designed to assess how the shape of TiO_2 nanoparticles influence its cytotoxic potential, HeLa cells were treated with 0-D nanoparticles (monodisperse particles <100 nm in size), 1-D nanorods (anisotropic, two dimensions, <100 nm in size), or 3-D aggregates (sterically bulky aggregates of nanoparticles or nanowires) of TiO_2 at concentrations up to 140 µg/mL (Chen et al., 2010). Whereas 1-D and 3-D nanoparticles were negative for cytotoxicity at all tested concentrations, 0-D particles decreased cell viability to 80% of untreated controls at ≥125 µg/mL. The researchers attributed these results not only to the increased surface area-to-volume ratio of 0-D particles compared to larger assemblies, but also to the increased access of smaller particles into cells via endocytosis. Upon irradiation with UV light, treatment with 0-D, 1-D, and 3-D nano-TiO_2 decreased cell viability by 37, 40, and 30%, respectively. Based on additional assays, the researchers concluded that the heightened cytotoxicity of irradiated TiO_2 resulted from increased ROS production.

The surface properties of nano-TiO_2 particles may also influence their cytotoxicity. In one study, human dermal fibroblasts were treated with rutile TiO_2 (oblate with an average width of 15 nm, aggregated into complexes several µms in diameter), coated rutile TiO_2 (polymer brush coating), or anatase TiO_2 (aggregates with an average diameter of 200 nm) at up to 0.8 mg/mL (Pan et al., 2009). Anatase TiO_2 produced greater cellular damage (characterized by reductions in cell surface area, cell proliferation, mobility, and ability to contract collagen) at lower concentrations and shorter durations than rutile TiO_2. By TEM, the researchers showed that TiO_2 nanoparticles penetrate cell membranes, and are sequestered in vesicles until they rupture. Consequently, levels of H_2O_2 were shown to be significantly increased in cells treated with uncoated anatase or rutile TiO_2 compared to untreated controls. Interestingly, rutile TiO_2 particles coated with a polymer brush did not adhere to cell membranes and did not penetrate cells; this led to decreased ROS production (relative to uncoated particles) and allowed normal cell function (i.e., viability comparable to untreated controls). Based on these data, the researchers suggested that functionalization can alter the toxicity of TiO_2 nanoparticles (in this case, functionalization served a protective function against cell injury).

In another study by Tiano et al. (2010), five surface-modified nano- TiO_2 particles (anatase, rutile, or a combination of anatase and rutile phases; modified with materials including manganese, aluminum hydroxide, alumina, sodium, hexametaphosphate, polyvinylpyrrolidone, aluminum phosphate, dimethicone, or glycerin) used in sunscreen formulations to prevent ROS generation under conditions of photoexcitation were tested for their ability to reduce cell viability in human skin fibroblasts following UVA exposure. Only cells treated with untreated TiO_2 (but not treated TiO_2 particles) compromised cell viability after UVA exposure. After treatment with UVA for 60 minutes, the cell viability of untreated (no TiO_2) cells was too strongly affected (decreased by UVA irradiation) to evaluate differences between control and TiO_2 samples. Nonetheless, the data allowed the authors to conclude that surface treatments (and

possibly phase composition) of TiO_2 nanoparticles affect their ability to reduce cell viability. In a cell-free system, Onuma et al. (2009) showed that TiO_2-2 particles (coated with $ZnO_2Al[OH]_3$ and stearic acid; hydrophobic), but not TiO_2-1 particles (coated with $ZnO_2Al[OH]_3$ alone; hydrophilic), generated ROS in a dose-dependent manner. Although both particle types generated high levels of ROS in QR-32 fibrosarcoma cells, TiO_2-2 particles elicited a stronger response, resulting in marked cytotoxicity (evaluated by trypan blue exclusion).

4.6.2.4. Animal Cell in Vitro Cytotoxicity

In most assays, animal cells treated with TiO_2 nanoparticles showed significant reductions in cell viability; however, TiO_2 nanoparticles tested negative for cytotoxicity in a few assays (Table 12). In one of these assays, neuro-2A (mouse neuroblastoma cells) were treated with TiO_2 nanoparticles (spherical and needle-shaped; <40 nm in size) at up to 200 µg/mL; significant levels of cytotoxicity were not observed (Jeng and Swanson, 2006). In a second assay, two types of TiO_2 nanoparticles (anatase particles, 20 nm in size or rutile particles, 20 nm in size and coated with AlOH and stearic acid) failed to induce cytotoxicity in rat liver epithelial (RLE) cells at concentrations up to 200 µg/cm^2; UV irradiation did not affect the results (Linnainmaa et al., 1997). It is unclear if the negative results obtained in these assays were related to the test systems used, the concentrations and/or durations of exposure, the characteristics (such as size, phase, or coating) of the particles utilized, or a combination of these factors.

In all other tests conducted in animal cells, TiO_2 nanoparticles tested positive for cytotoxicity (Table 12). Di Virgilio et al. (2010) observed dose-dependent cytotoxicity in nano-TiO_2-treated CHO-K1 cells; cytotoxicity was evidenced by changes in lysosomal and mitochondrial dehydrogenase activity. In another study, 40 nm TiO_2 particles showed marginal cytotoxicity in a rat liver-derived cell line (BRL 3A cells) at concentrations in the range of 100–250 µg/mL (Hussain et al., 2005). Positive results were also obtained in mouse fibroblast (L929) cells treated with homogeneous TiO_2 (5 nm in size) or weakly aggregated TiO_2 (20–30 nm in size); the response was dose-dependent at 3–600 µg/mL after 48 hours (Jin et al., 2008). In addition to decreased cell viability, increased oxidative stress, condensed chromatin, increased numbers of lysosomes, organelle damage, and increased levels of LDH and ROS were observed in nano-TiO_2-treated cells.

In a few studies, the cytotoxicity of TiO_2 nanoparticles with different shapes or surface coatings was evaluated. Bhattacharya et al. (2008) assessed cytotoxicity in V79 (human lung fibroblast) cells treated with uncoated TiO_2 nanoparticles (anatase, 30–50 nm in size) or coated TiO_2 nanoparticles (anatase, 30–50 nm in size, coated with vanadium pentoxide). Although both particle types decreased cell viability, the response was markedly enhanced by treatment with vanadium-coated nano-TiO_2; cytotoxicity coincided with heightened levels of free radical formation relative to uncoated particles. Based on these data, the researchers suggested that the vanadium pentoxide surface coating served to increase the reactivity of natural anatase TiO_2, thereby increasing cytotoxicity via increased free radical generation.

In another study, Hamilton et al. (2009) treated murine AMs with TiO_2 nanoparticles (60–200 nm in size), short TiO_2 nanobelts (60–300 nm width; 0.8–4 µm length) or long TiO_2 nanobelts (60–300 nm width; 15–30 µm length) at up to 200 µg/mL. Cytotoxicity was evaluated by trypan blue exclusion. Although all forms of nano-TiO_2 generated ROS, significant cytotoxicity was only induced by long TiO_2 nanobelts at ≥100 µg/mL. Treatment of cells with

long TiO_2 nanobelts also induced the release of cytokines, indicative of an inflammatory response. Based on these data, the researchers concluded that the capacity of TiO_2 nanoparticles to induce cytotoxicity can vary widely based on the shape of the TiO_2 particles used.

Taken together, the data show that TiO_2 nanoparticles are cytotoxic to human and animal cells in most assays. Studies conducted using several different types of TiO_2 nanoparticles indicate that no one particle characteristic is the best correlate for cytotoxicity; characteristics such as crystalline form, size, and surface coating all likely contribute to the cytotoxic potential of nano-TiO_2.

4.7. SUMMARY OF HAZARD AND DOSE-RESPONSE DATA

Inhalation Exposure: Most of the available data regarding toxicity of TiO_2 nanoparticles involve the inhalation route of exposure. Several short-term studies, several subchronic studies, a single chronic study (with rats and mice), and a developmental toxicity study were identified and are summarized in Table 4.13. In most of these studies, the aerosols produced from nanosized TiO_2 particles typically exhibited agglomeration, ultimately exposing animals to aerosols with MMAD values in the µm range. Currently available data are inadequate to assess whether the toxic potency of TiO_2 nanoparticles is significantly influenced by the degree of agglomeration within airborne aerosols (i.e., aerosol size).

Table 4.13. Summary of observed effects in animals repeatedly exposed by inhalation to aerosols made from TiO_2 nanoparticles

Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Short-term exposure							
Male Wistar rats (number not specified)	0 or 100 mg/m^3 (measured concentration 88 mg/m^3) for 6 hrs/d for 5 d	70% anatase/ 30% rutile; 20–30 nm particle size; surface area = 48.6 m^2/g; density 4.2g/cm^3; uncoated, shape not specified Aerosols: MMAD (σ) of 1.0 µm (2.2); 0.5% of total particle mass <100 nm	None	88 (lung)	Significant changes in BAL fluid parameters (including ↑ total cells, lymphocytes and neutrophils; ↑ protein, ↑ enzymatic activities), decreased lung weight, and histological changes to the lungs, mediastinal lymph nodes, and nasal cavity.	Endpoints evaluated included BAL fluid parameters (total and differential cell counts, protein content, enzyme activity), organ weights, and histological examination of respiratory tract tissues. Recovery was apparent for most endpoints by d 14 post-exposure.	van Ravenzwaay et al., 2009
Male Wistar rats (5–6 rats per endpoint per group)	0, 2, 10, or 50 mg/m^3 measured concentrations of 0, 2, 12, or 50 mg/m^3) for 6 hrs/d for 5 d	86% anatase/ 14% rutile; 31–71 nm particle size (mean 25 nm); surface area = 51.1 m^2/g; uncoated hydrophobic surface, shape assumed spherical if <100 nm in size Aerosols: MMAD (σ) of 0.8–1.1 µm (2.3–3.4); 0.1–0.4% of total particle mass <100 nm	None	2 (lung)	Changes in BAL fluid parameters (including ↑ total protein, ↑ enzymatic activities), increased cell proliferation in the large/medium bronchi and terminal bronchioli. Signs of pulmonary inflammation in BAL fluid and from cell proliferation measures increased in severity at higher concentrations.	Increased incidences of rats with histological inflammation lesions in lungs occurred at the two highest concentrations (e.g., diffuse alveolar infiltration with histiocytes, pigment-loaded macrophages in mediastinal lymph nodes). Endpoints evaluated included body weights, BAL fluid parameters (total and differential cell counts, protein content, enzyme activity), rates of cell proliferation and apoptosis in the lung, hematology parameters, organ weights, and histological examination of respiratory tract tissues.	Ma-Hock et al., 2009

Table 4.13. Summary of observed effects in animals repeatedly exposed by inhalation to aerosols made from TiO_2 nanoparticles

Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Male C57B1/6 mice (24/group)	0 or 8.9 mg/m^3 for 4 hrs/d for 10 d	Anatase; 3.5 nm particle size (mean 25 nm); surface area = 219 m^2/g; O, OH, and H_2O at the surface; shape not specified Aerosols: MMAD (σ) of 120–128 nm (1.6–1.7)	None	8.9 (lung)	Increased numbers of particle-laden macrophages in the lung tissues (indicative of inflammation).	Endpoints evaluated included body weights, BAL fluid parameters (total and differential cell counts, total protein, cytokine levels), and histopathological examination of lung tissues.	Grassian et al., 2007a

Table 4.13. Summary of observed effects in animals repeatedly exposed by inhalation to aerosols made from TiO_2 nanoparticles

Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Female BALB/c/Sca mice (8/group)	0 or 10 mg/m^3 for 2 hrs/d for 4 d; or 4 d/wk for 4 wks	Four types of nanoparticles used: (1) SiO_2-coated nano rutile TiO_2:10 × 40 nm (needle-shaped); surface area = 132 m^2/g; rutile; coated with silica, (2) nanoTiO_2 anatase: <25 nm, surface area = 222 m^2/g; anatase, (3) nano TiO_2 rutile/anatase: 30–40 nm; surface area = 23 m^2/g, rutile:anatase 9:1, and (4) nano TiO_2 anatase/brookite: ~21 nm, surface area = 61 m^2/g, anatase:brookite 3:1, in situ produced Aerosols: MMADs of about 100 and ≤82 nm for commercially-available and in situ-produced particles, respectively	None	10 (lung)	Increased neutrophils in BAL fluid, increased expression of mRNAs (CXCL1 and TNF-α) associated with inflammatory response in lung tissue. Effects observed for silica-coated rutile TiO_2 nanoparticles only. Other test materials induced no signs of pulmonary inflammation under the 2-hr/d exposure conditions used in this study.	Endpoints evaluated included BAL fluid parameters (differential cell counts and cytokine levels) and expression of mRNAs in lung tissue for gene products involved in inflammatory responses. Rossi et al. (2010) concluded that surface-coating was the most likely variable to explain potency difference observed across the test materials in this study. This was supported by citation of unpublished findings that 10 × 40 nm needle-shaped rutile nanoparticles with an alumina coating did not elicit inflammatory responses in an in vitro test system.	Rossi et al., 2010

Table 4.13. Summary of observed effects in animals repeatedly exposed by inhalation to aerosols made from TiO_2 nanoparticles

Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Subchronic exposure							
Male F344 rats (4–8/group)	0 or 23.5 mg/m^3 for 6 hrs/d, 5 d/wk for 12 wks	Anatase, 20–21 nm particle size; surface area = 50 m^2/g; density 4.28; uncoated Aerosols: MMADs (σ) of 0.71–0.78 µm (1.7–1.9)	None	23.5 (lung)	Variations in BAL fluid parameters (increased total cell and neutrophil counts, increased protein, changes in enzyme activity), increased expression of antioxidant enzymes (lung), and histopathological changes in the lungs (including increased alveolar epithelial thickness and septal fibrosis).	Endpoints evaluated included BAL fluid parameters (total and differential cell counts, total protein, enzyme activity), gene and protein expression of antioxidant enzymes in lungs, and histopathological examination of lung tissues. Animals were observed up to 64 wks post-exposure.	Baggs et al., 1997; Janssen et al., 1994; Oberdörster et al., 1994; Ferin et al., 1992
Female CDF (F344)/CrlBR rats (25/group)	0 or 0.5, 2.0, or 10 mg/m^3 for 6 hrs/d, 5 d/wk for 13 wks	P25 from Degussa; ~21 nm; typically 3:1 anatase:rutile; uncoated Aerosols: MMADs (σ) of 1.29–1.44 µm (2.5–3.4)	0.5	2.0	Decreased body weight gain, increased cell replication, and histopathological changes in the lung (aggregation of particle-laden macrophages, hypertrophy, and hyperplasia of type II alveolar epithelial cells).	Endpoints evaluated included mortality, clinical signs, body weights, BAL fluid parameters (total and differential cell counts, total protein, enzyme activity), cell proliferation in the lungs, and histopathological examination of the lungs. Groups of animals sacrificed immediately after exposure (13 wks), and after post-exposure periods of 4, 13, 26, or 52 (49 for hamsters) wks.	Bermudez et al., 2004
Female B3C3F1/CrlBR mice (25/group)			2.0	10 (lung)	Changes in BAL fluid parameters (total and differential cell counts, protein content, enzyme activity), increased cell replication, and histopathological changes in the lung (aggregation of particle-laden macrophages).		
Female Lak:LVG (SYR)BR hamsters			2.0	10 (lung)	Body weight loss, increased neutrophils in the BAL fluid, and increased bronchiolar cell replication.		

Table 4.13. Summary of observed effects in animals repeatedly exposed by inhalation to aerosols made from TiO_2 nanoparticles

Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Chronic toxicity							
Female Wistar rats (100/group)	0 or 10 mg/m^3 for 18 hrs/d, 5 d/wk for 24 mo	80% anatase/ 20% rutile; 15–40 nm particle size; surface area = 48 m^2/g; uncoated Aerosols: MMADs (σ) of 0.80 µm (1.8)	None	10 (systemic, lung)	Decreased lifespan, decreased body weight, changes in BAL fluid parameters, increased lung weights, and histopathological changes in lung (e.g., interstitial fibrosis in nearly all exposed rats and increased incidence of rats with lung tumors).	Endpoints evaluated included mortality, clinical signs of toxicity, body weights, BAL fluid parameters (including differential cell counts, total protein, and enzyme activity; in rats only), lung weights, and histopathological examination of nasal and prenasal cavities, larynx, trachea, and lung. Lung tumor incidence was significantly increased relative to controls in rats (but not mice) exposed to TiO_2.	Heinrich et al., 1995
Female NMRI mice (80/group)	0 or 10 mg/m^3 for 18 hrs/d, 5 d/wk for 13.5 mo		None	10 (systemic)	Decreased lifespan and increased lung weights.		
Reproductive/developmental toxicity							
Female C57BL/6BomTac mice (22–23/group)	0 or 40 mg/m^3 for 1 hr/d on GDs 8–18	Rutile; ~21 nm, 71% TiO_2 by weight; residual mass composed of zirconium, silicon, aluminum; coated with polyalcohols; surface area = 108 m^2/g Aerosols: geometric mean mass distribution 3.2 µm; <1% of total particle mass <100 nm	Maternal: None Fetal: None	Maternal: 40 Fetal: 40	Maternal: Changes in BAL fluid parameters (increased dead cells and increased neutrophils), increased lung weight. Fetal: Increased number of dead F1 pups during lactation, but not at birth; neurobehavioral effects (deficits in open field tests and prepulse inhibition response). No effects on reproductive performance by F1 offspring in cross-mating trials with nonexposed partners.	Endpoints evaluated included maternal body weights, BAL fluid parameters (total and differential cell counts) and organ weights, number of litters, body weights and sex distribution of pups, neurobehavioral assessments (pups), and fertility of F1 offspring in cross-mating trial (time-to-first-delivery of F2 litter, litter size, and gender). The litter was considered the unit for statistical analyses.	Hougaard et al., 2010

Pulmonary inflammation (i.e., changes in BAL fluid parameters and/or lung histopathological changes) is the principal effect noted in the short-term (5 days to 4 weeks) inhalation studies in rats and mice (Rossi et al., 2010; Ma-Hock et al., 2009; van Ravenzwaay et al., 2009; Grassian et al., 2007a; see summaries in Table 13). Aerosols were nanoparticle agglomerations in the 100–200 nm size range in studies by Grassian et al. (2007a) and Rossi et al. (2010) and in the µm size range in other studies (Ma-Hock et al., 2009; van Ravenzwaay et al., 2009). The available data, however, are inadequate to make conclusions about the relative potencies of nanosized versus µm-sized aerosols made from TiO_2 nanoparticles. All of these studies only included single exposure concentrations ranging from about 7 to 88 mg/m^3 (Rossi et al., 2010; van Ravenzwaay et al., 2009; Grassian et al., 2007a, b), except for the study by Ma-Hock et al. (2009), which exposed Wistar rats to three concentrations (2, 10, or 50 mg/m^3) 6 hours/day for 5 days; signs of pulmonary inflammation were apparent at 2 mg/m^3 and increased in severity at the higher concentrations (Table 13). Comparing across the short-term inhalation studies, the available data are inadequate to compare potencies of aerosols made from different crystalline forms of TiO_2 nanoparticles to induce pulmonary inflammation (Table 13). However, Rossi et al. (2010) observed signs of pulmonary inflammation following exposure of BALB/c/Sca mice to 10 mg/m^3 SiO_2-coated rutile TiO_2 nanoparticles 2 hours/day for 4 days or 4 days/week for 4 weeks, but found no evidence for pulmonary inflammation following short-term repeated exposure to 10 mg/m^3 concentrations of aerosols made from uncoated anatase, uncoated rutile/anatase (9:1), or uncoated anatase/brookite (3:1) nanoparticles (Table 13). These observations indicate that physical characteristics of nanoparticles, including surface characteristics from coatings, can influence potency to induce pulmonary inflammation under certain conditions.

Impairment of dilation in systemic arterioles has been observed in Sprague-Dawley rats following acute inhalation exposure for 4–12 hours to concentrations ranging from 1.5 to 20 mg/m^3 of aerosols made from fine rutile TiO_2 particles (1 µm primary particle size) or nanosized 80% anatase:20% rutile TiO_2 particles (21 nm primary particle size) (Nurkiewicz et al., 2009; 2008). Count mean diameters (GSDs) for aerosols made from fine and ultrafine particles were 402 nm (2.4) and 138 nm (2.2), respectively. Summaries of these acute-duration inhalation studies are not included in Table 13. Under the exposure conditions of these studies, significant microvascular dysfunction was observed without any histological signs of pulmonary inflammation. With both types of TiO_2 particles, the degree of microvascular dysfunction increased with increasing deposited mass of particles in the lung (Nurkiewicz et al., 2008). At equivalent lung mass burdens, however, rats exposed to TiO_2 nanoparticles showed a greater impairment than rats exposed to µm-sized TiO_2 particles (Nurkiewicz et al., 2008). The results indicate that acute inhalation exposure to insoluble TiO_2 particles in the µm- or nanosize range can induce systemic microvascular dysfunction at lung burdens producing no signs of pulmonary inflammation, and that nanoparticles are more potent than µm-sized particles. To date, however, exposure-response relationships for this effect from repeated exposure scenarios are not characterized.

Pulmonary inflammation is the principal effect identified in subchronic-duration inhalation studies of animals exposed to µm-sized aerosols made from anatase or 80% anatase:20% rutile TiO_2 nanoparticles (summarized in Table 13). Signs of pulmonary inflammation have been observed in F344 rats exposed to concentrations of about 23 mg/m^3

(Baggs et al., 1997; Janssen et al., 1994; Oberdörster et al., 1994; Ferin et al., 1992); in CDF (F344)/CrlBr rats exposed to 2 or 10 mg/m^3, but not 0.5 mg/m^3 (Bermudez et al., 2004), and in B3C3F1/CrlBR mice and Lak:LVG (SYR) BR hamsters exposed to 10 mg/m^3, but not 0.5 or 2 mg/m^3 (Bermudez et al., 2004). Each of these studies was designed to only examine respiratory tract endpoints, based on an assumption that no effects occur in nonrespiratory tract tissues (other than body weight changes) following repeated inhalation exposure to aerosols made from TiO_2 nanoparticles.

Results from the Bermudez et al. (2004) study clearly show the following order of species sensitivity to pulmonary inflammation from repeated inhalation exposure: rats > mice > hamsters. Species differences in sensitivity were associated with more severe pulmonary responses in rats than mice at equivalent lung burdens, and a high capacity of hamsters, compared with rats and mice, to rapidly clear particles from the lung. Similar species differences in sensitivity were observed in rats, mice, and hamsters exposed for 90 days to aerosols made from µm-sized (i.e., pigmentary) rutile TiO_2 particles at concentrations of 10, 50, or 250 mg/m^3 (Bermudez et al., 2002). Comparison of the results across these two studies supports the hypotheses that rats display more severe pulmonary responses than mice to conditions associated with high lung burdens of insoluble TiO_2 particles (regardless of primary particle size), that aerosols made from TiO_2 nanoparticles are more potent than aerosols made from µm-sized TiO_2 particles (signs of pulmonary inflammation occurred in rats and mice exposed to 10 mg/m^3 TiO_2 nanoparticles, but not to 10 mg/m^3 µm-sized TiO_2 particles), and that the hamster's relative lack of sensitivity is associated with relatively rapid lung clearance of particles.

Signs of pulmonary inflammation (increased number of neurtophils and dead cells in BAL fluid) have also been observed in pregnant C57BL/6BomTac mice exposed to µm-sized aerosols made from rutile TiO_2 nanoparticles at a single exposure concentration of 40 mg/m^3, 1 hour/day on GDs 8–18 (Hougaard et al., 2010).

In the only available chronic-duration study, female rats and mice were exposed to µm-sized aerosols made from 80% anatase:20% rutile TiO_2 nanoparticles at a single exposure concentration, 10 mg/m^3, for up to 2 years for rats and up to 13.5 months for mice (Heinrich et al., 1995). Exposed rats showed decreased lifespan, decreased body weights, increased lung wet weight, decreased ability to clear test iron oxide particles from the lungs, increased markers of inflammation in BAL fluid samples, bronchioalveolar hyperplasia, and interstitial fibrosis in nearly all rats exposed for 2 years, and increased incidence of lung tumors. Exposed mice showed decreased lifespan and body weights, but no increased incidence of lung tumors. The study clearly identifies 10 mg/m^3 as a chronic exposure concentration that produced nonneoplastic and neoplastic lung lesions in rats, but not in mice. However, exposure-response relationships for these noncancer and cancer lung effects from chronic exposure (most clearly observed in rats) have not been characterized.

Oral Exposure: Data regarding oral exposure to TiO_2 nanoparticles are limited to a few acute toxicity studies (Wang et al., 2007; Warheit et al., 2007) and two repeated-dose toxicity studies in rats and mice (Bu et al., 2010; Duan et al., 2010). Warheit et al. (2007) noted gray-colored feces and no other indications of toxicity in females (1/group) dosed with 1,750 or 5,000 mg/kg-day TiO_2 (median particle size, 140 nm). No mortality and no significant changes

in body weight or gross lesions were identified in rats dosed at up to 5,000 mg/kg-day; an oral LD_{50} >5,000 mg/kg-day was identified. In mice dosed once with 5,000 mg/kg-day TiO_2 nanoparticles via gavage, Wang et al. (2007) observed changes in clinical chemistry (including significant increases in ALT, LDH, α-HBDH, the ALT/AST ratio, and BUN concentration) and histopathology of the liver (hydropic degeneration and necrosis of hepatocytes) and kidneys (proteins in the renal tubules and swelling of the glomerulus).

In a repeated-dose study, rats were administered TiO_2 nanoparticles (mixture of anatase and rutile; <50 nm in size) at 0, 160, 400, or 1,000 mg/kg-day for 14 days; body weights, clinical chemistry (enzyme activities, and total levels of creatinine, cholesterol, bilirubin, and triglyceride) and hematological parameters (differential cell counts and hemoglobin concentration), metabonomic analyses of the of the urine and serum, and ultrastructural and histopathological examinations (10 tissues) were performed (Bu et al., 2010). Based on dose-related and statistically significant increases in serum enzyme activities and changes in hematological endpoints, the low dose of 160 mg/kg-day is identified as a LOAEL for this study; no NOAEL was identified.

In a second repeated-dose study, mice were administered TiO_2 nanoparticles (anatase, 5 nm in size) via gavage at 0, 62.5, 125, or 250 mg/kg-day every other day for 30 days (Duan et al., 2010). Endpoints evaluated included mortality and clinical signs of toxicity (monitored daily), body weights (at the start and end of treatment), clinical chemistry (including enzyme activities and total levels of protein, bilirubin, cholesterol, albumin, globulin, and triglycerides) and hematological parameters (including total and differential cell counts), selected organ weights, and histopathology (of the kidney and liver). Based on decreased body weight gain, increased relative organ weights (liver, kidney, spleen, and thymus), increased serum enzyme markers for hepatotoxicity (e.g., ALT, AST, ALP, LDH), marked increases in serum levels of NO, and marked decreases in several hematological endpoints including WBCs, RBCs, hemoglobin, and reticulocytes, the mid-dose level of 125 mg/kg-day was identified as a LOAEL for this study. Statistical changes in some hematological and immune endpoints at the low dose of 62.5 mg/kg-day were not considered to be biologically significant by the researchers; this dose level was identified as a NOAEL.

Although two studies in two different species are available, each has important study limitations. In one of the studies, Bu et al. (2010) suggested that significant changes in clinical chemistry parameters (dose-related increases in the activities of serum enzymes) and metabonomic profiles (of the serum and urine) in rats treated with TiO_2 at up to 1,000 mg/kg-day were potentially indicative of liver and/or heart toxicity; however, no histological changes to support this claim were apparent, and the duration of study was too short (14 days) to be used for the derivation of a subchronic toxicity value. In the other study (Duan et al., 2010), mice dosed every other day for 30 days with TiO_2 at up to 250 mg/kg-day showed decreased body weights and some dose-dependent changes in hematology and clinical chemistry parameters (including those associated with liver function); however, these data were poorly reported. For example, changes in IL-2 levels, reported to be statistically significant by the authors, were found to not be significant in unpaired *t*-tests performed for this review. The administered doses were also represented incorrectly in one of the data tables. No supporting data (studies of longer duration,

reproductive or developmental studies) regarding the oral toxicity of TiO_2 nanoparticles are available.

Dermal Exposure: Data regarding dermal exposure to TiO_2 nanoparticles are limited to one repeated-dose study in which mice were administered various formulations containing 5% TiO_2 particles (fine particles [300–500 nm in size] or nanoparticles [with primary particle sizes of 10, 21, 25, or 60 nm]) to a 3-cm^2 patch of the dorsal skin for 60 days (Wu et al., 2009). Extensive characterization of the particles utilized in the experiment was performed. Endpoints evaluated included mortality and clinical signs of toxicity (monitored daily), body weights (recorded weekly), levels of MDA and SOD (biomarkers of antioxidant response) in the skin and liver, HYP content of the skin, selected organ weights, and histopathology (eight tissues). Based on decreased body weight, changes in enzyme activity levels (liver and skin), and histopathological changes to the skin (including excessive keratinization, wrinkled epidermis, and liquefaction necrosis), which occurred mainly in mice treated with TiO_2 with primary particle sizes of 10, 21, and 25 nm, a LOAEL of 5% TiO_2 (or 6.7 mg/kg-day) was identified. An important limitation to this study is the use of a single exposure concentration; therefore, a LOAEL, but not a NOAEL, could be identified. Since a NOAEL was not identified, it is unclear if effects might occur at a lower dose of TiO_2. No supporting data regarding the dermal toxicity of TiO_2 nanoparticles are available.

4.8. DERIVATION OF POTENTIAL ACCEPTABLE DAILY INTAKES (ADIs)

For inhalation exposure, a potential ADI is derived using the NOAEL of 0.5 mg/m^3 for pulmonary inflammation in rats exposed to aerosols of TiO_2 nanoparticles (3:1 anatase:rutile; average primary particle size of 21 nm) for 13 weeks in Bermudez et al. (2004) as the point of departure. An inhalation ADI for TiO_2 nanoparticles of 0.9 $\mu g/m^3$ is derived by dividing the duration-adjusted NOAEL (0.09 mg/m^3 = 0.5 mg/m^3 × 6 hour/24 hour × 5 day/7 day) by a total uncertainty factor of 100 (10 for extrapolation from animals to humans and 10 for protection of susceptible populations).

Given the limited data available for oral exposure to TiO_2 (two acute toxicity studies and two repeated-dose studies with study limitations), and in the absence of supporting data (additional subchronic-duration, chronic duration, or reproductive, or developmental toxicity studies, an ADI for oral exposure to TiO_2 nanoparticles is not derived.

Similarly, only one repeated-dose study, with a single exposure level, is available to assess dermal toxicity to TiO_2 nanoparticles. In the absence of supporting data from subchronic, chronic, reproductive, or developmental studies, an ADI for dermal exposure to TiO_2 nanoparticles is not derived.

4.9. CONCLUSIONS

The available repeated-exposure toxicity data clearly identify pulmonary inflammation as a critical effect in rodents following inhalation exposure to aerosols made from TiO_2 nanoparticles. However, there are several areas of uncertainty to consider in extrapolating the observed effects in rodents to possible human exposure scenarios.

(1) There is a lack of data to describe exposure-response relationships for pulmonary inflammation in animals chronically exposed to aerosols made from any type of TiO_2 nanoparticle. The 2-year rat bioassay conducted by Heinrich et al. (1995) only included one exposure level, 10 mg/m^3, for aerosols made from 80% anatase:20% rutile TiO_2 nanoparticles. This exposure level produced pulmonary inflammation and lung tumors associated with a high lung burden of insoluble TiO_2 particles. In contrast, results from the 13-week study by Bermudez et al. (2004) provide adequate dose-response data to identify NOAEL and LOAELs for pulmonary inflammation in rats (0.5 and 2 mg/m^3, respectively), mice (2 and 10 mg/m^3), and hamsters (2 and 10 mg/m^3) exposed to aerosols made from anatase:rutile TiO_2 nanoparticles (see Table 13). The proposed inhalation ADI derived in Section 6 is based on an assumption that the dose-response relationships for pulmonary inflammation in rats exposed to aerosols are similar for subchronic and chronic durations of exposure. Results from the 5-day study by Ma-Hock et al. (2009) of rats exposed to aerosols made from anatase:rutile nanoparticles (see Table 13) indicate that duration of exposure may have minor influence on the exposure-response relationship for pulmonary inflammation from TiO_2 nanoparticles, because the mild signs of pulmonary inflammation at 2 mg/m^3 in the 5-day study were similar to those observed in the 13-week rat study at the same exposure concentration (Bermudez et al., 2004). The 5-day study, however, did not include an exposure level of 0.5 mg/m^3, an exposure level that was a NOAEL in the 13-week study. Nevertheless, any use of the proposed inhalation ADI to estimate risks for pulmonary inflammation from chronic exposure has an inherent uncertainty because it is based on subchronic-duration data.

(2) There are inadequate data to indicate the degree to which aerosol size and shape may influence exposure-response relationships for pulmonary inflammation in rodents. The available repeated-exposure inhalation bioassays exposed animals to µm-sized aerosols that were agglomerations of TiO_2 nanoparticles. The available data are adequate to conclude that µm-sized aerosols made from TiO_2 nanoparticles are more potent than similarly sized aerosols made from "fine" (µm-sized) TiO_2 particles (see Bermudez et al., 2004, 2002; Baggs et al., 1997; Janssen et al., 1994; Oberdörster et al., 1994; Ferin et al., 1992). In addition, available toxicokinetic data suggest that differences in lung clearance kinetics provide at least a partial explanation of the potency difference (Geiser et al., 2008, 2005; Sager et al., 2008a; Bermudez et al., 2004, 2002; Oberdörster et al., 1994; Ferin et al., 1992). No toxicity data are available, however, for aerosols in the nanosize range, with the exception of the short-term inhalation toxicity studies of mice by Rossi et al. (2010) and Grassian et al. (2007a). These short-term studies, however, only used single exposure concentrations and do not provide adequate description of exposure-response relationships for pulmonary inflammation from short-term exposures. Any use of the proposed inhalation ADI to estimate risks for pulmonary inflammation presented by exposure scenarios to nanosized aerosols made from TiO_2 nanoparticles would have an inherent uncertainty, because the ADI is based on toxicity data for rats exposed to µm-sized aerosols made from TiO_2 nanoparticles.

(3) There is suggestive evidence to indicate that TiO_2 nanoparticle characteristics such as crystalline form or coating may influence potency or exposure-response relationships for

TiO_2-induced pulmonary inflammation, but repeated exposure-response data are only available for aerosols made from uncoated anatase or anastase:rutile nanoparticles. Rossi et al. (2010) observed signs of pulmonary inflammation following short-term exposure of BALB/c/Sca mice to 10 mg/m^3 SiO_2-coated rutile TiO_2 nanoparticles, but found no evidence for pulmonary inflammation following exposure to aerosols made from other types of TiO_2 nanoparticles (uncoated anatase, uncoated rutile/anatase (9:1), or uncoated anatase/brookite (3:1) nanoparticles). These observations indicate that physical characteristics of nanoparticles, including surface coatings, can influence potency to induce pulmonary inflammation under certain conditions. In vitro genotoxicity and cytotoxicity studies have more extensively evaluated the influence of particle characteristics on toxicity. However, as discussed in Sections 4.1 and 4.2, no clear conclusions could be drawn from these data, because of the inconsistency of the results across studies. For example, some in vivo data (Renwick et al., 2004) suggest that the cytoxic potential of TiO_2 increases with decreasing particle size (from fine- to nanoscale), but other in vivo (Warheit et al., 2006) and in vitro data in human cell lines indicate that fine and nano-TiO_2 elicit cytotoxicity to comparable degrees (Lai et al., 2008; Park et al., 2007). Other in vitro data (L'Azou et al., 2008; Simon-Deckers et al., 2008) suggest that particle size, with other factors such as crystalline form, influences the cytotoxic response, with smaller particle size generally associated with a greater cytotoxic response (Chen et al., 2010; Hamilton et al., 2009; Aisaka et al. ,2008; L'Azou et al., 2008; Simon-Deckers et al., 2008; Sayes et al., 2006). Other studies provide evidence that surface modifications can also modulate the cytotoxic potency of TiO_2 nanoparticles in vivo and in vitro (Tiano et al., 2010; Onuma et al., 2009; Pan et al., 2009; Bhattacharya et al., 2008), and Warheit et al., 2007). Taken together, the data suggest that no single particle characteristic is the best correlate for toxicity; characteristics such as crystalline form, size, shape, and surface coating may contribute to the toxicity of TiO_2 nanoparticles, depending on the exposure conditions and endpoint evaluated. In summary, there is inherent uncertainty in the use of the proposed inhalation ADI to estimate risks for pulmonary inflammation from exposure to aerosols made from TiO_2 nanoparticles with different characteristics (e.g., surface coating, crystalline form) than those used in the available 13-week animal toxicity studies (i.e., nanoparticles of uncoated anatase or 80% anatase/20% rutile: Bermudez et al., 2004; Baggs et al., 1997; Janssen et al., 1994; Oberdörster et al., 1994; Ferin et al., 1992).

(4) Although pulmonary inflammation is clearly identified as a critical effect from acute-, short-term-, subchronic-, and chronic-duration toxicity studies of animals exposed to aerosols made from TiO_2 nanoparticles, results from acute-duration inhalation studies of rats indicate that impairment of dilation in systemic arterioles may occur under exposure conditions producing no signs of pulmonary inflammation (Nurkiewicz et al., 2009, 2008). The available repeated-exposure inhalation toxicity studies, however, have not examined endpoints associated with microvascular dysfunction, and exposure-response relationships for this effect from repeated exposure scenarios are not characterized. Without additional data, it is uncertain that the proposed ADI would provide protection for possible microvascular dysfunction from repeated exposures to aerosols made from TiO_2 nanoparticles.

ADI values for oral or dermal exposure to TiO_2 nanoparticles were not derived due to inadequate data to identify possible health hazards from these routes of exposure.

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5.0. TOXICITY DATA ON CARBON NANOTUBES (CNTs)

5.1. ABSORPTION, DISTRIBUTION, METABOLISM, AND ELIMINATION

Overview: Limited toxicokinetic data are available in animals exposed by oral administration, inhalation, intratracheal instillation, or pharyngeal aspiration of nonfunctionalized single- or multi-walled CNTs (SWCNTs or MWCNTs). In contrast, tissue distribution and elimination of surface-modified ("functionalized") CNTs following intraperitoneal (i.p.) or intravenous (i.v.) injection of animals has received considerable research attention, with an overall goal of developing water-soluble or water-dispersable CNTs as in vivo delivery vehicles for therapeutic or photoimaging agents targeted for specific organs or tissues (e.g., Villa et al., 2008; Guo et al., 2007; Liu et al., 2007; McDevitt et al., 2007a, b; Singh et al., 2006). The functional groups under investigation include hydroxyls, amines, phospholipids with polyethylene glycol (PEG), oligonucleotides, peptides, and antibodies.

Quantitative data on the rate and extent of absorption of inhaled, ingested, or dermally applied CNTs (functionalized or not) are not available for humans or animals.

Quantitative tissue distribution and elimination data in animals following oral exposure are limited to a study of mice exposed to single doses of water-soluble hydroxylated SWCNTs (Wang et al., 2004). These data indicate a rapid absorption and distribution of hydroxylated SWCNTs to tissues including the liver, kidney, and bone, but the extent of absorption by the gastrointestinal tract could not be estimated from the collected data (Wang et al., 2004). No tissue distribution and elimination data were located for animals orally exposed to other functionalized CNTs or poorly-soluble, nonfunctionalized CNTs.

Quantitative data on the tissue distribution of CNTs following inhalation exposure are limited to studies of rats following acute (Ellinger-Ziegelbauer and Pauluhn, 2009) and subchronic (Pauluhn et al., 2010a) inhalation exposures to agglomerated aerosols of a commercial nonfunctionalized MWCNT (Baytubes®). The results indicate that, like other inhaled particles with poor water solubility, retention of deposited material occurs in the lung, with only limited transport to non-portal-of-entry tissues and a time- and concentration-dependent transport of deposited material to lung-associated lymph nodes (LALNs) following subchronic exposure to concentrations $\geq$1.5 mg/m^3. The retention of nonfunctionalized MWCNTs in lung tissue and relatively slow movement into subpleural tissue is supported by observations of MWCNT-laden subpleural macrophages at 1 day, and 2 and 6 weeks following single 6-hour exposures of mice to MWCNT aerosols at 30 mg/m^3 (Ryman-Rasmussen et al., 2009a).

The persistence (i.e., deposition and slow clearance) of poorly-soluble, nonfunctionalized CNTs in respiratory tract tissues has been demonstrated following intratracheal instillation or pharyngeal aspiration of suspensions of nonfunctionalized SWCNTs or MWCNTs into animals (Mercer et al., 2010, 2008; Mutlu et al., 2010; Hubbs et al., 2009; Muller et al., 2005). Results from these experiments indicate that physical properties of agglomerated particles in the suspensions (e.g., length) and degree of agglomeration can influence kinetics of macrophage-mediated clearance from the lung and movement of deposited particles from airway surfaces to

the pleura and lymph nodes. For example, well-dispersed CNTs appear to be more widely dispersed in respiratory tissues, compared with agglomerated CNTs (Mercer et al., 2008), and shorter MWNCTs (average length = 0.7 μm) appear to be more rapidly cleared from respiratory tissues, compared with longer MWCNTs (average length = 5.9 μm) (Muller et al., 2005).

Results from tissue distribution and elimination studies of animals (principally mice) exposed by i.p. or i.v. injection indicate that poorly-soluble, nonfunctionalized CNTs (which aggregate in aqueous environments) can be distributed to specific organs (e.g., liver, lung, or spleen) where they accumulate and are slowly cleared (Kolosnjaj-Tabi et al., 2010; Lacerda et al. 2008a, b; Yang et al., 2008, 2007; Cherukuri et al., 2006). For example, in mice given single i.v. injections of [^{13}C]-labeled SWCNTs, the liver, lung, and spleen contained about 21, 9, and 2% of the injected dose, respectively, 28 days after injection; concentrations in other tissues were very low (Yang et al., 2007). Concentrations of SWCNTs in these accumulating tissues at 28 days were similar to concentrations at 1 day, indicative of very limited elimination (Yang et al., 2007). In contrast, some injected water-soluble or water-dispersable functionalized SWCNTs or MWCNTs are more rapidly distributed throughout tissues and eliminated through the kidneys in the urine, and through the bile in the feces. Water-soluble, functionalized CNTs that appear to be more rapidly eliminated (compared with nonfunctionalized CNTs) from mice after i.p. or i.v. injection include hydroxylated-SWCNTs (Wang et al., 2004), aminated SWCNTs (McDevitt et al., 2007a, b; Singh et al., 2006), aminated MWCNTs (Lacerda et al., 2008a, b), and glucosaminated MWCNTs (Guo et al., 2007). The available evidence indicates that surface modifications that prevent aggregates from forming in aqueous environments counteract the aggregation and accumulation of i.p. or i.v. injected CNTs in liver, lung, and spleen tissues and promote more rapid elimination in the urine via the kidneys and the feces, presumably via hepatic biliary excretion, compared with nonfunctionalized CNTs. Additional surface modifications with "targeting" functional groups, such as peptides and antibodies (which target tumor tissue components), can also influence the tissue distribution of injected CNTs (Villa et al., 2008; Liu et al., 2007; McDevitt et al., 2007b).

Studies on the in vivo or in vitro metabolism of nonfunctionalized CNTs in humans or animals were not located. The metabolism of functionalized CNTs has been investigated to a very limited extent. Evidence for defunctionalization of phopholipid polyethylene glycosylated SWNCTs was reported in the livers, but not the spleens, of mice following i.v. administration of the material into mice (Yang et al., 2009). In vitro incubation of carboxylated SWCNTs with a plant peroxidase and hydrogen peroxidase led to degradation of the CNTs over a 12–16-week period (Allen et al., 2008).

5.1.1. Absorption

Quantitative data on the rate or extent of absorption of inhaled, ingested, or dermally applied CNTs are not available for humans or animals.

Limited qualitative evidence of absorption and distribution is available in reports of detection of CNTs in non-portal-of-entry tissues in animals following repeated inhalation exposure to aerosols of nonfunctionalized MWCNTs (Ellinger-Ziegelbauer and Pauluhn, 2009) and acute oral exposure to water-soluble hydroxylated SWCNTs (Wang et al., 2004).

Evidence of very limited absorption and distribution to non-portal-of-entry tissues was found in a study of male Sprague-Dawley rats exposed to aerosols of nonfunctionalized MWCNTs (containing 0.53% cobalt) for 6 hours; 7 days following exposure, the liver (but not LALNs, brain, kidneys, or testes) showed elevated concentrations of cobalt, compared with control means (Ellinger-Ziegelbauer and Pauluhn, 2009; see Section 5.1.2 for more study details).

In contrast, Wang et al. (2004) reported that 3 hours following gavage administration of aqueous solutions containing 1.5 µg ^{125}I-labeled hydroxylated SWCNTs to male KM mice, elevated levels of radioactivity were detected in the stomach, bone, kidney, lung, blood, heart, liver, and spleen, but very little radioactivity was detected in the brain (see Section 5.1.2 for more details about this study). This study was not designed to provide quantitative estimates of absorption; during post-exposure periods, urine and feces were not collected for determination of radioactivity.

5.1.2. Tissue Distribution and Elimination

No studies were located examining the tissue distribution and elimination of CNTs in humans.

Oral Exposure Studies: No tissue distribution and elimination data were located for animals orally exposed to poorly-soluble, nonfunctionalized CNTs. Only one oral exposure study with data for a functionalized CNT was located (Wang et al., 2004).

Wang et al. (2004) administered aqueous solutions of ^{125}I-labeled hydroxylated SWCNTs to male KM mice (5/group; each weighing about 25 g) at a dose of 1.5 µg/mouse by i.p. injection, subcutaneous (s.c.) injection, gavage, or i.v. injection. The hydroxylated SWCNTs were labeled by reacting them with Na^{125}I and N-chloro-p-toluenesulfonamide (chloramine-T). The test material was reported to be soluble in water at the test concentration of 15 µg/mL. As characterized by laser scattering, the hydroxylated SWCNTs had a mean length of 340 nm (range 280–450 nm); an average diameter of about 1.4 nm was reported for the SWCNTs. Animals were sacrificed 3 hours after dosing and the following tissues were dissected, weighed, and analyzed for radioactivity: skin, muscle (leg), bone (shank), brain, heart, lung, liver, kidney, spleen, stomach (emptied), and intestine (emptied). Excreta were not collected for analysis of radioactivity. The time-course of tissue distribution and elimination in excreta following i.p. administration was investigated in separate experiments discussed later in this report (see Table 5.1). Three hours following oral administration, estimated mean concentrations of SWCNT (ng/g wet weight of tissue) showed the following order: stomach (~120) > bone (~75) > kidney (~60) > lung (~50) > blood or skin (~20–25) > heart, liver, spleen, or intestine (~15) > muscle (~10) > brain (<5). Similar distribution profiles were observed with i.v., i.p., or s.c. injection, with the exceptions that 1) lung concentrations were similar to heart, liver, and spleen concentrations following any of the injection routes, and 2) skin concentrations were similar to bone, kidney, and stomach concentrations following s.c. injection. The time course data, following i.p. injection, showed peak concentrations at 6 hours after dosing, with the highest peak concentrations in the bone, kidneys, and stomach (~100 ng/g wet weight in each). The

results indicate that hydroxylated SWCNTs are distributed preferentially to the stomach, bone, kidneys, and lungs following oral, i.v., i.p., or s.c. administration of single doses to mice.

Inhalation Exposure Studies: Ellinger-Ziegelbauer and Pauluhn (2009) exposed male Wistar rats (nose-only; 6 rats/group) to aerosols of 0, 11, or 241 mg/m^3 MWCNTs (containing 0.53% cobalt) for 6 hours and sacrificed rats at 7 or 90 days following exposure to determine cobalt concentrations as a surrogate for MWCNT concentrations in lung, LALNs, brain, liver, kidneys, and testes. MWCNT (Baytubes®) were reported to have diameters of 10–16 nm and surface areas of 253 m^2/g. Measured mass median aerodynamic diameters (MMADs; with geometric standard deviations [GSD]) for aerosols (described as assemblages of coiled, intertwined structures) in test atmospheres were 2.9 (1.8) and 2.0 (1.8) µm for 11 mg/m^3 and 2.2 (2.6) and 1.9 (1.6) µm for 241 mg/m^3. Seven days after exposure, mean cobalt concentrations were elevated in the lungs of rats exposed to 11 mg/m^3 (0.113 µg Co/g) and 241 mg/m^3 (0.418 µg Co/g), compared with control means (<0.03 µg Co/g); the liver in rats exposed to 241 mg/m^3 was the only other tissue showing an elevated mean concentration (0.062 versus 0.044 µg Co/g), compared with control means. At 90 days after exposure, the lungs of rats exposed to 241 mg/m^3 were the only tissue showing an elevated concentration (0.237 versus <0.03 µg Co/g). Assuming first-order elimination kinetics, estimated elimination half-times of cobalt from the lungs at 11 and 241 mg/m^3 were 60 and 140 days, respectively. The results suggest that inhaled MWCNTs are deposited in the lung in a concentration-dependent manner following acute exposure. The results also provide evidence that deposited MWCNTs are slowly cleared from the lungs, but not widely distributed to non-portal-of-entry tissues; 7 days following exposure, the liver (but not LALNs, brain, kidneys, or testes) showed elevated concentrations of cobalt, compared with controls.

Pauluhn (2010a) also evaluated MWCNT biokinetics in the lungs and LALNs of male Wistar rats (6/dose/time period) exposed to 0, 0.1, 0.4, 1.5, or 6 mg/m^3 (6 hours/day, 5 days/week) for 13 weeks by evaluating cobalt burdens in these tissues at 1 day, and 4, 13, and 26 weeks after exposure. Cobalt was used as a surrogate measure of the MWCNTs (Baytubes®) used in this study, which contained 0.53% cobalt. Aerosols in test atmospheres were described as coiled, tangled assemblages of MWCNT with MMADs ranging from 1.67 to 2.19 µm (GSD, 1.68–1.76). Mean lung burdens at the post-exposure periods showed the following approximate ranges in the low- to high-concentration groups, respectively: below the limit of quantification; ~50–100; ~400–500; and ~1,500–2,000 ng Co/lung; respective elimination half-times, assuming first-order kinetics, were 151, 350, 318, and 375 days. In rats exposed to 1.5 or 6 mg/m^3, mean cobalt burdens in LALNs increased with concentration and time after exposure. The results indicate that deposited MWCNTs are slowly cleared from the lung following subchronic inhalation exposure, and that post-exposure translocation and accumulation of MWCNTs to and in lymph nodes was pronounced following subchronic exposure to concentrations of 1.5 and 6 mg/m^3.

Ryman-Rasmussen et al. (2009a) exposed male B57BL6 mice (10/group) to nonfunctionalized MWCNT aerosols at 0, 1, or 30 mg/m^3 for 6 hours and then collected lung tissues at 1 day, and 2, 4, and 14 weeks after exposure for light and electron microscopic examinations. MWCNTs used to make aerosols showed widths between 10 and 50 nm. Aerosols were a mixture of agglomerated and individual nanotubes with lengths ranging from

<100 nm to >10 µm, and MMADs of 183 and 164 nm for the low- and high-exposure group (GSD <2), respectively. Microscopy showed MWCNT-laden macrophages in the subpleural regions at 1 day, and 2 and 4 weeks after exposure to the high concentration. Focal and regional subpleural fibrotic lesions (with MWCNT-laden macrophages present beneath the lesions) were apparent at 2 and 4 weeks (but not 14 weeks) after exposure to the high concentration only. The results indicate that following inhalation of aerosols of nonfunctionalized MWCNTs, materials accumulated in lung tissue and slowly moved into subpleural tissue

Intratracheal Instillation or Pharyngeal Aspiration Studies: Following single intratracheal instillations of suspensions (1% Tween in 0.9% saline) of unground MWCNTs (average length = 5.9 µm) or ground MWCNTs (average length = 0.7 µm) in female Sprague-Dawley rats (0.5 mg instilled/rat), approximately 80 or 40% of the administered dose, respectively, remained in the lung 60 days after instillation (Muller et al., 2005). These findings demonstrate slow clearance of deposited MWCNTs from the lung, but indicate that shorter MWCNTs are cleared more quickly than longer MWCNTs. Histological examinations of lung sections shortly after exposure revealed that the ground MWCNT were more widely dispersed in the lung, whereas the unground MWCNTs showed localized accumulation of agglomerates in bronchial airways.

Similarly, following pharyngeal aspiration of suspensions of untreated nonfunctionalized MWCNTs (with average area equivalent diameter of 15.2 µm) or well-dispersed, nonfunctionalized MWCNTs (with average area equivalent diameter of 0.69 µm) to mice (10 µg/mouse), electron microscopy showed that the untreated MWCNTs were deposited as large agglomerations near the proximal alveolar region, whereas the dispersed MWCNTs were widely distributed throughout alveolar interstitial spaces and in the vicinity of the pleura (Mercer et al., 2008).

In a study currently available only as an abstract, Hubbs et al. (2009) noted that, following exposure of mice by pharyngeal aspiration to 20 or 80 µg MWCNTs per mouse, histological examination of lungs and tracheobroncial lymph nodes collected 7 and 56 days after exposure showed MWCNTs principally accumulating in macrophages, granulomatous inflammation, and pleural inflammation in 7/8 and 4/8 MWCNT-exposed mice at 7 and 56 days, respectively. Indicative of movement of MWCNTs to the tracheobronchial lymph nodes and the pleura, dilated subpleural and peribronchiolar lymphatics were observed at 56 days in mice exposed to 80 µg. Mean dimensions of the MWCNTs were reported to be 4.2 µm × 49 nm, but further characterization of the administered material and suspension vehicle was not reported. The observations are consistent with translocation within the lung by alveolar macrophages and movement of particles to the pleura.

Mercer et al. (2010) used light microscopic morphometry to determine the distribution and numbers of MWCNT particles in alveolar epithelium, intrapleural space, and subpleural tissue at 1, 7, 28, and 56 days following exposure of male C57BL/6J mice (n = 8/dose and timepoint) by pharyngeal aspiration to 0, 10, 20, 40, or 80 µg MWCNTs. The nonfunctionalized MWCNTs (purchased from Mitsui and Company) were well dispersed in a medium (Ca^{+2}- and Mg^{+2}-free phosphate-buffered saline [PBS], pH 7.4, with 5.5 mM D-glucose, 0.6 mg/mL mouse serum albumin, and 0.01 mg/mL 1,2-dipalmitoyl-*sn*-glycero-3-phospholine choline [1,2-DSPC])

and determined to contain 0.78% metal contaminants, with sodium (0.41%) and iron (0.32%) being the major metal contaminants. Median length of the MWCNTs was 3.86 µm and count mean diameter was 49 nm. Particles (i.e., "fibers") penetrating into various regions of lung sections (alveolar epithelium, subpleural tissue, and intrapleural space) were counted by means of an eyepiece counting grid method. At 1 day post-aspiration of the 80-µg dose, particles in airways, alveoli, and subpleural tissue represented 18, 81, and 0.6% of the total lung burden, respectively; most particles in the alveolar region were associated with macrophages (about 62% of total lung burden). Particle penetrations into alveolar Type I epithelial cells at 1 day were frequently detected, but estimated to be about 18-fold less than penetrations into alveolar macrophages. Fifty-six days after aspiration, the number of particle penetrations into alveolar epithelial cells per lung showed an apparent sigmoidal dose-response relationship with an apparent ED_{50} of about 15 µg. Fifty-six days after aspiration, the number of particle penetrations per lung into intrapleural space and subpleural tissue increased with increasing doses of 20, 40, and 80 µg; no particle penetrations into these tissue regions were detected at this timepoint in mice exposed to 10 µg. At the highest dose, the numbers of particle penetrations into intrapleural space and subpleural tissue were highest 1 day after aspiration and declined by 7 days to about 10–15% of the 1-day mean number (e.g., about 28,000 penetrations per lung into subpleural tissue at 1 day versus about 3,000 at day 7). At 28 and 56 days, the numbers of penetrations in intrapleural tissue were about 30% of the 1-day mean value, indicative of continued transport into the pleura from other lung regions. The results indicate that aspirated MWCNTs moved into the visceral pleura of mice rapidly (within 1 day) and were cleared to a large extent by 7 days. However, particle penetrations were still evident in intrapleural space and subpleural tissue 56 days after aspiration. The results indicate that the penetrations of aspirated MWCNTs into alveolar macrophages, alveolar epithelium, and visceral pleura of mice are rapid, frequent, and persistent, and that movement of MWCNT particles into the visceral pleura continued throughout a 7–56-day post-aspiration period.

Mutlu et al. (2010) reported that intratracheal instillation of well-dispersed, nonfunctionalized SWCNTs suspended in 1% aqueous solution of a nonionic surfactant, called Pluronic 108NF, in mice (40 µg/mouse) produced a diffuse pattern of deposition throughout the lung and no histological evidence of inflammation or fibrosis in respiratory tissue 30 days after exposure, whereas the same SWCNTs suspended in phosphate buffered saline were agglomerated in suspension and produced areas of chronic inflammation surrounding aggregates of SWCNTs deposited on the surface of medium sized airways. Sections of lungs sampled 24 hours after exposure showed alveolar macrophages containing structures thought to be SWCNTs, and lungs sampled 90 days after exposure to the dispersed SWCNT showed minimal light microscopic evidence of SWCNTs. The latter observations suggest that considerable macrophage-mediated clearance of the dispersed SWCNTs occurred within 90 days.

Other studies have had difficulties detecting nonfunctionalized MWCNTs or SWCNTs in extrarespiratory tract tissues following instillation or aspiration of suspensions of these materials in animals (Al Faraj et al., 2010; Mercer et al., 2009; Elgrabli et al., 2008a).

In an abstract, Mercer et al. (2009) noted that only lung and gastrointestinal tract tissue contained detectable gold-labeled SWCNTs at intervals of 1 hour and 1, 3, 7, and 28 days following pharyngeal aspiration of nonfunctionalized SWCNTs in C57BL/6 mice (40 µg

suspended in phosphate buffered saline/mouse). Other tissues examined included heart, brain, liver, kidneys, right cranial mediastinal lymph node, and blood. Gold content in lung tissue decreased by 49% between days 7 and 28.

Elgrabli et al. (2008a) detected MWCNTs (containing 0.53% nickel) by inductively coupled plasma (ICP) optical emission spectrometry (OES) quantification of nickel in lungs at 1, 7, 30, 90, and 180 days following exposure of rats (n = 6 per time point) by intratracheal instillation to suspensions of 100 µg nonfunctionalized MWCNTs, but could not detect elevated nickel levels in liver, kidneys, spleen, heart, brain, thymus, or testis tissues at any time point. MWCNTs were suspended in saline solution with bovine serum albumin (concentrations not specified). The MWCNTs in the suspensions were agglomerated with >80% of agglomerates showing "a size smaller than 10 µm", but additional characterization of the administered material was not presented in the report. Nickel detected in the lung samples (bronchoalveolar lavage fluids [BALFs] were collected from animals before each sampling period) accounted for the following percentages of the administered nickel: 53, 54, 55, 26, and 16% at 1, 7, 30, 90, and 180 days after exposure. Nickel detected in the cellular fraction of BAL samples accounted for the following percentages of the administered nickel for the 1–180-day samples: 10, 24, 14, 12%, and below the detection limit. Nickel levels in the fluid fraction of bronchoalveolar lavage (BAL) samples were below the detection limits at all time points. These results indicate a slow clearance from the lung, presumably via macrophage- and mucociliary-mediated transport to the gastrointestinal tract; the absence of increased nickel levels in the examined extrarespiratory tissues indicates minimal migration to these tissues following exposure.

Al Faraj et al. (2010) used non-invasive magnetic resonance imaging (MRI) techniques to qualitatively detect metal impurities in nonfunctionalized SWCNTs in lungs, liver, spleen, and kidneys following intratracheal administration of suspensions of nonfunctionalized SWCNTs to rats (0.1, 0.5, or 1 mg SWCNTs suspended in saline solution with bovine serum albumin per rat). Metals in SWCNTs in lungs sampled 1 and 30 days after exposure could be detected with this technique, but no SWCNTs were detected in the liver, spleen, or kidneys. Definitive conclusions about the transport of SWCNT from the lung to the liver, spleen, or kidneys cannot be made from these observations, because the quantitative limits of the MRI technique are uncharacterized.

i.p. or i.v. Injection Studies: Summaries of available animal studies are presented in Table 5.1. Studies of poorly water soluble, nonfunctionalized CNTs are presented first, followed by studies of water-soluble or water-dispersable functionalized CNTs.

The most quantitative data concerning the tissue distribution and elimination of water insoluble nonfunctionalized CNTs come from a study of mice given single i.v. doses (600 µg/mouse) of [^{13}C]-labeled SWCNTs (Yang et al., 2007). At 1, 7, and 28 days after dose administration, [^{13}C] isotope ratio mass spectrometry showed that SWCNTs accumulated principally in the liver, lung, and spleen, with the liver being the predominant tissue of accumulation (see Table 5.1). Concentrations in liver, lung, and spleen did not show marked decreases between days 1 and 28 after injection. SWCNTs were "hardly detected" (by transmission electron microscopy [TEM] or [^{13}C] analysis) in urine and feces samples collected during this post-exposure period (Yang et al., 2007). The results indicate that i.v. injected

nonfunctionalized SWCNTs accumulate in liver, lung, and spleen of mice and are eliminated very slowly. Supportive, less quantitative information showing the persistence of nonfunctionalized CNTs in animals following injection include (see Table 5.1 for more details): (1) detection of SWCNTs in liver, lung, and spleen (by TEM and Raman spectroscopy) 90 days after i.v. injection of mice with 1,000 µg SWCNTs (Yang et al., 2008); (2) detection of SWCNTs by near-infrared (IR) fluorescence microscopy in liver sections from rabbits sacrificed 24 hours after i.v. injection of 75 µg SWCNTs (Cherukuri et al., 2006); (3) detection, by TEM, of aggregated CNTs in liver and spleen macrophages and renal tubule epithelial cells, 14 days after i.p. injection of mice with 20 mg SWCNTs (either raw or "purified" to remove metal contaminants) or 10 mg SWCNTs with decreased lengths (ultra-short-tubes) (Kolosnjaj-Tabi et al., 2010); and (4) detection, by TEM, of bundles of MWCNTs in glomerular capillaries and no detection of individual MWCNTs crossing the renal filtration membrane, 5 and 30 minutes following i.v. injection of mice with 200 µg nonfunctionalized MWCNTs (Lacerda et al., 2008a, b).

Water-soluble, functionalized CNTs, which appear to be more rapidly eliminated (compared with nonfunctionalized CNTs) from mice after i.p. or i.v. injection, include hydroxylated-SWCNTs (Wang et al., 2004), aminated SWCNTs (McDevitt et al., 2007a, b; Singh et al., 2006), aminated MWCNTs (Lacerda et al., 2008a, b), and glucosaminated MWCNTs (Guo et al., 2007). Details of tissue distribution and elimination studies of these materials are summarized in Table 5.1. Following i.p. injection of mice with radiolabeled hydroxylated SWCNTs, 80% of the injected dose was detected in urine and feces samples collected for 11 days (Wang et al., 2004). In studies of i.v. injection of radiolabeled aminated CNTs into mice, <1% of the injected dose of aminated SWCNTs remained in tissues 24 hours after injection in one study (Singh et al., 2006); concentrations in kidney, liver, and spleen declined by about 70, 45, and 30% between days 1 and 15 days after injection in another study (McDevitt et al., 2007b); and radioactivity was barely discernable in the kidneys and bladder, 24 hours after injection of aminated MWCNTs in a third study (Lacerda et al., 2008b). In mice given single i.p. injections of radiolabeled glucosaminated MWCNTs, radioactivity in urine and feces collected for 24 hours accounted for 70% of the injected dose (Guo et al., 2007).

The effects on tissue distribution and elimination of additional functional groups that might recognize tumor components (e.g., peptides or antibodies) or allow in vivo self-assembly of nanoparticles (e.g., oligonucleotides) to water-soluble, functionalized SWCNTs has been examined. Attachment of a tumor-recognizing peptide (cyclic RGD) to phospholipid-PEG SWCNTs (Liu et al., 2007; see Table 5.1) or to oligonucleotide-aminated SWCNTs (Villa et al., 2008; see Table 5.1) showed greater accumulation in tumor tissues in tumor-bearing mice following i.v. injection, compared with respective SWCNTs without the peptide attachment. Similarly, injection of 1,4,7,19-tetraazacyclododecane-1,4,7,10-tetracacetate (DOTA)-aminated SWCNTs, covalently modified with antibodies to detect tumors, showed enhanced accumulation in mice with tumors in multiple tissues, compared with mice without tumors (McDevitt et al., 2007b; Table 5.1).

In summary, the available evidence indicates that surface modifications that prevent aggregates from forming in aqueous environments counteract the aggregation and accumulation of i.p. or i.v. injected CNTs in liver, lung, and spleen tissues and promote more rapid elimination

in the urine via the kidneys and the feces, presumably via hepatic biliary excretion, compared with nonfunctionalized CNTs. Additional surface modifications with targeting functional groups, such as peptides and antibodies, can also influence the tissue distribution of injected CNTs (Villa et al., 2008; Liu et al., 2007; McDevitt et al., 2007b).

Table 5.1. Summary of tissue distribution and elimination studies of animals exposed to CNTs by i.v. or i.p. injection

Injected material	Exposure route, species, dose	Detection technique(s)	Pertinent findings	Reference
Nonfunctionalized CNTs				
Nonfunctionalized SWCNTs; bundles suspended in aqueous 1% Tween; 10–30 nm diameter, 2–3 µm length	i.v.; mouse; 600 µg/mouse; single injection	[^{13}C] isotope ratio mass spectrometry and TEM	*Tissue distribution*: Tissues were sampled at 1, 7, and 28 d after injection. Accumulations of SWCNTs were primarily in lungs, liver, and spleen at 1 d; high accumulations were still apparent at 28 d. *% of ID per organ (from [^{13}C] analysis)*: *1 d*: 21.1% liver; 15.0% lung; 1.2% spleen *7 d*: 18.5% liver; 13.3% lung; 1.6% spleen *28 d*: 21.3% liver; 9.4% lung; 1.9% spleen *Elimination*: Urine and feces were collected daily. SWCNTs were "hardly detected" in urine and feces by TEM and [^{13}C] analyses. Concentrations in liver, lung, and spleen did not show marked decreases between d 1 and 28 after injection, indicating very limited or slow elimination.	Yang et al., 2007
Nonfunctionalized SWCNTs; bundles suspended in aqueous 1% Tween; 10–30 nm diameter, 2–3 µm length	i.v.; mouse; 0 or 1,000 µg/mouse; single injection	TEM and Raman spectroscopy	Blood and tissues (liver, lung, spleen) were sampled 90 d after injection. SWCNTs were qualitatively detected in liver, lung, and spleen samples from exposed mice, but not in controls (in tissue homogenates by Raman spectroscopy and in digested tissue samples by TEM).	Yang et al., 2008
Nonfunctionalized SWCNTs; dispersed in aqueous 1% Pluronic F108 (a nonionic surfactant); individual SWCNTs were reported to be suspended as indicated by near-IR fluorescence microscopy	i.v., rabbits; 0 or 75 µg/rabbit; single injection	Near-IR fluorescence microscopy	Tissues were sampled 24 hrs after exposure and examined for the presence of SWCNTs by near-IR fluorescence microscopy. Examined tissues: liver, kidneys, lungs, spleen, heart, brain, spinal cord, bone, muscle, pancreas, intestine, and skin. Fluorescence emissions indicative of SWCNTs were only seen in the liver.	Cherukuri et al., 2006

Table 5.1. Summary of tissue distribution and elimination studies of animals exposed to CNTs by i.v. or i.p. injection

Injected material	Exposure route, species, dose	Detection technique(s)	Pertinent findings	Reference
Nonfunctionalized SWCNTs; bundles suspended in saline solution with 0.1% Tween; three test materials before suspension (diameters ~1 nm): raw SWCNTs; 1–2 μm length, 25% Fe; purified SWCNTs: 1–2 μm length, ~4% Fe; and ultra-short SWCNTs: 0.02–0.08 μm length, ~1.5% Fe (cut by fluorination and pyrolysis).	i.p; mouse; 0, 50, 300, or 1,000 mg/kg for ultra-short-tubes (0, 1, 6, or 20 mg/mouse); 0, 50, 300, or 500 mg/kg for raw or purified SWCNTs (0, 1, 6, or 10 mg/ mouse); single injection Mice weighed about 0.02 kg	TEM	*Tissue distribution*: Tissues (stomach, intestine, lung, heart, brain, kidney, spleen, lung) were sampled 14 d after injection and examined for SWCNTs by TEM. For each test material, TEM showed aggregates of CNTs in liver and spleen macrophages and renal tubule epithelial cells. TEM provided no evidence of injected materials in the other organs. *Elimination*: TEM and near-IR fluorescence microscopy of urine and feces samples collected 3 d after exposure to the highest doses showed structures suggestive of individual and aggregated SWCNTs in urine and feces samples. The authors concluded that these findings indicate that nonfunctionalized SWCNTs can be eliminated in urine (via the kidneys) and in feces (via biliary excretion) following i.p. administration.	Kolosnjaj-Tabi et al., 2010
Nonfunctionalized MWCNTs; bundles suspended in saline solution; diameter and length of the bundles were not reported.	i.v., mouse, 400 μg/mouse; single injection	TEM of ultrathin renal cortex sections (70 nm)	*Elimination*: TEM of kidney sections (sampled at 5 and 30 mins after i.v. injection) showed bundles of MWCNT in glomerular capillaries, but did not show individual MWCNT translocating through the renal filtration membrane. In contrast, water-soluble DTPA-amine- MWCNTs were well dispersed and individualized in the renal capillary lumen, and individual MWCNTs were shown crossing the renal filtration membrane (with longitudinal axis vertically oriented to the endothelial fenestrations).	Lacerda et al., 2008a, b
Functionalized CNTs (water soluble or water dispersable)				
Hydroxylated SWCNTs; water soluble; diameter, 1.4 nm; length, 280–450 nm (340 nm mean).	i.p.; mouse; 1.5 μg/mouse; single injection	^{125}I radioactive label	*Tissue distribution*: Tissues sampled at 1, 3, and 6 hrs and 1, 3, 6, 11, and 18 d after injection and assayed for radioactivity. Peak concentrations in tissues were observed at 6 hrs with highest concentrations in bone, kidneys, and stomach (concentrations in these tissues were at least 7-fold higher than concentrations in blood, skin, liver, spleen, lungs, heart, muscle, brain, and muscle). At 3 or 6 d after injection, hydroxylated-SWCNTs were detected in bone (>50% ↓ in concentration at d 6, compared with hr 6) and kidney (about 90% ↓ in concentration at d 6 versus hr 6), and concentrations in other tissues were very low. *Elimination*: 80% of injected dose was detected in urine and feces collected for 11 d after injection: 94% of detected radioactivity was in urine and 6% was in feces.	Wang et al., 2004

Table 5.1. Summary of tissue distribution and elimination studies of animals exposed to CNTs by i.v. or i.p. injection

Injected material	Exposure route, species, dose	Detection technique(s)	Pertinent findings	Reference
DTPA-aminated SWCNTs; water soluble; bundles (10–40 tubes) formed on TEM grid for observation: 13–40 nm diameter; variable lengths >400–600 nm due to "roping" in bundles.	i.v.; mouse; 60 µg/mouse; single injection	^{111}In radioactive label; TEM	*Tissue distribution*: Tissues sampled at 0.5, 3, and 24 hrs after injection and assayed for radioactivity. Peak levels of radioactivity in tissues were observed at 30 mins. Highest levels were in muscle, skin, kidney, and skin. At 30 mins in one experiment: 20, 8.5, 9, and 1.3% of ID was in kidneys, muscle, skin, and lung, respectively; in a second experiment respective values were 10.5, 6, 2, and <0.5%. By 24 hrs, all tissues showed very low levels of radioactivity; <1% of ID was detected in all tissue combined. *Elimination*: 400 µg DTPA-aminated SWCNTs or DTPA-aminated MWCNT injected per mouse (no radioactive label). TEM examination of urine collected for 18 hrs showed abundant presence of intact SWCNTs or MWCNTs.	Singh et al., 2006
DOTA-aminated SWCNTs; water soluble; individual SWCNTs: 1.1 nm diameter and mean length 42 (standard deviation, 17) nm.	i.v., mouse; 12 g/mouse; single injection	^{86}Y positron label; PET whole-body imaging	*Tissue distribution*: PET estimated tissue concentrations (% ID/g): *3 hrs*: kidney, 8.3; liver 17.8; spleen, 14.3; bone, 2.2 *24 hrs*: kidney, 6.0; liver, 15.8; spleen, 13.2; bone, 2.0. Results are indicative of clearance from tissues, but at rate lower than that observed with DTPA-aminated-SWCNTs (Singh et al., 2006). At 24 hrs, activity in kidney, liver, and spleen accounted for about 6, 15, and 0.8% of the ID. *Elimination*: Technique did not provide quantitative measure of rate of elimination from body in excreta.	McDevitt et al., 2007a

Table 5.1. Summary of tissue distribution and elimination studies of animals exposed to CNTs by i.v. or i.p. injection

Injected material	Exposure route, species, dose	Detection technique(s)	Pertinent findings	Reference
DOTA-aminated SWCNTs; water soluble; aminated-SWCNTs before covalent attachment of DOTA showed 1.4 nm diameter and lengths ranging from 200 to 1,000 nm.	i.v., mouse; 10 µg/mouse; single injection	^{111}In radioactive label	*Tissue distribution*: Tissues were sampled at 1 and 24 hrs and 4, 9, and 15 d after injection and assayed for radioactivity. Peak levels of radioactivity were at 1 (kidney) or 24 hrs (liver, spleen, and bone). Highest peak concentrations were kidneys > liver > spleen > blood ≈ bone ≈ lung > heart > adipose > brain. After 1 hr, very low concentrations were noted in all tissues, except kidneys, liver, spleen, and bone; clearance was evident in all of these "high-accumulating" tissues between 1 and 15 d. Concentrations in kidney, liver, and spleen were about 40, 18, and 7%ID/g, 1 d after injection, and about 12, 10, and 5%ID/g on d 15. Thus, respective concentrations decreased by about 70, 45, and 30% between d 1 and 15 after injection. *Elimination*: No quantitative measurements of radioactivity in urine were reported, but radioactivity was reported to be detected in urine collected during the first hr after injection. Injection of DOTA-aminated SWCNT "constructs", which were covalently modified with antibodies to detect tumors, showed enhanced accumulation in mice with tumors in multiple tissues, compared with mice without tumors.	McDevitt et al., 2007b

Table 5.1. Summary of tissue distribution and elimination studies of animals exposed to CNTs by i.v. or i.p. injection

Injected material	Exposure route, species, dose	Detection technique(s)	Pertinent findings	Reference
DOTA-PL-PEG SWCNTs; water soluble; PL-PEG SWCNTs (2000 or 5400 MW) before DOTA attachment showed diameters 1–5 nm and lengths 100–300 nm. The aim of this project was to modify water-soluble, functionalized SWCNTs so that they accumulated in tumor tissues.	i.v. mouse; 200–300 µCi/mouse; single injection; mass injected was not specified Each mouse had a s.c. U87MG tumor	^{64}Cu positron label, detected by PET whole-body imaging	*Tissue distribution*: Tissues and tumors were assayed at 24 hrs for radioactivity by PET imaging. Accumulation of radioactivity occurred principally in the liver (~35%ID/g with PL-PLPEG$_{2000}$-SWCNT and ~20%ID/g with PL-PEG$_{5400}$ and spleen (~8%ID/g with PL-PEG$_{2000}$ and ~4%ID/g with PL-PEG$_{5400}$). 24-hr concentrations in other tissues (blood, kidneys, skin, muscle, intestine, heart, lung, stomach, bone, pancreas) and the tumors were low (<2%ID/g). *Elimination*: No quantitative measurements of radioactivity in urine were reported and the time course of radioactivity elimination was not monitored. When mice were injected with PL-PEG SWCNTs linked with an arginine-glycine-aspartic acid peptide (cyclic RGD) expected to bind to tumor components, similar accumulations in liver and spleen were observed at 24 hrs, but 24-hr concentrations in tumors were elevated (~5%ID/g with PL-PEG$_{2000}$ and ~13%ID/g with PL-PEG$_{5400}$), compared with PL-PEG-SWCNTs without the peptide.	Liu et al., 2007

Table 5.1. Summary of tissue distribution and elimination studies of animals exposed to CNTs by i.v. or i.p. injection

Injected material	Exposure route, species, dose	Detection technique(s)	Pertinent findings	Reference
Oligonucleotide-DOTA-aminated SWCNTs; water dispersable; dynamic light scattering indicated an apparent hydrodynamic radius of 230 nm; TEM showed small bundles with apparent lengths of 50–100 nm. Aminated SWCNTs were modified with oligonucleotides as a group which would direct in vivo assembly through hybridization to complementary oligonucleotide sequences.	i.v.; mouse; 10 µg/mouse; single injection	^{111}In radioactive label	*Tissue distribution*: Tissues were sampled at 1, 24, and 96 hrs after injection and assayed for radioactivity. Radioactivity quickly cleared from the blood. Peak levels of radioactivity in tissues were detected at 1 hr after injection. Highest peak concentration were kidney (~21%ID/g) > liver (~15%ID/g) > spleen (~5%ID/g) >>> bone, lung, stomach, intestine, muscle, and heart (each <1%ID/g). Peak concentrations in kidney declined to about 12%ID/g by 96 hrs, indicating some clearance, presumably via urine. *Elimination*: Urine or feces samples were not collected or analyzed. Concentrations in liver and spleen remained approximately constant through 96 hrs, indicating accumulation in, and limited elimination from, these tissues. No evidence for accumulation in other tissues was found. Oligonucleotide-DOTA-aminated SWCNTs with a tumor-recognizing peptide attached to the amine groups showed enhanced binding to human coronary artery endothelial cells expressing αVβ3-integrin in a flow cytometric assay, compared with oligonucleotide-DOTA-aminated SWCNTs without the peptide.	Villa et al., 2008
Glucosaminated MWCNTs; water soluble; before functionalization, MWCNTs showed 20–40 nm diameter and lengths of "several tens of micrometers".	i.p., mouse; 0.5 mC_i/mouse; single injection mass of injected material was not reported	^{99m}Tc radioactive label	*Tissue distribution*: Tissues were sampled at 1, 3, 6, 10, and 24 hrs after injection and assayed for radioactivity. Peak levels of radioactivity were at 1 (most tissues), 3 (liver, stomach), or 6 hrs (enterogastric area). Peak concentrations (%ID/g) showed the following order: enterogastric (54) > stomach (16) > liver, blood, lung (2.1–2.4) > kidney, intestines, spleen, heart, muscle (0.8–1.4). Following attainment of peak concentrations, all concentrations declined through the last sample at 24 hrs [e.g., 24-hr concentrations were (%ID/g): 0.8 enterogastric; 2.1 stomach; 0.4 liver; 0.1 blood; 0.3 heart]. *Elimination*: Radioactivity in urine and feces collected for 24 hrs accounted for about 70% of ID. Distribution of injected radioactivity between the urine and feces could not be determined from the reported data.	Guo et al., 2007

Table 5.1. Summary of tissue distribution and elimination studies of animals exposed to CNTs by i.v. or i.p. injection

Injected material	Exposure route, species, dose	Detection technique(s)	Pertinent findings	Reference
DTPA-aminated-MWCNTs; water soluble; diameters 20–30 nm; length "few hundreds nm".	i.v., mouse, 400 µg/mouse; single injection	TEM of ultrathin renal cortex sections (70 nm)	*Elimination*: TEM of kidney sections (sampled at 5 and 30 mins after i.v. injection) showed water-soluble DTPA-functionalized MWCNTs were well dispersed and individualized in the renal capillary lumen, and individual MWCNTs were shown crossing the renal filtration membrane (with longitudinal axis vertically oriented to the endothelial fenestrations).	Lacerda et al., 2008a
DTPA-aminated-MWCNTs; water soluble; diameters 20–30 nm; length "few hundreds nm".	i.v., mouse, 300 µg/mouse; single injection	^{111}In radioactive label; microSPECT whole-body imaging	*Tissue distribution*: MicroSPECT whole-body imaging showed radioactivity quickly distributing throughout body during the first 5 mins, followed by accumulation in kidneys and bladders up to 30 mins after injection. At 6 hrs, activity in kidneys and bladder had diminished and activity was barely discernable in kidneys at 24 hrs. *Elimination*: Quantitation of radioactivity in harvested tissues at 24 hrs (and in urine collected within 24 hrs) showed elevated levels of radioactivity only in the kidneys (about 0.6%ID/g) and that the mean level of radioactivity in the collected urine was >10-fold higher (about 11%ID/g).	Lacerda et al., 2008b

DTPA = diethylenetriaminepentaacetate, a chelator for the radioactive label; ID = injested dose; microSPECT = microsingle photon emission tomography; PET = positron emission tomography; PL = phospholipids

5.1.3. Metabolism

Studies on the in vivo or in vitro metabolism of nonfunctionalized CNTs in humans or animals were not located.

The only information located about in vivo metabolism of functionalized CNTs was a report that following i.v. injection of phospholipid polyethylene glycosylated-SWCNTs in mice, SWCNTs without the functional group (indicated by Raman spectroscopy) were qualitatively evident in liver samples 4 weeks after injection, but not at 1 or 7 days after injection (Yang et al., 2009). In contrast, evidence for SWCNTs without the functional group was not found in spleen samples collected at 1 day, and 1, 4, and 8 weeks after injection (Yang et al., 2009). These results suggest that the liver, but not the spleen, was capable of metabolically removing the functional group.

Allen et al. (2008) reported that CNTs, functionalized with carboxylic acid groups, could be degraded in vitro in the presence of a plant peroxidase and low concentrations (about 40 μM) of hydrogen peroxide over a 12–16-week period.

5.2. ACUTE TOXICITY

5.2.1. Acute Oral Toxicity

Overview: Limited testing in animals indicates that nonfunctionalized CNTs have a low potential for toxicity following acute oral exposure. Administration of single, oral, 1,000-mg/kg doses of three types of SWCNTs (differing in iron content and lengths) to male Swiss mice produced no deaths, no body-weight changes, no changes in serum biochemistry variables or hematological variables, and no histological changes in several tissues including the stomach, intestines, brain, liver, and kidneys within 14 days after exposure (Kolosnjaj-Tabi et al., 2010). No changes in serum biochemistry or hematological variables were observed in CD-1 mice within 30 days of administration of single oral doses of 1, 2.5, or 5 mg/kg of acid-functionalized MWCNTs (Carrero-Sanchez et al., 2006).

Kolosnjaj-Tabi et al. (2010) evaluated the potential influence of nanotube length, surface area, surface chemistry, and metal catalyst contamination on CNT toxicity by administering a single dose of three different types of SWCNTs to groups of 10 male Swiss mice (22 g, age not reported) via gavage at 0 or 1,000 mg/kg. The three types of SWCNTs used in the study were referred to as raw SWCNTs (25% iron, 1 nm diameter, >1–2 μm length, no data on surface area or carbon or oxygen content), pristine SWCNTs (<4% iron, 1 nm diameter, 1–2 μm length, 574 m^2/g BET surface area, 97.85% carbon content, 2.85% oxygen content), and ultra-short SWCNTs, <1.5% iron, 1 nm diameter, 20–80 nm length, 980 m^2/g BET surface area, 91.7% carbon content, 8.3% oxygen content). The authors reported that the ultra-short SWCNTs had many defects (ends and sidewall) from the chemical cutting procedure. The mice were observed for clinical signs and changes in body weight for up to 14 days. At the end of 14 days, the mice were sacrificed. Blood samples were collected for serum biochemistry and complete blood counts. Stomach, intestines, lungs, heart, brain, kidneys, spleen, and the right liver lobes were collected and analyzed microscopically. No significant changes in body weights, behavior, or

pathology were observed among the treated mice with any of the three SWCNTs used in this study when compared to controls. The authors concluded that their findings show that SWCNTs, irrespective of length, surface area, surface interaction, or iron content, have no acute oral toxicity after single bolus administration of up to 1,000 mg/kg in mice.

Carrero-Sanchez et al. (2006) administered a single dose of two different types of MWCNTs to groups of 10 male inbred CD-1 mice (4 weeks old) via gavage at 0, 1, 2.5, or 5 mg/kg and evaluated gross changes in lungs, liver, intestines, spleen, and heart at 24 hours, 48 hours, 72 hours, 7 days, and 30 days post-treatment (two mice from each treatment group and one control were sacrificed at each time point). Serum samples were also obtained from each animal one day prior to sacrifice (it is unclear if serum was collected prior to each sacrifice or only the terminal sacrifice) for evaluation of clinical chemistry (glucose, creatinine, amylase, alanine aminotransferase [ALT], total protein, albumin, globulins, and cholesterol). There were two types of MWCNTs synthesized by the authors for use in this study: undoped pristine MWCNTs and nitrogen-doped MWCNTs (CNx MWCNTs, 2–4 weight% nitrogen). Both types of nanotubes were reported to contain between 2 and 2.5 weight% of iron. Scanning electron microscopy (SEM) revealed differences in size dimensions between the two types of nanotubes as follows: undoped MWCNTs were generally longer (up to 450 μm in length) and slightly wider (<50 nm) than CNx MWCNTs (maximum length of 300 μm with average diameters between 20 and 40 nm). Additionally, high-resolution TEM revealed a higher degree of crystallinity within the grapheme cylinders and a smoother surface among the undoped MWCNTs compared to the CNx MWCNTs. Before administration to the mice, both types of MWCNTs were treated with acid to create hydroxyl, carboxyl, carbonyl, and sulfate groups on their surfaces and increase dispersion in a PBS solution. The authors did not report the results of oral exposure to MWCNTs quantitatively, but indicated that no significant treatment-related changes were observed in mice at any dose at any time point based on the endpoints evaluated.

5.2.2. Acute Inhalation Toxicity

Overview: The poorly soluble and fibrous (i.e., lengths > widths) nature of CNTs has prompted research in animals on the potential for nonfunctionalized CNTs to cause adverse lung effects including pulmonary inflammation and fibrosis, and effects associated with occupational exposure to other airborne particles in workplaces, including ultrafine carbon black and asbestos (NIOSH, 2010). Short-term inhalation exposure has been associated with signs of pulmonary inflammation in BALF and/or histological lesions (e.g., epithelial hyperplasia, granulomas, or fibrosis) in bronchoalveolar, alveolar, or subpleural regions of the respiratory tract in: male Wistar rats exposed to aerosols of MWCNTs at concentrations ≥2 mg/m^3 (Ma-Hock et al., 2009) or 11 mg/m^3 (Ellinger-Ziegelbauer and Pauluhn, 2009); female C57BL/6 mice exposed to aerosols of SWCNTs at 5 mg/m^3(Shvedova et al., 2008a); and male C57BL/6 mice exposed to aerosols of MWCNTs at 1 and 30 mg/m^3 (Ryman-Rasmussen et al., 2009a) or 100 mg/m^3 (Ryman-Rasmussen et al., 2009b).

Two other short-term inhalation studies in rats provide evidence that inhaled nonfunctionalized CNTs also may target nonrespiratory tissues, but confirmatory studies of these targets are not available. Impaired systemic immunosuppresson was observed in male C57BL/6 mice exposed for 14 days to aerosols of MWCNTs at 0.3, 1, or 5 mg/m^3, although a clear

monotonic dose-response relationship was not evident (Mitchell et al., 2007). Neuroinflammation and induced cellular stress in certain brain regions were reported in male C57BL/6 mice exposed for 12 days to aerosols of MWCNTs at 10 mg/m^3, but only an abstract report of this study is available (Sriram et al., 2009).

In a 5-day range-finding study, Ma-Hock et al. (2009) head-nose exposed groups of male Wistar rats (group size not specified) to MWCNTs (97% pure) dust aerosol for 6 hours/day on 5 consecutive days at target concentrations of 0, 2, 8, or 32 mg/m^3. Actual analyzed concentrations were 2.4 ± 0.7, 8.4 ± 1.6, and 29.8 ± 2.1 mg/m^3. Cascade impactor measurements of particle size found MMADs between 0.5 and 1.3 μm with GSDs between 3.1 and 5.4. The calculated mass fractions of particles <3 μm aerodynamic size ranged between 77.4 and 86.3%. Rats were observed for clinical signs of toxicity and changes in body weight. Five rats per concentration were evaluated for BALF, blood clinical chemistry and hematology 3 days after the last exposure. Histological examination of the respiratory tract (nasal cavity, larynx, oropharynx, trachea, lung, and mediastinal lymph nodes) was performed on three rats per concentration. All remaining rats were subjected to the same examinations following a 24-day recovery period to evaluate potential reversibility or progression of effects. The results of the range-finding study are only reported qualitatively by Ma-Hock et al. (2009). No exposure-related clinical signs or significant changes in body weight or food consumption were observed among the low- or mid-exposure groups. However, body weight gain was reduced in animals exposed to 32 mg/m^3. Treatment-related changes in BALF characterized by increases in total cell counts, total protein content, and enzyme activities (LDH, γ-GT, βNAG, and ALP) were observed in all treatment groups. However, there were no significant changes on BALF eosinophil or macrophage cell counts. For the most part, these effects remained following the 24-day recovery period. No significant effects were observed on hematology. Lung weights were increased in the mid- and high-exposure groups. Histology revealed diffuse or focal histiocytosis, particle laden macrophages, and bronchoalveolar hypertrophy and hyperplasia at the lowest concentration tested. It is not clear whether these specific changes were also noted in the other concentration groups, but the authors reported small alveolar septal granulomas composed mainly of macrophages in the lungs of rats at the mid- and high- exposure concentrations. Upper respiratory tract irritation, minimal to mild diffuse pulmonary histiocytosis and minimal infiltration with neutrophils were also observed at the highest concentration. The exposure-related changes were not resolved in rats, 3 weeks after the last exposure.

Ellinger-Ziegelbauer and Pauluhn (2009) nose-only exposed groups of six male Wistar rats to a pristine MWCNT (0.53% cobalt, 10–16 nm diameter, MMAD of 2.0–2.9 μm) respirable, solid aerosol for 6 hours at concentrations of 0, 11, or 241 mg/m^3. An additional group of six Wistar rats were similarly exposed to 11 mg/m^3 of MWCNTs depleted of residual materials (0.12% cobalt, MMAD of 2.0–2.5 μm). Pulmonary response was characterized at 7, 28, and 90 days post exposure by BAL (lactate dehydrogenase [LDH], protein, and absolute polymorphonuclear neutrophil [PMN] counts, γ-glutamyltransferase [γ-GT], β-N-acetyl-glucosaminidase [β-NAG], collagen), lung and LALN weights, lung histopathology, and gene expression analyses of lung homogenates with emphasis on extracellular matrix components. BAL-cell counts (total and PMN) were significantly elevated over controls at 7 days post exposure in rats exposed to 11 mg/m^3 and at all time points in rats exposed to 241 mg/m^3 (data

shown graphically). Similarly, measurements of collagen and LDH were significantly elevated over controls at 7 days post exposure in the low exposure group and at all time points in the high exposure group (except collagen measured at 90 days). These increases in BAL parameters were maximal at 7 days and decreased with increasing observation time. These responses were more pronounced in rats exposed to the pristine MWCNTs than in rats exposed to the cobalt-depleted MWCNTs. Evidence of alveolar phospholipoproteinosis characterized by foamy-looking alveolar macrophages was seen at the high-dose, was maximal on day 7, and declined to the level of the controls on day 90 (data not shown). Rats in the low exposure group demonstrated discoloration of LALNs at the day 7 sacrifice, but not at the 28 or 90 day sacrifices, whereas rats in the high exposure group demonstrated enlargement and discoloration of the LALNs at all time points. Lung weights of both pristine MWCNT exposure groups were significantly elevated over controls at the 7 and 90 day sacrifices (data shown graphically). LALN weights of both pristine MWCNT exposure groups rats were significantly elevated over controls at the 7 day sacrifice, but not at the 28 or 90 sacrifices. Both lung and LALN weights in the cobalt-depleted MWCNT group were comparable to controls. Histopathology revealed macrophages with dark cytoplasmatic spots in all MWCNT exposure groups. Other histopathological findings were similar between rats exposed to pristine MWCNTs at 11 mg/m^3 and controls. Rats exposed to pristine MWCNTs at 241 mg/m^3 demonstrated significant increases in hypercellularity of the bronchiole-alveolar tissues, focal septal thickening, and focal increase in septal collagen (6/6 rats compared to 0/6 controls). Only rats exposed to cobalt-depleted MWCNTs demonstrated a significant increase in the incidence of dark alveolar macrophages in bronchus-associated lymphatic tissue (4/6 compared to 0/6 controls). Gene expression analysis of MWCNT-exposed rats revealed an upregulation in genes encoding inflammatory regulators and a parallel induction of genes with an anti-inflammatory control function. MWCNT exposure also resulted in higher expression of genes known to be induced in response to oxidative stress, general stress, mechanical stress, and cell injury or cell damage, as well as genes controlling endothelial or epithelial permeability and genes involved in alveolar fluid clearance. The upregulation of genes encoding collagens was not observed. Based on the analysis of gene regulation, significant differences between cobalt-depleted and pristine MWCNTs did not occur.

Shvedova et al. (2008a) whole-body exposed groups of female C57BL/6 mice (20 g, 8–10 weeks old) to aerosolized unpurified SWCNTs (82% pure; 17.7% iron, 0.16% cobalt, 0.049% chromium, and 0.046% nickel) at target concentrations of 0 or 5 mg/m^3 for 5 hours/day for 4 consecutive days. The actual measured concentration of SWCNTs tested in this study was 5.52 ± 1.37 mg/m^3. The SWCNTs used in this experiment had reported measured diameters of 0.8–1.2 nm and measured lengths of 100–1,000 nm, respectively. MMAD was approximately 4.2 μm. The authors estimated the pulmonary deposited dose to be approximately 5 μg per mouse in this study. The effect on pulmonary function was evaluated by monitoring breathing patterns using whole body plethysmography. Animals were euthanized at 1, 7, and 28 days (12/sacrifice) post-exposure for evaluation of pulmonary toxicity by BAL and collagen deposition. SWCNT inhalation caused minor changes in breathing patterns characterized by elevated breathing frequency in treated mice in comparison to controls. This change was accompanied by decreases in the inspiratory, expiratory, and relaxation time. Counts of PMNs, alveolar macrophages, and total cells in BALFs were significantly elevated over controls ($p < 0.05$) at 7 and 28 days post-exposure, indicative of an inflammatory response. Significantly more PMNs were seen as early as 1 day post-exposure in treated mice over controls. Significant

elevations in the levels of protein and LDH activity and of cytokine accumulation were observed at all time points in treated mice compared with controls ($p < 0.05$). In all cases, the values decreased toward control values with increased time after dosing. Significant changes in alveolar wall collagen characterized by increased thickness (indicative of fibrosis) were observed to increase with increased observation time, and a significant increase in collagen deposition was observed in treated mice when compared with controls. Microscopy showed a broad distribution of SWCNTs in the lungs of treated mice, from alveoli at the bronchiole junction to the more distal regions of the lungs where granuloma development was observed on day 1 postexposure. Histopathology revealed pulmonary inflammation, bronchiolar epithelial cell hypertrophy, and the presence of green-brown foreign material in the interstitium, within macrophages or free of lung tissue, and most frequently, aggregated near bronchoalveolar junctions. Shvedova et al. (2008a) compared these pulmonary responses to inhaled SWCNTs with responses in the same strain of mice to pharyngeal aspiration of 5, 10, or 20 μg of the same SWCNT material suspended in PBS. A similar pattern was observed showing an acute pulmonary inflammatory reaction followed by a deposition of collagen and progressive fibrosis, but the inhalation conditions appeared to be more potent on a basis of estimated deposited mass. For example, collagen deposition in lung sections at 28 days post-exposure (detected by Sirius-red staining and expressed as a percentage of control values) was higher in mice exposed by inhalation (with an estimated deposited dose of 5 μg per mouse), compared with mice exposed to a bolus dose of 10 μg by pharyngeal aspiration (313 versus 188% of control).

Ryman-Rasmussen et al. (2009a) exposed groups of 10 male C57BL6 mice to an aerosol of MWCNTs (>94% pure, 5.5% nickel based on energy dispersive x-ray analysis (EDX) and 0.34% nickel based on ICP-Auger electron spectroscopy (AES), 30–50 nm average diameter, 0.3–50 μm length) at 1 (MMAD 164 nm) or 30 (MMAD 183 nm) mg/m^3 for 6 hours and then collected lung tissues at 1 day, 2, 6, and 14 weeks post exposure to evaluate whether inhaled CNTs cause injury within the pleura. TEM analysis of the bulk material showed widths ranging from 10 to 50 nm. Aerosolized MWCNTs were found to comprise a mixture of agglomerated and individual nanotubes with lengths from <100 nm to >10 μm. Microscopic examination revealed MWCNTs embedded in the subpleural wall and within subpleural macrophages. Mononuclear cell aggregates were observed on the pleural surface, composed mainly of lymphocytes with some monocytes/macrophages (see Table 5.2). The number of these aggregates was minor at 1 mg/m^3 (data not shown) and significantly ($p < 0.05$) increased at 30 mg/m^3 1 day and 2 weeks after inhalation (data shown graphically). The size of the cell aggregates was also significantly ($p < 0.05$) increased at the high concentration (data shown graphically). Subpleural fibrosis was observed in mice of both exposure groups (see Table 5.3), and exposure to 30 mg/m^3 caused a significant ($p < 0.05$) increase in fibrosis at 2 and 6 weeks post exposure (data shown graphically). The subpleural fibrotic lesions observed in these mice were focal and regional. In the high exposure group, pleural mononuclear cell aggregates and fibrotic lesions appeared to resolve with increasing observation time. The authors reported that most of the inhaled MWCNTs appeared to be cleared from the lung by 14 weeks. Since the deposition of nanoparticles in the lung tissue can lead to chronic inflammation, the removal of MWCNTs could explain the diminished incidence of fibrosis over time.

Table 5.2. Incidence of pleural mononuclear cell aggregates in C57BL6 mice after inhalation with MWCNTs for 6 hours

Observation period	Exposure concentration (mg/m^3)		
	0	1	30
1 d	2/10	4/10	8/10
2 wks	3/10	4/10	7/10
6 wks	4/10	5/10	5/10
14 wks	4/10	2/10	5/10

Source: Ryman-Rasmussen et al. (2009a).

Table 5.3. Incidence of subpleural fibrosis in C57BL6 mice after inhalation with MWCNTs for 6 hours

Observation period	Exposure concentration (mg/m^3)		
	0	1	30
1 d	0/10	0/10	0/10
2 wks	0/10	1/10	9/10
6 wks	0/10	1/10	6/10
14 wks	0/10	3/10	1/10

Source: Ryman-Rasmussen et al. (2009a).

In reports only available as abstracts, pulmonary toxicity was observed in mice following MWCNT or SWCNT inhalation exposures (Kisin et al., 2009; Porter et al., 2009), and an inflammatory response was observed in the brains of mice inhaling MWCNTs for up to 12 days (Sriram et al., 2009). Porter et al. (2009) exposed 6-week-old male C57BL6/J mice (group size not reported) to aerosolized MWCNT at 0 or 10 mg/m^3 for 5 hours/day for 2, 4, or 8 days. The MMAD was 1.3 μm. Mice were sacrificed at one-day post-exposure for evaluation of pulmonary toxicity by BAL (PMNs, LDH activity, and albumin) and lung histopathology. A time-dependent increase in BAL cell counts was observed. Histological indications of pulmonary inflammation were reported after 4 and 8 days of exposure, and some mice demonstrated histopathological evidence of fibrosis at sites of MWCNT deposition at 8 days. Kisin et al. (2009) exposed C57BL/6 mice (gender and group size not reported) to non-purified SWCNT (17.7% iron) at 5 mg/m^3 for 5 hours/day for 4 days. The count mode aerodynamic diameter for these particles was 240 nm. Exposed mice developed multifocal granulomatous pneumonia and interstitial fibrosis. Sriram et al. (2009) whole-body exposed male C57BL/6 mice (age and group size not reported) to MWCNT (details on particles not reported) at 10 mg/m^3 for 5 hours/day for 2, 4, 8, or 12 days. Mice were evaluated for neurological changes at 1 day post-exposure. MWCNT exposure induced neuroinflammation, altered blood-brain barrier integrity and induced cellular stress in discrete brain areas.

Ryman-Rasmussen et al. (2009b) evaluated the effects of MWCNT in normal and ovalbumin (OVA) sensitized mice to determine if inhaled MWCNTs would increase airway fibrosis in mice with allergic asthma. Groups of 40 adult male C57BL/6 mice (20 sensitized to OVA and 20 unsensitized) were nose-only exposed to MWCNTs (>94% purity, up to 5.5% nickel measured by EDX and only 0.34% nickel measured by ICP-AES, 30–50 nm diameter, 0.3–50 μm length, MMAD of 714 ± 328 nm) at a target concentration of 100 mg/m^3 for 6 hours.

Following a 1 or 14 day observation period (number of mice observed at each time point was not clearly specified), mice were sacrificed and BALF and lung tissue were collected for analysis of collagen deposition and thickness and measurement of cytokines and chemokines (numbers of mice evaluated for each BALF parameter varied). The actual average measured concentration in the inhalation chamber was 103 ± 8.34 mg/m^3. TEM analysis primarily found MWCNTs as amorphous aggregates 2 μm in diameter or less with protruding tubes extending beyond the nexus with smaller aggregated and individual MWCNTs also present. A significant reduction in LDH release was observed in BALF at 14 days (but not at 1 day) in both non-sensitized (0.72 U/mg compared to 1.04 U/mg in controls) and sensitized (0.65 U/mg compared to 0.97 U/mg in controls) treated mice compared with controls. Neutrophil counts were significantly elevated over controls in treated mice one day after inhalation, but returned to basal levels by 14 days. There was also a trend ($p < 0.1$) for increased neutrophils in sensitized animals over non-sensitized animals. No significant differences in total protein levels were observed at 1 or 14 days in any treatment group. Histopathology showed agglomerates of MWCNTs in the lungs of treated mice at 1 and 14 days, primarily localized in macrophages. Exposure by non-sensitized mice to MWCNTs did not result in a significant increase in airway collagen deposition or collagen thickening. However, sensitized mice exposed to MWCNTs demonstrated an apparent increase in the thickness of collagen in the reticulum around the basement membrane of the bronchioles at 14 days post exposure. These findings in sensitized mice exposed to MWCNTs were accompanied by significant increases in the profibrogenic growth factors, platelet-derived growth factor (PDGF)-AA and transforming growth factor (TGF)-β1 simultaneously. OVA alone only induced TGF-β1, whereas MWCNT exposure alone only induced PDGF-AA. IL-13 was increased in the lavage fluid of both treated and control sensitized mice. IL-5 messenger ribonucleic acid (mRNA) was increased only in mice that received both OVA challenge and MWCNT inhalation. Ryman-Rasmussen et al. (2009b) concluded that these findings indicate that inhaled MWCNT require pre-existing inflammation to cause airway fibrosis.

Mitchell et al. (2007) whole-body exposed groups of six male C57BL/6 mice (8 weeks old) to aerosolized MWCNTs at concentrations of 0, 0.3, 1, or 5 mg/m^3 6 hours/day for 7 or 14 consecutive days. Characterization of the MWCNTs by SEM and TEM showed a high purity (>95%) with little indication of impurities due to metal catalysts (0.5% nickel, 0.5% iron), oxide (2.1%) or ash (<0.2%) (measured by x-ray photoemission and EDX). The particles used in this study measured 10–20 nm in diameter and 5–15 μm in length with a measured surface area of 100 m^2/g. Electron microscopy revealed that the MWCNT aerosols generated in this study were a mixture of material in varying states of agglomeration, including some tubes that were not agglomerated. Agglomeration increased with increasing aerosol concentration. Cascade impactor measurements of particle size resulted in MMADs of 0.7–1 μm (GSD of 2.0 μm) for the low and mid concentrations, respectively, and 1.8 μm (GSD of 2.5 μm) for the high concentration. The animals were sacrificed on the morning following the last exposure day and blood, lungs (with the trachea intact) and spleen were collected. Lungs were processed for histopathological examination as well as for white blood cell count in BALF and measurements of oxidative stress and cytokine induction. Spleens were processed to evaluate immune suppression by analyzing for T-cell-dependent antibody response (assessed by the Jerne-Nordin Plaque assay), T- and B-cell proliferative ability (assessed by the mitogenesis assay), and innate immune function (assessed by the natural killer [NK] cell assay). Gene expression analysis of

selected cytokines (interleukin 6 [IL-6], interleukin 10 [IL-10]) and an indicator of oxidative stress (nicotinamide adenine dinucleotide phosphate [NADPH] oxidoreductase 1 [NQO1]) were evaluated using real-time polymerase chain reaction (RT-PCR) in lung tissue and spleen. Splenic IL-10 gene expression was further validated with IL-10 protein analysis by enzyme-linked immunosorbent assay (ELISA). Mitchell et al. (2007) only showed the 14-day data. Particle-laden and enlarged macrophages were observed in BALF, but there was no increase in inflammatory cells in the lungs of any treated mice. No significant change in the distribution of cell types (macrophages, PMNs, lymphocytes, eosinophils) in BALF were reported in treated mice relative to controls. Lung histopathology revealed MWCNTs distributed throughout the lung primarily as aggregates, although a few free MWCNTs were also observed. Most of the visible material was present within alveolar macrophages, with lesser amounts noted extracellularly on surfaces. The amount of material observed in the lung tissue was qualitatively correlated with exposure concentration. However, there were no indications of treatment-related fibrosis, increased cellularity, or granuloma formation. There was no significant effect of MWCNT treatment on immune function among any treatment group at 7 days. However, mice exposed for 14 days demonstrated a suppressed T-cell-dependent antigen response and reduced T-cell proliferative ability (results shown graphically). These effects did not demonstrate clear concentration-dependent responses, as the responses decreased with increasing concentration in the low and mid exposure groups, and increased in the high exposure group to levels comparable or higher than observed in the low exposure group. Innate immune response measured by NK cell-mediated lysis was suppressed by MWCNT inhalation only in the mid-concentration group. No effects were observed on gene expression in the lung at 7 or 14 days (data now shown). However, spleen mRNA levels of IL-10 and NQO1 were significantly increased ($p < 0.05$) in the 14-day exposure group (data shown graphically). Protein analysis confirmed this finding with an increase in IL-10 protein at 1 and 5 mg/m^3 (data shown graphically).

5.2.3. Toxicity from Short-term Intratracheal Instillation or Pharyngeal Aspiration

Overview: Acute and persistent lung effects (pulmonary inflammation, granulomas, and fibrosis) have been reported in many studies of rodents exposed to nonfunctionalized or functionalized SWCNTs or MWCNTs by intratracheal instillation or pharyngeal aspiration (see Tables 5.4 and 5.5 for references). Results from these studies suggest a sequence of dose-related events starting with an acute inflammatory response, evident within a day after exposure, and followed by a fibrotic response culminating in increased collagen deposition, granulomas, and fibrotic tissue in lungs, which can persist for 28 days or longer following single exposures (Porter et al., 2010; Shvedova et al., 2008a, b, c, 2005). Results from available studies support the idea that the acute inflammatory and persistent fibrotic lung responses to acute exposure to CNTs are a complex function of a number of factors including deposited dose, metal content, particle dimensions including length, degree of agglomeration of administered material, structural defects in the graphene framework, and the presence and properties of functional groups added to the graphene framework. In contrast to the extensive evidence for lung effects from intratracheal instillation or pharyngeal aspiration of MWCNTs or SWCNTs, there is only limited evidence that the cardiovascular system, liver, kidneys, or brain may be toxicity targets from CNTs administered to the respiratory tract of animals.

5.2.3.1. *Lung Effects*

Acute and persistent lung effects including pulmonary inflammation, granulomas, and fibrosis have been reported in many studies of animals exposed to nonfunctionalized or functionalized SWCNTs or MWCNTs by intratracheal instillation or pharyngeal aspiration (see Tables 5.4 and 5.5 for study summaries). Lung effects observed after single or a few intratracheal instillations of SWCNTs include pulmonary epitheloid granulomas and interstitial inflammation in male B6C3F1 mice instilled with 3- or 17-mg/kg doses of 3 types of SWCNTs with varying metal contents (Lam et al., 2004); non-dose dependent pulmonary granulomas and transient signs of inflammation in BAL samples in Crl:CD rats instilled with 1 or 5 mg/kg SWCNTs containing 5% nickel and 5% cobalt (Warheit et al., 2004); pulmonary collagen and granulomas and large-airway fibrosis in male C57BL/6 mice in mice instilled with aggregates of SWCNTs containing 8.5% iron (Mutlu et al., 2010); bronchoalveolar pulmonary inflammation, granulomas, and fibrosis in rats of unspecified strain instilled with 0.4, 2, or 4 mg/kg SWCNTs containing 10% iron (Al Faraj et al., 2010); signs of inflammation in BAL samples in male ICR mice instilled with 4 mg/kg SWCNTs of unspecified metal content (Inoue et al., 2008); and alveolar granulomas in ICR mice instilled with 0.5 mg/kg SWCNTs of unspecified metal content (Chou et al., 2008). A similar array of persistent lung effects have been observed in rats (Cesta et al., 2010; Muller et al., 2008a, 2005), guinea pigs (Huczko et al., 2005), and mice (Crouzier et al., 2010; Li et al., 2009a, 2007a; Park et al., 2009) following intratracheal instillation of MWCNTs (see Table 5.4), and in rats (Mangum et al., 2006) and mice (Porter et al., 2010; Mercer et al., 2008; Shvedova et al., 2008a, b, c, 2005) following pharyngeal aspiration of SWCNTs or MWCNTs (see Table 5.5). In addition, Porter et al. (2010—see Table 5.5) reported that inflammation extended to the visceral pleura in more than half of exposed mice at 28 and 56 days following pharyngeal aspiration of 10, 20, 40, or 80 μg of a well dispersed MWCNT containing <0.78% metal contaminants.

The relatively fast development (on the order of days) and long-lasting nature (on the order of weeks to months) of the lung effects has been demonstrated in a number of studies following single intratracheal instillations (Al Faraj et al., 2010; Cesta et al., 2010; Park et al., 2009; Muller et al., 2008a, 2005; Li et al., 2007a; Huczko et al., 2005; Lam et al., 2004—see Table 3.4) or pharyngeal aspirations (Mercer et al., 2010; 2008; Porter et al., 2010; Shvedova et al., 2008a, b, c, 2005; Mangum et al., 2006—see Table 5.5). The time course of these effects is consistent with results from deposition and clearance studies (see Section 5.1.2) indicating that CNTs are retained in, and slowly cleared from, lung tissues and move into pleural tissues following inhalation exposures (Pauluhn et al., 2010a; Ellinger-Ziegelbauer and Pauluhn, 2009; Ryman-Rasmussen et al., 2009a), intratracheal instillations (Mutlu et al., 2010; Hubbs et al., 2009; Muller et al., 2005) or pharyngeal aspirations (Mercer et al., 2010, 2008). These observations are also consistent with the hypothesis that noncleared particles retained in the lung or parietal pleura can lead to macrophage-mediated development of chronic inflammation and fibrosis (Donaldson et al., 2010, 2006; Donaldson and Poland, 2009). Based on observations in mice following single pharyngeal aspiration of SWCNTs, Shvedova et al. (2008a, b, c, 2005) have described the lung response as an acute inflammatory response peaking at days 1–7 (e.g., with increased levels of proinflammatory cytokines in BALF samples), followed by a fibrotic response, beginning with the peak elevation of a profibrotic cytokine, TGF-β1, in BALF samples at 7 days and culminating with increased collagen deposition in the lung, persisting to up to

28 days or longer after aspiration. Porter et al. (2010) have described a similar sequence of events in lungs of mice following pharyngeal aspiration of MWCNTs.

The manufacture of SWCNTs and MWCNTs utilizes metal catalysts (such as iron, cobalt, or nickel), leading to metal contamination of these materials. Such metals can participate in redox reactions leading to generation of reactive oxygen species (ROS) in biological systems, and have been proposed to contribute to the pulmonary inflammation response to SWCNTs and MWCNTs (Shvedova et al., 2008a, b, c, 2005). For example, Shvedova et al. (2008a, 2005—see Table 5.5) compared the pulmonary responses of mice to pharyngeal aspiration of suspensions of SWCNTs containing 17.7 or 0.2% iron and reported that the higher iron content MWCNTs induced a greater depletion of low molecular weight thiols in lung homogenates, a greater release of LDH in BALF samples, and a greater deposition of collagen in alveolar walls, compared with the iron-depleted SWCNTs. Nevertheless, studies of pharyngeal aspiration of metal-depleted CNTs indicate that there are intrinsic acute pulmonary inflammatory responses and persistent and progressive fibrotic responses in mice to SWCNTs (Shvedova et al., 2005) and MWCNTs (Porter et al., 2010—see Table 5.5) containing very small amounts of metal contaminants. Three different SWCNT forms caused dose-dependent increase in pulmonary granulomas and interstitial inflammation 90 days after mice were exposed by intratracheal instillation (I: 0.53% iron, 26% nickel, 5% yttrium; II: 26.9% iron, 0.78% nickel, 0% yttrium: and III: 2.14% iron, 0% nickel, 0% yttrium); the findings for the metal depleted form (III) provide support for the intrinsic inflammatory potential of CNTs deposited in the respiratory tract (Lam et al., 2004—see Table 5.4). Similarly, Ellinger-Ziegelbauer and Pauluhn (2009) reported that acute inhalation exposure of rats to aerosols of MWCNTs containing 0.53 or 0.12% cobalt produced signs of pulmonary inflammation in BALF samples, and that the responses were more pronounced in rats exposed to the MWCNTs with higher cobalt content.

The degree of agglomeration of CNTs in suspensions (i.e., dispersability) is another factor that may influence pulmonary inflammatory or fibrotic responses to these materials following intratracheal instillation or pharyngeal aspiration. Increased lung collagen and granulomas and mild fibrosis in large airways were observed in mice at 30 and 90 days following intratracheal instillation of 0.4 or 1.8 mg/kg aggregated nonfunctionalized SWCNTs, but these effects were not observed in mice instilled with well dispersed suspensions of SWCNTs at the same dose levels (Mutlu et al., 2010—see Table 5.4). Following pharyngeal aspiration of aggregated or well-dispersed nonfunctionalized SWCNTs to mice (10 µg/mouse; estimated dose of 0.5 mg/kg), both treatments increased indices of inflammation in BALF samples at 1 and 7 days after aspiration (increased PMNs and macrophages) to similar degrees, which were resolved by 30 days (Mercer et al., 2008—see Table 5.5). Both treatments also produced areas of abnormal, high collagen deposition at 30 days, detectable by Sirius red or Lucifer yellow staining and indicative of fibrotic reactions, but lungs treated with dispersed SWCNTs showed collagen deposition widely distributed within alveolar walls throughout the lungs without granulomas, whereas collagen deposition in lungs treated with aggregated SWCNTs was typically associated with agglomerates and granulomas (Mercer et al., 2008). The abstract-only reports by Castranova (2009, 2007—see Table 5.5) appear to refer to the results presented by Mercer et al. (2008). The available results indicate that both agglomerated and well dispersed CNTs can produce time- and dose-dependent pulmonary inflammation and fibrotic responses,

especially following pharyngeal aspiration, and that the qualitative nature of the fibrotic response (e.g., localized or widely distributed) is influenced by the degree of dispersion.

The length of nonfunctionalized CNTs is also expected to influence inflammatory and fibrotic responses to CNTs deposited in respiratory tissues. Following intratracheal instillation of nonground or ground MWCNTs with average respective lengths of 5.9 or 0.7 μm to rats, both types of MWCNTs were present in lungs at 60 days, both types induced signs of inflammation in BALFs collected at 3 and 15 days after instillation, and both types induced fibrotic lung lesions detected by light microscopy at 90 days after instillation (Muller et al., 2005—see Table 5.5). However, the shorter, ground MWCNTs appeared to be more effectively cleared from the lung (at 60 days about 40% of the mass detected at day 0 was found in lungs, compared with 80% for the longer, nonground MWCNTs). The inflammatory responses induced by the shorter, ground MWCNTs at 3 and 15 days were stronger than responses induced by the longer, nonground MWCNTs, whereas the fibrotic response induced by the shorter MWCNTs, assessed by collagen accumulation on a whole lung basis, was less strong than the response to the longer MWCNTs at equivalent doses (Muller et al., 2005). The difference in the fibrotic response was correlated with the relatively more rapid clearance of the short, ground MWCNTs, compared with the longer, nonground MWCNTs (Muller et al., 2005).

Further studies by Muller et al. (2008a—see Table 5.5) with ground MWCNTs showed that the acute inflammatory and persistent fibrotic responses to short, ground MWCNT were counteracted by pre-treatment at 2,400°C to remove metals and anneal structural defects in the graphene molecular framework. The structural defects were introduced as a result of grinding and were shown to be associated with increased surface oxygen and water adsorption potential of the MWCNTs; the latter properties were proposed to be due to the reaction of unsaturated, dangling bonds (in the damaged graphene framework) with oxygen in air during the grinding process (Fenoglio et al., 2008). The results support the idea that the acute inflammatory and persistent fibrotic lung responses to acute exposure to CNTs are dependent on a number of factors including deposited dose, metal content, particle length, degree of agglomeration of administered material, and structural defects in the graphene framework.

The influence of water-soluble functional groups added to MWCNTs or SWCNTs on lung effects following intratracheal instillation or pharyngeal aspiration also has been examined in a few studies. Acute or persistent pulmonary inflammatory responses have been observed in male Kunming mice exposed to 0.125, 0.25, 0.5, or 1 mg/kg MWCNTs functionalized with water-soluble taurine groups (Wang et al., 2007). Likewise, pharyngeal aspiration of carboxylated SWCNTs into C57BL/6 mice (40 μg/mouse) produced signs of pulmonary inflammation (increased PMNs in BALF) at 1 and 7 days after instillation and lung granulomas at 7 days after instillation (Kagan et al., 2010). Acid functionalization of SWCNTs or ultrafine carbon black particles was shown to increase acute pulmonary responses in female CD-1 mice exposed to intratracheal instillation doses of 10 or 40 μg/mouse, compared with nonfunctionalized SWCNTs or ultrafine carbon black particles (Tong et al., 2009—see Table 4.4). An earlier study by this group of investigators showed that increased pulmonary toxicity of acid functionalized dispersed SWCNTs could be counteracted by neutralizing the surface charge, although this also decreased the dispersability of the SWCNTs (Saxena et al., 2007 as cited by Tong et al., 2009). Functionalization of MWCNTs with N atoms introduced

into the carbon network (N-doping), followed by acid functionalization, counteracted the lethal and lung effects of single intratracheal instillations of 1, 2.5, or 5 mg/kg into male CD-1 mice, compared with acid functionalized MWCNTs (Carrero-Sanchez et al., 2006—see Table 5.4). Both types of MWCNTs induced acute pulmonary inflammation and granulomatous fibrotic reactions in the lung interstitium 30 days after instillation, but the responses to N-doped, acid functionalized MWCNTs only occurred at the highest dose, and were less severe than those caused by acid functionalized MWCNTs. Acid functionalization is thought to add hydrophilic hydroxyl, carboxyl, carbonyl, and sulfate groups to the surface of CNTs, and increase dispersability compared with nonfunctionalized CNTs; however, the lesser toxicity of the acid functionalized, N-doped MWCNTs was associated with better dispersal than the acid-functionalized MWCNTs (Carrero-Sanchez et al., 2006). In summary, the available data indicate that intratracheal instillation or pharyngeal aspiration of CNTs functionalized with water-soluble functional groups cause pulmonary effects qualitatively similar to those induced by nonfunctionalized CNTs (Kagan et al., 2010; Wang et al., 2007; Carrero-Sanchez et al., 2006). Limited comparative data indicate that addition of water-soluble functional groups may increase the potential of CNTs to cause acute pulmonary responses (on a mass basis); it is uncertain if the increased toxic potency is due to increased surface charge or increased dispersability and wider distribution in lung tissues (Tong et al., 2009; Saxena et al., 2007 as cited by Tong et al., 2009).

5.2.3.2. *Extrarespiratory Effects*

Extra-respiratory tissues have been examined in a few studies following intratracheal instillation or pharyngeal aspiration of nonfunctionalized MWCNTs (liver and kidney: Reddy et al., 2010; brain: Sriram et al., 2009) or SWCNTs (cardiovascular tissue: Tong et al., 2009; Erdely et al., 2009; Li et al., 2007b). In contrast to the extensive evidence for pulmonary toxicity from intratracheal instillation or pharyngeal aspiration of MWCNTs or SWCNTs, there is only limited evidence that the cardiovascular system, liver, kidneys, or brain may be toxicity targets from CNTs administered to the respiratory tract of animals.

5.2.3.3. *Liver, Kidney, or Brain Effects*

Dose-related periportal lymphocytic infiltration of ballooning foamy degenerated hepatocytes, fatty changes, focal inflammation, and necrosis were reported in livers of male Wistar rats up to 3 months after intratracheal instillation of 0.2, 1, or 5 mg/kg MWCNTs; high-dose rats also showed elevated serum levels of creatinine and renal tubular necrosis and interstitial nephritis (Reddy et al., 2010—see Table 5.4). Histological examination of heart tissue was reported to produce no exposure-related changes (Reddy et al., 2010). Reporting of details for this study was inadequate to independently evaluate the findings, and confirmatory findings in additional studies are not available. In an abstract-only report, neuroinflammation, altered blood-brain barrier integrity, and induced cellular stress in discrete brain areas were reported in male C57BL/6 mice, 1, 7, 28, or 56 days following pharyngeal aspiration of MWCNTs at doses ranging from 10 to 80 µg (Sriram et al., 2009).

5.2.3.4. *Cardiovascular Effects*

Increases in several markers of cardiovascular disease (activation of heme oxygenase-1 in aorta and heart tissue, damage to mitochondrial deoxyribonucleic acid (DNA) in aorta, or depleted levels of aortic mitochondrial reduced glutathione [GSH]) were observed in mice given single pharyngeal aspirations of 10 or 40 µg/mouse of acid-functionalized SWCNTs with low levels of metal contaminants (Li et al., 2007b—see Table 5.5). In Apo $E^{-/-}$mice, a mouse strain that lacks apolipoprotein E and develops atherosclerotic plaques spontaneously, exposure to a high fat diet and 20 µg/mouse of the same SWCNTs, once every other week for 8 weeks, increased plaque areas in aortas and brachiocephalic arteries and increased mitochondrial DNA damage in aortas (Li et al., 2007b). Increased expression of genes encoding mediators of inflammation, oxidative stress, tissue remodeling, or thrombosis were observed in lungs, blood, aorta, heart, liver, and kidneys of male C57BL/6 mice, 4 hours after pharyngeal aspiration of 40 µg SWCNTs containing 8.8% iron or MWCNTs containing 0.27% iron (Erdely et al., 2009—see Table 5.5). In another study, perfused hearts isolated from female CD-1mice, 24 hours after pharyngeal aspiration of 40 µg acid-functionalized SWCNTs, had significantly lower cardiac functional recovery following ischemia, greater infarct size following ischemia, higher coronary flow rates, and more commonly observed myofiber degeneration, compared with hearts from saline controls (Tong et al., 2009—see Table 5.5). These cardiac effects were not observed in mice exposed to nonfunctionalized SWCNTs (Tong et al., 2009).

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Intratracheal instillation studies with nonfunctionalized SWCNTs						
Male Crl:CD rats (8 wks old, 240–255 g), group size not specified	Intratracheal instillation of 0, 1, or 5 mg/kg SWCNTs	Survival, lung weights, BALF analysis and lung pathology at 24 hrs, 1 wk, and 3 mo post-instillation	SWCNTs (5% Ni, 5% Co), referred to as "soot", 30 nm diameter (usually exist as agglomerated ropes)	15% mortality at high dose thought to be due to treatment method rather than SWCNT toxicity. Significant increases in lung weights at high dose up to 1 mo, but comparable to controls by 3 mo. BALF analysis revealed only transient inflammatory and cell injury effects. Non-dose-dependent multifocal granuloma formation, not progressive beyond 1 mo post-instillation.		Warheit et al., 2004
Male B6C3F1 mice (8 wks old, 30 g), 9/group	Intratracheal instillation of 0, 0.1, or 0.5 mg SWCNTs (estimated doses of 0, 3, or 17 mg/kg based on average body weight of 30 g)	Clinical signs and survival, and lung pathology at 7 and 90 d post-instillation	Three SWCNTs with varying metal contents were tested: I: 0.53% Fe, 26% Ni, 5% Y; II: 26.9% Fe, 0.78% Ni, 0% Y III: 2.14% Fe, 0% Ni, 0% Y. Length and size distributions were not reported.	Test compound I caused mortality at high dose (5/9). Deaths were generally preceded by lethargy, inactivity, and body-weight loss and occurred between d 4 and 7. II and III caused no deaths. All SWCNT forms caused time- and dose-dependent increase in pulmonary granulomas and interstitial inflammation. These lesions were detected at 7 d and persisted through 90 d (more pronounced at 90 d).	Intratracheal instillation with carbon black at equivalent doses caused no deaths and no granulomas or inflammation in lung tissue.	Lam et al., 2004
Male C57BL/6 mice (8–12 wks old, 20–25 g), group size not reported	Intratracheal instillation of 0, 10, or 40 μg/mouse (approximately 0, 0.4, or 1.8 mg/kg based on an average body weight of 22.5 g)	BALF analysis, histology of the trachea, lungs and heart 30 and 90 d post-instillation	Dispersed SWCNTs or aggregated SWCNTs (8.5% Fe), 1–2 nm diameter, 100–2,000 nm length	Mild increases in lung collagen and granulomas and mild fibrosis in the large airways of mice treated with aggregated SWCNTs; these changes were not observed in mice treated with dispersed SWCNTs.	Aggregates observed in the mid to small sized airways following exposure to aggregated SWCNTs; uptake of dispersed SWCNTs by macrophages with gradual clearance over time.	Mutlu et al., 2010

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Rats, sex and strain not specified, 3–4/group (only one control rat per observation time point)	Intratracheal instillation of 0, 0.4, 2, or 4 mg/kg SWCNTs	Body weights, MRI image analysis of lungs and lung pathology at 1, 7, 30, and 90 d post-instillation	SWCNTs (10% Fe), size characteristics not reported	No effect on weight gain, alveolar thickening at 7 d post-instillation accompanied by cell infiltration, multifocal granulomatous lesions containing macrophages, lymphocytes, fibroblasts, and collagen deposition formed around the sites of SWCNT deposition by d 30, similar changes observed at d 90 with membrane thickening and alveolar collapse.	Although sonication and albumin were used to obtain a homogenous distribution, SWCNTs tended to aggregate into clumps in bronchioles and alveoli.	Al Faraj et al. 2010
Apolipoprotein E knockout mice (ApoE-/-)	Intratracheal instillation of 0 or 54 μg SWCNTs (estimated dose of 2 mg/kg assuming a body weight of 30 g)	Protein concentration in BALF, mRNA cytokine expression at 3 and 24 hrs post-instillation	SWCNTs, data on purity and size could not be obtained	Significant increases in total protein in BALF indicative of lung cell injury and significant increases in IL-6 and Mcp-1 mRNA at 3 and 24 hrs.		Jacobsen et al., 2009
Male ICR mice (6 wks old, 28–33 g), 20–25/group	Intratracheal instillation of 4 mg/kg SWCNTs or MWCNTs alone or following exposure to LPS	BALF analysis and lung pathology at 24 hrs post-instillation	SWCNTs, 1.2–1.4 nm diameter, 2–5 μm length; MWCNTs, 2–20 nm diameter, 100 nm-several micrometers length	Both SWCNTs and MWCNTs significantly increased the number of neutrophils, but only SWCNTs significantly increased total cells and protein levels in BALF and lung weight. Lungs from both groups demonstrated moderate infiltration of neutrophils. Chemokines were significantly elevated in both groups (to a greater extent with SWCNTs). Only SWCNTs significantly increased coagulatory parameters. MWCNTs significantly increased the level of MCP-1.	Purity not reported; moderate enhancement of effects by LPS.	Inoue et al. 2008
ICR mice, sex and group size not specified	Intratracheal instillation of 0 or 0.5 mg/kg SWCNTs	Inflammatory responses evaluated at 3 and 14 d post-instillation	SWCNTs (characteristics not specified)	At d 3, foamy-like macrophages loaded with SWCNTs accumulated in alveoli to give rise to multifocal macrophage-containing granulomas around the sites of SWCNT aggregation by d 14.		Chou et al., 2008

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Intratracheal instillation studies with nonfunctionalized MWCNTs						
Female Kunming mice (30 g), 5/group	Intratracheal instillation of 0.05 mg MWCNTs suspended in 0.1 mL tween-saline (0.9% saline containing 1% Tween-80), (estimated dose of 2 mg/kg based on an average body weight of 30 g)	Lung pathology at 8, 16, and 24-d post-instillation	Pristine MWCNTs (95% pure), ash (La, Ni) <0.2 wt%, diameter 50 nm, length 10 μm	Clumps deposited on lining wall of bronchi and alveoli; no obvious signs of inflammation to the lining wall of bronchi at 8 or 16 d, but inflammation observed at 24 d; increasing severity of destruction of alveolar netted structure with increased time after exposure.		Li et al., 2007a
Female Sprague-Dawley rats (200–250 g), group size not specified	Intratracheal instillation of 0, 0.5, 2, or 5 mg MWCNTs (estimated doses of 0, 2.2, 8.9, or 22 mg/kg based on an average body weight of 225 g)	BALF analysis at 3 and 15 d post-instillation, soluble collagen and hydroxyproline levels and lung pathology at 60 d post-instillation	Intact MWCNTs (97.8% pure), 5.2 nm inner diameter, 9.7 nm outer diameter, 5.9 μm length or ground MWCNTs (98% pure), 5.1 nm inner diameter, 11.3 nm outer diameter, 0.7 μm length	Dose-dependent increase in LDH release which was more marked with ground MWCNTs, increased total protein levels in BALF, accumulation of granulocytes, neutrophils, and eosinophils at both time points. Significant elevation in hydroxyproline and collagen at the highest dose. Collagen-rich granulomas in the bronchi which blocked partially or completely the bronchial lumen. Ground MWCNTs were better dispersed in the parenchyma and induced granulomas in the interstitial tissue. Significant increases in TNF-α at 9 mg/kg for intact MWCNTs and at 2 and 9 mg/kg for ground MWCNTs.	The fibrotic response induced by 22 mg/kg of ground MWCNTs was equivalent to that induced by 9 mg/kg intact MWCNTs.	Muller et al. 2005

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Female Wistar rats (200–250 g), 5/group	Intratracheal instillation of 2 mg/rat (approximately 9 mg/kg based on 225 g body weight)	BALF analysis, collagen deposition, lung pathology at 3 and 60 d post-instillation	Ground MWCNTs, 2% aluminum, 0.5% cobalt, 0.5% iron, 20–50 nm diameter; 0.7 μm length. Surface oxygen was 1.95%.	Increased LDH activity and total protein levels, accumulation of macrophages and neutrophils in the lung, and increased levels of IL-1β and TNF-α after 3 d, compared with control rats. Numerous granulomas containing collagen well-dispersed in the parenchyma observed after 60 d.	Grinding caused ↓ length; ↑ structural defects in graphene framework, and ↑ surface oxygen, compared with unground CNTs (Fenoglio et al., 2008).	Muller et al., 2008a
Same as above	Same as above	Same as above	Ground MWCNTs modified by heating at 600°C (CNTg600), 2.3% aluminum, 0.5% cobalt, 0.5% iron, 20–50 nm diameter, 0.7 μm length. Surface oxygen was 0.46%.	Increased LDH activity and total protein levels, but less marked than with ground MWCNTs; increased accumulation of macrophage and neutrophiles in the lung but to a lesser extent than ground MWCNTs, and no increase in IL-1β and TNF-α after 3 d. Granulomas containing collagen after 60 d, but smaller than ground MWCNTs. Heating at 600°C annealed some of the structural defects in the graphene framework.	Heating at 600°C caused no change in metal content, but ↓ surface oxygen and potential to adsorb water, compared with ground MWCNTs (Fenoglio et al., 2008).	
Same as above	Same as above	Same as above	Ground MWCNTs modified by heating at 2,400°C (CNTg2400), 0.37% aluminum, trace levels of cobalt and iron, 20–50 nm diameter, 0.7 μm length. Surface oxygen was 0.10%.	No effect on LDH activity or total protein levels, almost no cellular accumulation in lungs, and no increase in IL-1β and TNF-α after 3 d. Granulomas containing collagen after 60 d, but smaller than ground MWCNTs. Heating of ground MWCNTs at 2,400°C removed metals, annealed structural defects, and ↓ surface oxygen and potential to adsorb water (Fenoglio et al., 2008).	Removal of metals and annealing of structural defects prevented acute inflammatory response at administered dose, but fibrotic response was still apparent.	

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Same as above	Same as above	Same as above	MWCNTs heated at 2,400°C and ground MWCNTs (CNTg2400), 0.06% aluminum, 0.07% cobalt, 0.08% iron, 20–50 nm diameter, 0.7 μm length. Surface oxygen was detected at 0.73%.	Same acute effects as observed with ground MWCNTs, but to a greater extent. At 60 d, granulomas similar to those induced by CNTg2400 were observed. Structural defects (and associated properties) were reintroduced by grinding (after heating). This treatment reintroduced the acute inflammatory response.	Grinding after heating at 2,400°C reintroduced structural defects in graphene framework, and ↑ surface oxygen and potential to adsorb water (Fenoglio et al., 2008).	
Guinea pigs (sex and strain not specified), 5–10/group	Intratracheal instillation of 0 or 15 mg MWCNTs (estimated dose of 0 or 60 mg/kg based on an average body weight of 250 g)	Lung function and BALF examination at 90 d post-instillation	MWCNTs (95% pure), size characteristics not reported, two types: one made by chemical vapor deposition and one made by carbon arc	Organizing pneumonitis with focal nonspecific desquamative interstitial pneumonia-like reaction without fibrosis or with mild peribronchiolar fibrosis. Simultaneous significant increase in lung resistance in some of the animals treated with carbon-arc MWCNTs. Infiltration of inflammatory cells in BALF. Histopathology revealed lung inflammation characterized by infiltration of inflammatory cells, central and peripheral atelectasis, and emphysema and alveolar exudation.	MWCNTs observed inside of alveolar macrophages and inside some phagocytes.	Huczko et al., 2005
Male Dunkin Hartley guinea pigs (250 g), group size not specified	Intratracheal instillation of 0 or 25 mg CNT-soot (estimated dose of 0 or 100 mg/kg based on average body weight of 250 g)	Lung function and BAL examination at 4 wks post-instillation	CNTs containing soot, characteristics not reported (likely MWCNT)	No significant changes in lung function, no significant differences in cell distribution and protein concentration in BAL.	Huczko et al. (2005) reported that the number of test animals was limited in this study.	Huczko et al., 2001

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Male Sprague-Dawley rats (180–220 g), 6/group	Intratracheal instillation of 0, 1, 10, or 100 μg MWCNTs dispersed (estimated doses of 0, 0.005, 0.05, or 0.5 mg/kg based on an average body weight of 200 g)	Body weights, plethysmography, collagen deposition, cytokine expression, and lung pathology at 1, 7, 30, 90, and 180 post-instillation	MWCNTs, 20–50 nm diameter, 0.5–2 μm length, purity not reported	No effect on body weights or plethysmography parameters, increase in total numbers of alveolar cells at 100 μg at 7 and 180 d, no significant pathological changes in the lungs, apoptosis of alveolar macrophages after 30, 90, and 180 d with 100 μg and after 30 and 90 d with 10 μg, significant induction of caspase 3 protein at 100 μg	Phagocytosis of CNT agglomerate was also observed by alveolar macrophages.	Elgrabli et al., 2008b
Male Sprague-Dawley rats (6–8 wks old), 5/group	Intratracheal instillation of 0 or 4 mg/kg MWCNTs (alone or following exposure to LPS)	BALF analysis, immunohistochemistry for PDGF-AA and lung pathology at 1 and 20 d post-instillation	MWCNTs (>94% pure, 0.34% Ni, 0.03% La), 10–30 nm diameter, 0.3–50 μm length	Two-fold increase in LDH and total protein level in BALF at 24 hrs, these increases were enhanced in rats pre-exposed to LPS. PDGF-AA levels were increased at 24 hrs, but returned to control levels by 21 d. Significant fibrosis in the interstitium associated with nanoparticle-induced fibroproliferative lesions in the lungs at 21 d, these lesions were present in rats exposed with or without LPS, but the lesions were larger and more fibrotic in rats pre-exposed to LPS.	Also reported that LPS enhanced MWCNT-induced PDGF mRNA expression in cultured lung macrophages and fibroblasts in vitro.	Cesta et al. 2010
Male Swiss mice (30–35 g), 40 mice used in test	Intranasal instillation of 0 or 1.5 mg/kg double-walled CNTs	Serum cytokines and lung pathology at 6, 24, and 48 hrs post-instillation	Double-walled CNTs (80% double-walled CNTs, 20% SWCNTs and a few triple-walled CNTs), 2 nm mean outer diameter, several micrometer length, detected to be self-organized in ropelike structures with a diameter up to 80 nm, or in globular masses	Lung inflammation detected macroscopically at 24 hrs near bronchioles, macrophages detected more frequently in treated mice, thickening of alveolar walls focally noted at 6 hrs and as a major part of the tissue at 24 and 48 hrs post-instillation. Significant increase in leptin concentration at all time points and in IL-6 at 48 hrs post-instillation.	Small clusters of dense material detected in lumen of lobar bronchus at 6 hrs, large clusters detected in bronchioles and alveoli at 24 hrs, only some material observed in the lumen of bronchus at 48 hrs.	Crouzier et al. 2010

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Male Kunming mice (7 wks old, 30 g), 18/group	Intratracheal instillation of 0, 6.67, 2.67, or 9.34 mg/kg of PBS, MWCNTs, benzene, or MWCNT-benzene combo, respectively	BALF analysis and lung pathology at 3 and 7 d post-instillation	MWCNTs (>95% pure, 1.25% Ni, 0.07% Fe, 0.01% Co, 0.005% Cu), 50 nm diameter, 5–15 μm length	Significant dose-dependent increase of total protein and LDH in BALF, and slight increased in ALP and ACP in BALF at 3-d, but no obvious changes in BALF at 7 d. At 3 d, inflammatory cells surrounded the MWCNT aggregations in the alveolar area and the alveolar netted structure was destroyed. At 7 d, the alveolar netted structure around MWCNT aggregations was almost kept integrated. MWCNT aggregations adsorbed to inner wall of bronchi at 3 d and some thinner bronchi were almost blocked; quantity of aggregations significantly decreased by 7 d.	Benzene alone did not induce pulmonary toxicity, but enhanced MWCNT toxicity.	Li et al., 2009a
Male ICR mice, 10–12/group	Intratracheal instillation of 0, 5, 20, or 50 mg/kg MWCNTs	BALF analysis, cytokine analysis and immunophenotyping on splenocytes, and lung pathology at 1, 3, 7, and 14 d	MWCNTs (>90% pure), 110–170 nm outer diameter, 5–9 μm length	Significant increase in the total numbers of immune cells in BALF at all doses and increased distribution of neutrophils at all time points. Dose-dependent increase in pro-inflammatory cytokines (IL-1, TNF-α, IL-6, IL-4, IL-5, IL-10, IL-12, and IFN-γ) in BALF and in blood (highest levels at d 1 post-instillation decreasing with time). Increased distribution of B cells in spleen and blood. Dose-dependent increase in granuloma formation in lungs that peaked at d 3, persisting throughout the 14 d.	MWCNTs found in the bronchiole and alveoli at d 1; number of MWCNTs in fibrous tissues decreased slightly with time; some MWCNTs found in the cytoplasm of alveolar macrophages, in the alveolar epithelial cells and also in the interstitium of the alveoli.	Park et al., 2009

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Male Wistar albino rats (8 wks old, 200–225 g), group size not specified	Intratracheal instillation of 0.2, 1, or 5 mg/kg MWCNTs	Serum biochemistry and histopathology of liver, kidney, and heart at 24 hrs, 1 wk, 1 mo, and 3 mo post-instillation	MWCNTs produced by chemical vapor deposition, 60–80 nm diameter, or MWCNTs produced by electric arc, 90–150 nm diameter; both well-dispersed in PBS+Tween 80 solution	Liver: dose-dependent increase in serum ALT activities (significant increases at ≥1 mg/kg at all time points), dose-dependent periportal lymphocytic infiltration, congestion of sinusoids, and hemorrhage; ballooning foamy degeneration of hepatocytes, fatty changes, focal inflammation, and necrosis at all post-installation time periods. Kidney: dose-dependent increase in creatinine levels (significant increases at 5 mg/kg at 1 mo post-installation), "significant" tubular necrosis and interstitial nephritis with 5 mg/kg at 1 mo post-installation Heart: No significant pathological changes were found.	Purity of MWCNTs and metal content were not reported. Histopathology findings were presented qualitatively with representative photomicrographs. Incidence data and severity scores were not presented.	Reddy et al., 2010
Intratracheal instillation studies with functionalized SWCNTS or MWCNTs						
Male CD-1 mice (4 wks old), 10/group	Intratracheal instillation of 0, 1, 2.5, or 5 mg/kg MWCNTs in PBS	Gross and microscopic evaluation of lung, heart, liver, spleen, and intestines, at 24 h, 48 h, 72 h, 7 d, and 30 d post treatment and serum biochemistry 1 d prior to sacrifice	Acid-functionalized MWCNTs, 2–2.5% Fe, diameter up to 50 nm, length up to 450 µm. Acid treatment conferred dispersability in PBS, and was reported to introduce hydrophilic hydroxyl, carboxyl, and sulfate groups.	Mortality (30, 60, and 90% at 1, 2.5, and 5 mg/kg, respectively), signs of pulmonary distress (dyspnea) in dead mice; time and dose-dependent increases in lung changes: granulomas in the lung interstitium and goblet cell hyperplasia, granulomatous inflammatory reaction in the lumen of the bronchi, atypical hyperplasia of the bronchiolar epithelium	Lung deposition observed at all doses, with the level of deposition proportional to the dose; clumps of MWCNTs observed in bronchi, aggregates observed to have broken the bronchiolar wall and infiltrated the interstitium.	Carrero-Sanchez et al. 2006

Table 5.4. Summary of studies of animals exposed to SWCNTs or MWCNTs by intratracheal instillation

Animal (gender, strain, group size)	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Male CD-1 mice (4 wks old), 10/group	Intratracheal instillation of 0, 1, 2.5, or 5 mg/kg MWCNTs in PBS	Gross and microscopic evaluation of lung, heart, liver, spleen, and intestines, at 24 h, 48 h, 72 h, 7 d, and 30 d post treatment and serum biochemistry 1 d prior to sacrifice.	Nitrogen-doped, acid functionalized MWCNTs, 2–4% nitrogen, 2–2.5% Fe, 20–40 nm diameter, 100–300 μm length. Pristine MWCNTs were treated to introduce N atoms into the hexagonal carbon network, either as pyridine-type N (each N bound to two C atoms) or as substitional N (each N atom bound to three C atoms). These were then acid treated before instillation into mice.	No mortality, no effect on body weight, external appearance, or behavior, no significant histological changes in heart, liver, spleen, or intestines; high-dose mice exhibited a time-dependent increase in lung inflammation (cuboidal or flattened epithelial cells, absence of epithelial cells in bronchiolar wall), lung lesions (extensive papillomatous hyperplasia with hypercromatic muclei and leomorphic cells), and granulomatous reaction containing macrophages, lymphocytes, fibroblasts, and collagen deposition (also abundant in the peribronchiolar interstitium), hyperplastic lymph nodes (without deposition), reactive fibrosis in the peribronchiolar interstitium.	Lung deposition observed at ≥2.5 mg/kg, small aggregates in bronchioles. N-doped MWCNTs did not cause the acute lethality observed with acid functionalized MWCNTs at the same dose levels. Pulmonary responses to MWCNTs were more severe than responses to N-doped MWCNTs.	Carrero-Sanchez et al. 2006
Kunming mice (sex not specified), 5/group	Intratracheal instillation of 0, 0.125, 0.25, 0.5, or 1 mg/kg MWCNTs	Serum biochemistry and lung pathology at 1, 7, 14, and 28 d post-instillation	MWCNTs functionalized with taurine (tau-MWCNTs)	All exposed groups showed elevations in serum ALP and LDH at 1 and 7 d after instillation Significant elevation in LDH at 28 d only in high-dose group. No significant exposure-related changes in angiotensin converting enzyme. Histopathology revealed a significant increase in pulmonary inflammation and lung cell proliferation in all groups.	Chinese study, English abstract. Many tau-MWCNTs were found in some alveolar macrophages and bronchial epithelial cells.	Wang et al., 2007

ACP = acid phosphatase; ALP = alkaline phosphatase; LPS = lipopolysaccharide; TNF-α = tumor necrosis factor-alpha

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Pharyngeal aspiration studies with nonfunctionalized SWCNTs						
Adult female C57BL/6 mice (12/group)	Once. 0, 5, 10, or 20 µg/mouse SWCNTs suspended in PBS. Mice were 8–10 wks old at exposure, with average body weight of 20.0 g. Calculated doses were about 0.25, 0.5, or 1.0 mg/kg.	Pulmonary inflammation was assessed (BALF analysis, lung homogenates analysis for collagen, and lung histopathology) in mice killed on d 1, 7, and 28 post-exposure.	SWCNTs had 82% elemental carbon and 17.7% Fe. The diameter was 0.8–1.2 nm and length was 100–1,000 nm. SWCNTs were not processed (i.e., acid treated) to remove metal contaminants.	Significant dose-related inflammatory response was detected on d 1 and appeared to be maximal at this time.	Comparison of aspiration versus inhalation exposure showed that the inhalation was more potent than aspiration of an equivalent mass of SWCNTs.	Shvedova et al., 2008a
6-wk-old female F344 rats (number not specified)	Once. 0 or 2 mg/kg of SWCNT suspended in 0.1% Pluronic F-68 in PBS.	Lung histopathology, cell proliferation, and growth factor mRNA evaluated at 1 and 21 d after exposure.	SWCNT particles had external diameters of <2 nm, and 0.5–40 µm length. Purity was >90%. Metal concentrations not reported.	SWCNTs caused no overt inflammatory response at 1 or 21 d, but induced focal interstitial fibrosis in the alveolar region of the lung at 21 d. SWCNTs also induced formation of unique carbon bridge structures between macrophages in situ.	The carbon bridge structures linking macrophages could serve as biomarkers of SWCNT exposure.	Mangum et al., 2006

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Adult male C57BL/6 mice (6/group)	Once. 0 or 10 µg/mouse of dispersed SWCNT in PBS. Mice were 25 wks old at time of exposure. Average body weights were not reported. Assuming a body weight of 0.02 kg, a dose of 0.5 mg/kg is calculated.	Initial deposition pattern, inflammatory response, chronic fibrotic response, fate of the CNTs, connective tissue, and alveolar responses were evaluated at 1 hr and 1, 7, and 30 d after aspiration.	Dispersed SWCNTs prepared from purified SWCNTs with <2% contaminants; mean diameter was 0.69 µm.	Electron microscopy showed highly dispersed, interstitial distribution of dispersed SWCNT deposits on d 1. Macrophage phagocytosis of dispersed SWCNTs was rarely observed. Lung sections and lavage cells showed early transient inflammation that resolved by d 30 (dispersed SWCNTs). Granulomas apparent at 7 and 30 d with nondispersed, but not with dispersed SWCNTs. Thickness of connective tissue in alveolar regions progressively increased between d 1 and 30.	Dispersed SWCNTs caused more interstitial collagen accumulation (evidence of fibrosis) than nondispersed SWCNTs. Dispersed SWCNTs caused pulmonary inflammation and interstitial fibrosis, but no granulomas.	Mercer et al., 2008
10-wk-old male C57BL/6 mice	Once. 40 µg SWCNTs dispersed in 0.3 mg/mL mouse serum albumin and 5 µg/mL 1,2-DSPC. Mice were 10 wks old at time of exposure. Average body weights were not reported.	Mice were sacrificed 4 hrs postexposure. Blood was collected for antigen analysis and whole blood gene expression. Gene expression changes in lung, aorta, and blood were measured with a microarray including genes involved in inflammation (37%), oxidative stress (21%), growth factors (17%), tissue remodeling (12%), endothelial function (8%), and coagulation (5%).	SWCNTs with 8.8% iron content; 0.8–1.2 nm diameter, 0.1–1 µm in length. All genes upregulated in blood and aorta were confirmed by RT-PCR. A subset of genes upregulated in aorta were evaluated in heart, liver, and kidney by RT-PCR.	Numbers of genes upregulated in lung by MWCNT was greater than the number upregulated by SWCNTs; both types upregulated genes involved in coding mediators of inflammation, oxidative stress, remodeling, and thrombosis. Upregulation of genes involved in inflammation and coagulation was found in blood. Also activation of inflammatory genes in aorta, heart, liver, and kidney.	CNT induced activation of an endothelial specific cell adhesion molecule (“E-selectin”) in aorta that facilitates recruitment of leucocytes into the vessel wall.	Erdely et al., 2009

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Pharyngeal aspiration studies with nonfunctionalized MWCNTs						
10-wk-old male C57BL/6 mice	Once. 40 µg dispersed in 0.3 mg/mL mouse serum albumin and 5 µg/mL 1,2-DSPC. Mice were 10 wks old at time of exposure. Average body weights were not reported.	Mice were sacrificed 4 hrs postexposure. Blood was collected for antigen analysis and whole blood gene expression. Gene expression was examined also in lung, aorta, heart, liver, and kidney.	MWCNT with 0.27% iron content; ~80 nm in diameter, 10–20 µm in length. Report did not clarify if other metal contaminants were in the MWCNTs, or if acid treatment was used to deplete iron.	Gene expression changes similar to those obtained with SWCNTs (see above), but more pronounced with the MWCNT sample.	Uncertain whether the stronger strong gene expression effect in the lung may be related to the more rigid and better dispersed MWCNTs, compared with the SWCNTs.	Erdely et al., 2009
8-wk-old female C57BL mice (12/group)	Once. 0 or 20 µg MWCNTs suspended in PBS. Mice were 8 wks old at time of exposure. Average body weights were not reported.	Interaction between O_3 and MWCNTs was studied. Mice were exposed to 0.5 ppm O_3 12 hrs after a single dose of MWCNTs. Broncoalveolar lavage fluid was analyzed 5 and 24 hrs after exposure to O_3.	MWCNTs had a diameter of 20–30 nm and were up to 50 µm long. Purity was >95%.	Exposure to MWCNTs increased cytotoxicity-inflammatory markers in the lungs. Sequential exposure to MWCNT/O_3 had neither additive nor synergistic effect.	The results were interpreted as the development of possible cross-tolerance for sequential exposure to MWCNT and O_3.	Han et al., 2008
8-wk-old female C57BL mice (8/group)	Once. 0, 20, or 40 µg MWCNTs in 40 µL PBS. Mice were 8 wks old at time of exposure. Average body weights were not reported.	BALFs, serum, and lung tissues were analyzed for inflammatory and oxidative stress markers 1 and 7 d after treatment.	Mean outer and inner diameter of acid-treated MWCNTs were 31 and 6 nm, respectively; mean length was 20 µm; surface area was 50 m^2/g. MWCNTs formed bundles with diameter 30–300 nm.	MWCNTs induced acute inflammation in the lung greater at d 1and began to resolve at d 7. Effects were generally dose-related.	Indicators of localized oxidative stress in the lung were not significantly altered.	Han et al., 2010

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
7-wk-old male C57BL/6J mice (4/group). Average body weights were not reported.	Once. 0, 10, 20, 40, 80 µg well-dispersed MWCNTs in Ca^{+2}- and Mg^{+2}-free PBS, pH 7.4, with 5.5 mM D-glucose, 0.6 mg/mL mouse serum albumin, and 0.01 mg/mL 1,2-DSCP.	BALF was examined 1, 7, 28, and 56 d post-dosing. Lung histopathology examined 7 and 28 d post-exposure. Mice in the 20 and 80 µg groups also examined at 56 d.	Number of walls in the MWCNTs ranged from 20–50. Na (0.41%) and Fe (0.32%) were the major metal contaminants. Median length of MWCNTs was 3.86 µm and count mean diameter was 49 nm.	Pulmonary inflammation and damage markers in BALF increased dose-dependently and peaked 7 d post-exposure and almost totally recovered by d 56. Histopathology showed rapid development of pulmonary inflammation and fibrosis by d 7 (all doses, dose-dependent) which persisted until 56 d after aspiration. At each sampling, inflammation extended to the pleura in over half of the CNT-exposed mice.	Inflammation in pleura is consistent with companion morphometric study showing movement of MWCNT particles into intrapleural space and subpleural tissue (Mercer et al., 2010).	Porter et al., 2010
Pharyngeal aspiration studies with SWCNTS or MWCNTs: abstract reports only						
Mice (number, gender, and strain not specified)	Once. Dose was not specified.	Lung histopathology.	Purified SWCNTs (<0.25% iron).	Granulomas present at deposition sites of large agglomerates; interstitial fibrosis was associated with deposition of more dispersed nanotube structures.		Castranova, 2007 (abstract only)
Mice (number, gender, and strain not specified)	Once. Dose was not specified.	Lung histopathology.	Poorly dispersed versus well dispersed SWCNT; no further details provided.	The more dispersed SWCNT structures rapidly crossed the alveolar epithelial lining, entered the interstitium initiating a persistent fibrotic response.	Pharyngeal aspiration of dispersed SWCNT mimics more closely inhalation of a dry aerosol of SWCNT than aspiration of a poorly dispersed suspension.	Castranova, 2009 (abstract only)

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Mice (strain, gender, and number not specified)	Four doses (unspecified) of SWCNTs in 28 d.	The pulmonary response was assessed by global proteonomics and genomics of lung tissue and microarray of BALF. Results were compared to exposure to crocidolite asbestos and ultrafine carbon black.	No details were provided regarding the SWCNT particles.	SWCNTs induced cytokine biomarkers of inflammation. Microarray analysis of lung tissue revealed over 3,000 genes significantly changed by SWCNT exposure, compared to only 469 with asbestos and 71 with carbon black.	The significant gene lists for carbon black and asbestos were complete subsets of the SWCNT data set.	Teeguarden et al., 2009 (abstract only)
C57BL/6 mice (number and gender not provided)	Once. 0, 10, or 40 µg/mouse SWCNTs in PBS.	Lung response was examined in mice exposed to SWCNTs alone and in mice exposed to SWCNTs and 3 d later exposed to LM.	No details were provided regarding the SWCNT particles.	Combined exposure to SWCNTs and Listeria amplified lung inflammation and fibrosis. Despite this response, pre-exposure to SWCNTs significantly decreased the pulmonary clearance of LM 3–7 d after infection. Collagen deposition in the lung was also increased with the combined exposure.	Combined exposure also caused changes in breathing rate patterns suggesting decline in lung function.	Murray et al., 2008 (abstract only)
Male C57BL/6J mice (group size not specified)	Once. MWCNT in doses 10–80 µg/ mouse.	Brain areas were examined 1, 7, 28, or 56 d post-exposure. Results were compared to whole-body inhalation exposure to 10 mg/m^3 5 hrs/d for 2, 4, 8, or 12 d.	No details were provided regarding the MWCNT particles.	MWCNTs induced neuroinflammation, altered blood-brain barrier integrity, and induced cellular stress in discrete brain areas. Some effects persisted until d 56.	Exposure by inhalation induced similar responses.	Sriram et al., 2009 (abstract only)

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
C57BL/6 mice (number and gender not provided)	Once. CNTs 0, 10, 20, or 40 µg/mouse.	Histology of the lung was examined at 1 and 7 d and 1 and 2 mo after dosing. Size of the deposited CNTs and of the granulomatous lesions was also measured.	No details provided regarding the CNT particles.	D 1: CNT deposits infiltrated with alveolar macrophages. D 7: connective tissue accumulation within CNT deposits. 2 mo: lesions accounted for up to 4.6% of alveolar parenchyma (high dose).	CNTs induced a rapid response in the alveolar region with both focal granulomatous lesions and more generalized fibrosis. Effects were dose-dependent.	Mercer et al., 2005 (abstract only)
Pharyngeal aspiration studies with functionalized SWCNTs or MWCNTs						
Adult female C57BL/6 mice (6/group)	Once. 0, 10, 20, or 40 µg/mouse SWCNTs in PBS. Mice were 7–8 wks old at exposure, with average body weight of 20.3 g (0.0203 kg). Calculated doses were about 0.5, 1.0, or 2 mg/kg.	Pulmonary toxicity, inflammation, and fibrogenic responses were examined (BALF analysis, lung homogenates analysis for collagen, and lung histopathology) in mice killed 1, 3, 7, 28, or 60 d after dosing.	SWCNTs comprised 99.7% elemental carbon and 0.23% iron. The mean diameter and surface area were 1–4 nm and 1,040 m^2/g. SWCNTs were purified by acid treatment to deplete metal contaminants.	Exposure to SWCNTs induced a dose-dependent robust acute inflammatory reaction involving an early fibrogenic response and formation of granulomas. Formation of granulomas was associated mainly with deposition of dense SWCNT aggregates.	SWCNTs also caused interstitial fibrosis in pulmonary regions distant from deposition sites of SWCNT aggregates. This occurred in the absence of persistent inflammation.	Shvedova et al., 2005

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Adult female C57BL/6 mice (6/group), WT and NADPH oxidase-deficient [gp91phox$^{(-/-)}$]	Once. 0 or 40 µg/mouse SWCNTs in PBS. Mice were 7–8 wks old at exposure. Dose is estimated at 2 mg/kg.	Pulmonary toxicity, inflammation, and fibrogenic responses were examined (BALF analysis, lung homogenates analysis for collagen, and lung histopathology) in mice killed at 1, 3, 7, or 28 d after dosing.	SWCNTs comprised 99.7% elemental carbon and 0.23% Fe. The mean diameter and surface area were 1–4 nm and 1,040 m^2/g. SWCNTs were purified by acid treatment to deplete metal contaminants.	gp91phox$^{(-/-)}$ mice showed a greater acute inflammatory response than WT mice, evidenced by: increased PMNs and total cells in BALF, particularly on d 7 and 28; and higher levels of pro-inflammatory cytokines in BALF in d 1, 7, and 28. In contrast, gp91phox$^{(-/-)}$ mice showed a decreased fibrotic response compared with WT mice: decreased levels of a pro-fibrotic cytokine, TGB-β, in BAL and decreased collagen levels in lung homogenates at 28 d. gp91phox$^{(-/-)}$ mice also showed elevated numbers of apoptotic cells in lungs at 7 and 28 d, compared with WT mice.	NADPH oxidase deficiency was associated with enhancement of the acute inflammatory phase and suppression of the fibrotic phase. The results suggest a role for NADPH oxidase-dependent ROS generation in determining the shift from the acute inflammatory phase to the fibrotic phase of the lung response to SWCNTs.	Shvedova et al., 2008b
Adult female C57BL/6 mice (6/group)	Once. 0, 10, or 40 µg/mouse SWCNTs in PBS. Mice were 8–9 wks old at exposure, with average body weights of 20.3 g. Calculated doses were about 0.5 or 2.0 mg/kg.	Pulmonary toxicity was assessed in mice exposed to SWCNTs alone and in combination with LM.	SWCNT comprised 99.7% elemental carbon and 0.23% Fe. More than 99% of carbon content in the SWCNTs was present in a CNT morphology. Mean diameter and surface area were 1–4 nm and 1,040 m^2/g, respectively. Length was 1–3 µm. SWCNTs were purified by acid treatment to deplete metal contaminants.	Exposure to SWCNT followed by LM amplified lung inflammation and collagen formation caused by SWCNTs alone. SWCNTs decreased pulmonary clearance of LM. Some effects were significant at the low SWCNT level and 3 d after dosing. Body weight gain was significantly decreased 3, 8, and 10 d after dosing with 40 µg/mouse SWCNT relative to treated with Listeria only.	Enhanced acute inflammation and pulmonary injury with delayed bacterial clearance may lead to increased susceptibility to lung infection.	Shvedova et al., 2008c

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Male C57BL/6 or *Ho1-luc* reporter (at least four per group)	Once. 0, 10, or 40 µg/mouse SWCNTs in PBS. Exposure conditions corresponded to those described by Shvedova et al. (2005).	Measures of oxidative stress in aorta and heart tissues, measured at 1, 7, 28, or 60 d after aspiration.	SWCNTs comprised 99.7% elemental carbon and 0.23% iron. The mean diameter and surface area were 1–4 nm and 1,040 m^2/g. SWCNTs were purified by acid treatment to deplete metal contaminants.	High dose activated heme oxygenase-1, a marker of oxidative stress) in aorta, heart, and lung in *Ho-1-luc* mice, 7 d after aspiration. Exposed C57BL/6 mice showed mitochondrial DNA damage in aortic tissue at 7, 28, and 60 d and depleted levels of reduced GSH in aortic mitochondria (high dose only) at 7 d.	Findings of oxidative stress in vascular tissue prompted the study of effects of SWCNTs in $ApoE^{-/-}$ mice, which lack apolipoprotein E and develop atheroscelortic plaques spontaneously.	Li et al., 2007b
Male $ApoE^{-/-}$ mice, which develop atherosclerotic plaques spontaneously due to lack of apolipoprotein E (10 per group)	0 or 20 µg/mouse SWCNTs in PBS, once every other wk for 8 wks fed either a regular fat diet or a high fat diet.	Body weight, plasma cholesterol, triglycerides, glucose, LDH, and several cytokines. Plaque formation (quantification of lesion areas) in thoracic aortas and brachiocephalic arteries, and mitochondrial DNA damage in aortic tissue.	SWCNT comprised 99.7% elemental carbon and 0.23% Fe. The mean diameter and surface area were 1–4 nm and 1,040 m^2/g. SWCNTs were purified by acid treatment to deplete metal contaminants.	Exposed, high-fat mice showed greater areas with atherosclerotic lesions in thoracic aortas and brachiocephalic arteries, compared with low-fat exposed or control mice, and increased damage to mitochondrial DNA in aorta.	No exposure-related changes in other endpoints.	Li et al., 2007b

Table 5.5. Summary of short-term studies of animals exposed to SWCNTs or MWCNTs by pharyngeal aspiration

Animal	Exposure	Endpoints examined	Nanotube description	Results	Notes	Reference
Adult female CD-1 mice (6/group)	Once. 0, 10, or 40 µg/mouse SWCNTs or acid-functionalized SWCNTs.	Pulmonary inflammatory response and histological changes in the lung and heart were monitored 24 hrs after dosing. Physiological functions of isolated perfused hearts from exposed (40 µg) and control mice: left ventricle developed pressure, heart rate, flow rate time to ischemic contracture, infarct size after 20 mins of ischemia.	Acid functionalization of SWCNTs lowered C content by 20%. It also lowered the content of some transition metals by 20–33%. Acid-functionalized SWCNT particles were in the range of 22–138 nm. Surface area of acid-functionalized SWCNTs was 1/3 that of SWCNTs.	Exposure to 40 µg acid-functionalized SWCNTs induced significant lung inflammation and cellular infiltration and increased markers of injury, edema, and cellular activation in BALF. It also significantly reduced red blood cells, but did not affect white blood cells or platelets. Acid-functionalized SWCNTs (40 µg) caused histological alterations in the heart, and decreased recovery of heart functions following ischemic injury. Particles were not detected by light microscopy in heart tissue.	In perfused hearts from 40 µg exposed mice, the average severity score for myofiber degeneration was 1 (on a scale of 1 [minimal] to 5 [severe], based on the number of affected cells and size of cluster). Acid functionalized ultrafine carbon black particles at 40 µg did not cause cardiovascular effects.	Tong et al., 2009
C57BL/6 mice (sex and group size not specified)	Once. 50 µL, SWCNTs at a dose of 40 µg/mouse.	Neutrophil and cytokine levels in BAL and lung pathology 1 and 7 d after dosing.	Short, carboxylated SWCNTs, non-biodegraded, partially degraded, or degraded.	Non-degraded carboxylated SWCNTs induced an acute inflammatory response and formation of granulomas, but no changes in BAL and no granulomas were observed with degraded SWCNTs. Partially degraded SWCNTs induced less inflammation than non-biodegraded SWCNTs.	Characteristics of nanotubes not reported in this paper.	Kagan et al., 2010

LM = listeria monocytogenes

5.2.4. Toxicity from Acute Injection of SWCNTs or MWCNTs

Overview: Numerous studies have evaluated the acute lethality and systemic toxicity (including lung toxicity) of CNTs following injection into rodents (see Table 5.6 for study summaries).

Injected CNTs appear to have low acute lethality potential based on observations of no deaths in: male Swiss mice exposed to single i.p. doses as high as 500 mg/kg of SWCNTs with lengths of 1–2 μm containing 25 or <4% iron or 1,000 mg/kg of short SWCNTs with lengths of 20–80 nm (Kolosnjaj-Tabi et al., 2010); male CD-ICR mice exposed to single i.v. doses as high as 40 mg/kg SWCNTs with lengths of 2–3 μm containing 0.4% iron, 3.0% nickel, and 1.3% yttrium (Yang et al., 2008); and male CD-1 mice exposed to single i.p. doses up to 5 mg/kg of MWCNTs with lengths of 100–450 μm containing 2–2.5% iron (Carrero-Sanchez et al., 2006).

The liver, lung, spleen, and kidney have been proposed toxicity targets of concern due to the detection and accumulation of CNTs in these organs following injection of animals with nonfunctionalized SWCNTs or MWCNTs (Kolosnjaj-Tabi et al., 2010; Lacerda et al., 2008a, b, c, Yang et al., 2008; Cherukuri et al., 2006—see discussion in Section 5.1.2). Evidence for degenerative liver effects has been reported in mice following i.v. injection of 60 mg/kg nonfunctionalized MWCNTs; these effects were not produced by MWCNTs functionalized by acid oxidation or polyethylene glycosylation (Zhang et al., 2010; Ji et al., 2009; Schipper et al., 2008). Several other studies found no evidence for liver effects in mice injected with nonfunctionalized SWNCTs (Kolosnjaj-Tabi et al., 2010; Yang et al., 2008) or nonfunctionalized or functionalized MWCNTs (Lacerda et al., 2008b, c; Carrero-Sanchez et al., 2006). Lung inflammation was reported in mice 90 days following i.v. injection of 40 mg/kg nonfunctionalized SWCNTs (Yang et al., 2008), but histological examinations in other studies showed no exposure-related lung lesions in mice after i.p. injection of nonfunctionalized SWCNTs at higher doses (Kolosnjaj-Tabi et al., 2010) or i.v. injection of nonfunctionalized MWCNTs (Lacerda et al., 2008b, c). Histological examinations showed no adverse lesions in kidneys or spleen in mice following i.p. injection (Kolosnjaj-Tabi et al., 2010) or i.v. injection (Deng et al., 2009; Lacerda et al., 2008b, c; Yang et al., 2008) of nonfunctionalized or functionalized SWCNTs or MWCNTs. In summary, available data do not provide consistent evidence for a high potential for SWCNT or MWCNT toxicity in these organs following acute injection exposure.

5.2.4.1. *Liver Effects*

Microscopic examination of liver sections revealed no exposure-related lesions in: male Swiss mice 14 days after exposure to i.p. doses as high as 500 mg/kg of nonfunctionalized SWCNTs with lengths of 1–2 μm containing 25 or <4% iron or 1,000 mg/kg of short nonfunctionalized SWCNTs with lengths of 20–80 nm (Kolosnjaj-Tabi et al., 2010); male CD-ICR mice 90 days after exposure to single i.v. doses as high as 40 mg/kg nonfunctionalized SWCNTs with lengths of 2–3 μm containing 0.4% iron, 3.0% nickel, and 1.3% yttrium (Yang et al., 2008); and female BALB/c mice 24 hours after exposure to single i.v. doses up to 400 μg/mouse of nonfunctionalized MWCNTs with lengths of 0.5–2 μm containing 10.6% metal

contaminants (iron and nickel) (Lacerda et al., 2008b, c). In contrast, Ji et al. (2009) and Zhang et al. (2010) reported severe inflammatory cell infiltration in hepatic portal regions, focal hepatocellular necrosis, and elevated serum activities of aspartate aminotransferase (AST) in male Kunming mice, 15 and 60 days after i.v. exposure to single 60-mg/kg doses (but not 10 mg/kg) of Tween-80-dispersed nonfunctionalized MWCNTs with an average length of 0.356 μm containing 0.86% nickel, 0.06% iron, and 0.04% cobalt. Although Kolosnjaj-Tabi et al. (2010) found no histological evidence for degenerative liver lesions or elevated serum enzyme activities indicative of liver damage (ALP or ALT) at 14 days or 5 months following i.p. exposure to nonfunctionalized SWCNTs at doses as high as 500 mg/kg, they observed granulomas associated with agglomerated SWCNTs on the surface of livers from exposed mice. No exposure-related increased serum ALT activities were found in male CD-1 mice at 1, 2, 3, 7, or 30 days after i.p. exposure to single doses up to 5 mg/kg of acid functionalized MWCNTs (Carrero-Sanchez et al., 2006). Comparison of results reported by Kolosnjaj-Tabi et al. (2010), Zhang et al., (2010), Ji et al. (2009), and Carrero-Sanchez et al. (2006) provides evidence that high doses (≥60 mg/kg) of injected MWCNTs, but not SWCNTs, may cause persistent degenerative liver lesions. Functionalization of MWCNTs by acid oxidation (Ji et al., 2009) or polyethylene glycosylation (Zhang et al., 2010) appears to prevent the development of degenerative liver changes following i.v. injection of MWCNT doses ≥60 mg/kg. This change in hepatoxicity may be due to more efficient elimination due to increased water solubility of the functionalized CNTs (see Section 5.1.2).

5.2.4.2. *Lung Effects*

Microscopic examination of lung sections revealed no exposure-related lesions in: male Swiss mice 14 days after exposure to i.p. doses as high as 500 mg/kg of SWCNTs with lengths of 1–2 μm containing 25 or <4% iron or 1,000 mg/kg of short SWCNTs with lengths of 20–80 nm (Kolosnjaj-Tabi et al., 2010); male Swiss mice 150 days after exposure to i.p. doses of 300 mg/kg of short or relatively long nonfunctionalized SWCNTs (Kolosnjaj-Tabi et al., 2010); and female BALB/c mice 24 hours after exposure to single i.v. doses up to 400 μg/mouse of MWCNTs with lengths of 0.5–2 μm containing 10.6% metal contaminants (iron and nickel) (Lacerda et al., 2008b, c). In contrast, inflammatory cell infiltration was observed in lungs of male CD-ICR mice 90 days after exposure to single i.v. doses of 40 mg/kg SWCNTs (but not 8 mg/kg) with lengths of 2–3 μm containing 0.4% iron, 3.0% nickel, and 1.3% yttrium (Yang et al., 2008).

5.2.4.3. *Kidney and Spleen Effects*

Microscopic examination of kidney or spleen sections revealed no exposure-related adverse lesions in male Swiss mice 14 or 150 days after i.p. injection with SWCNTs (Kolosnjaj-Tabi et al., 2010); male CD-ICR mice 90 days after i.v. injection of up to 40 mg/kg SWCNTs (Yang et al., 2008); female BALB/c mice 24 hours after i.v. injection of up to 400 μg/mouse of nonfunctionalized or ammonia-functionalized MWCNTs (Lacerda et al., 2008b, c); and female Kunming mice 15, 30, or 60 days after i.v. injection of up to 100 mg/kg taurine-functionalized MWCNTs (Deng et al., 2009).

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Male Swiss mice (20 ± 2 g), 6/group	Raw SWCNTs with ~25% Fe 1 nm diameter, >1–2 μm length; no data on surface area, carbon content, or oxygen content	i.p. of a single dose of 0, 50, 300, or 500 mg/kg SWCNTs administered in Tween suspension (for biocompatibility)	Survival, growth, behavior, serum biochemistry, and total blood counts, microscopic examination of stomach, intestines, lungs, heart, brain, kidneys, spleen, and the right liver lobe examined at 14 d post-treatment	No effect on survival, growth, or behavior; increased ALT activity at high dose (attributed to the metal catalyst); organs at all doses exhibited normal morphology; liver granulomas (on the surface) increased in size with increased dose (reported in 1/6 mice); very small granulomas also observed inside the liver sinusoids of high-dose mice (1/6).		Kolosnjaj-Tabi et al., 2010
Same as above	Purified SWCNTs with <4% Fe; 1 nm diameter; 1–2 μm length; 574 m^2/g BET surface area, 870 m^2/g langmuir surface area, 97.85% carbon content, 2.85% oxygen content	Same as above	Same as above	No effect on survival, growth or behavior; no significant changes in biochemistry; organs at all doses exhibited normal morphology; liver granulomas (on the surface) increased in size with increased dose (reported in 2/6 mice); very small granulomas also observed inside the liver sinusoids of high-dose mice (2/6).	Responses to "raw" or "purified" nonfunctionalized SWCNTs were similar (i.e., histological changes restricted to surface granulomas), with the exception of increased ALT with the high-Fe SWCNTs.	Kolosnjaj-Tabi et al., 2010

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Same as above	Ultra-short SWCNTs with <1.5% Fe; 1 nm diameter, 20–80 nm length; 980 m2/g BET surface area, 1,478 m^2/g Langmuir surface area, 91.7% carbon content, 8.3% oxygen content	i.p. injection of a single dose of 0, 50, 300, or 1,000 mg/kg SWCNTs administered in Tween suspension (for biocompatibility)	Same as above	No effect on survival or growth; high-dose animals exhibited inactivity, lethargy, and pilo-erection; increased ALT activity at high-dose; dose-dependent increase in number and size of granulomas on surfaces and inside of livers and spleens.	Maximum length of bundles in suspension was <300 nm; short, compact aggregates >10 μm with fiber-like structures detected in granulomas; numerous aggregates smaller than 2 μm observed within phagocytic cells of liver and spleen without subsequent granulomatous reaction.	Kolosnjaj-Tabi et al., 2010
Same as above	Purified SWCNTs or ultra-short SWCNTs (see above rows for characteristics)	i.p. injection of 300 mg/kg CNTs in Tween suspension (for biocompatibility)	Serum biochemistry, and gross and microscopic evaluation of stomach, intestines, lungs, heart, brain, kidneys, spleen, and right liver lobes at 150 d post-treatment	Purified SWCNTs induced elevated creatinine levels without indications of kidney damage and some well-circumscribed granulomas surrounded by a thin easily-removable membrane on and inside the liver; ultra-short SWCNTs induced large and diffuse granulomas inside both liver and spleen, tightly bound to the surrounding tissues.	Characteristics of granulomas were similar to those observed at 14 d.	Kolosnjaj-Tabi et al., 2010

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Male CD-ICR mice (25 g), 5/group	SWCNTs (> 95% pure, metal impurities are 0.4 wt% Fe, 3.0 wt% Ni, and 1.3wt% Y, 10–30 nm diameter, 2–3 μm length	i.v. (tall vein) injected SWCNT at a dose of 0, 40, 200, or 1,000 μg/mouse (0, 1.6, 8, or 40 mg/kg based on an average body weight of 25 g)	Body weights and behavior every 3 d up to 90 d post-exposure; serum biochemistry, liver, lung, and spleen weights, and histology at 90 d post treatment	No significant effects on body weights or behavior. Significant increases in spleen weights at low and mid dose, but not at high dose. Significant increase in lung weight at high dose. Dose-dependent increases in ALT, AST, and LDH, with ALT and AST levels significantly higher than controls at mid and high doses, and LDH levels significantly higher than controls at all doses. Inflammatory cells around trapped SWCNTs in lung; dose-dependent increases in lung inflammation with inflammatory cell infiltration at high dose. No changes in liver or spleen pathology. No obvious changes of immunological indicators were induced.	Size dimensions based on TEM analysis, which showed SWCNT bundles; no signs of cell apoptosis evaluated in vitro. Activities of AST and ALT in mid- and high-dose groups, although elevated, were no more than 20% higher than control values. LDH activities in all exposed groups were about 60–70% higher than control values.	Yang et al., 2008
Female BALB/c mice (6–8 wks old), 5/group	Nonfunctionalized purified MWCNTs (pMWCNTs) (94% pure, 10.6% metal impurities (Fe and Ni), 20–320 nm diameter, 0.5–2 μm length	Tail-vein i.v. injection with 200 or 400 μg of purified MWCNTs	Urine production (pooled), water consumption, serum biochemistry (for 200 μg group only), organ weights, gross and microscopic pathology of kidneys, liver, spleen, heart, and lungs evaluated at 24 hrs post treatment	Subdued behavior, hunched posture, pilo-erection, and signs of respiratory distress including tachypnea. These signs diminished over the 24-hr period at 200 μg, but were persistent at 400 μg. Decreased ALP at 200 μg. No significant changes in organ weights. No significant histological changes, but gross changes including general discoloration of the lungs and liver were noted at the high dose.	Accumulation of MWCNTs in the lung and liver detected by light microscopy, but no MWCNTs found in kidney sections.	Lacerda et al., 2008b, c

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Female C57BL/6 mice (6–8 wks old), group size not specified	MWCNTs (M1) (20–60 nm diameter, 5–15 μm length); MWCNTs (M2) (60–100 nm diameter, 1–2 μm length); MWCNTs (M3) (<10 nm diameter, 1–2 μm length); SWCNTs (S4) (<2 nm diameter, 5–15 μm length)	i.p. injected with 0 or 50 μg CNT; results measured 24 hrs post treatment	Number of inflammatory cells in abdominal cavity lavage fluid	M1 and M2 induced a significant increase in the total number of inflammatory cells in abdominal lavage fluid; M1 induced a greater number of infiltrating cells than M2; M3 and S4 were comparable to controls.	Metal impurities not reported.	Yamashita et al., 2010
Male CD-1 mice (4 wks old), 10/group	Acid functionalized MWCNTs, diameter up to 50 nm, length up to 450 μm; MWCNTs doped with nitrogen (CNx MWCNTs); 2–4 wt% nitrogen; 20–40 nm diameter, 100–300 μm length; both CNTs reported to contain between 2 and 2.5% Fe.	i.p. injection of 0, 1, 2.5, and 5 mg/kg acid functionalized or N-doped MWCNTs. Pristine MWCNTs were treated to introduce N atoms into the hexagonal carbon network, either as pyridine-type N (each N bound to two C atoms) or as substitional N (each N atom bound to three C atoms). The N-doped MWCNTs were then acid treated before instillation into mice.	Gross necropsy of lungs, liver, intestines, spleen, and heart and serum biochemistry evaluated after 24 h, 48 h, 72 h, 7 d, and 30 d	No signs of inflammation or tissue damage and biochemical parameters by either acid functionalized or N-doped MWCNTs. The report did not mention microscopic examination of tissues.	Aggregates were reported to be dispersed between the intestinal loops without evidence of inflammation or tissue damage.	Carrero-Sanchez et al., 2006

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Female BALB/c mice (6–8 wks old), 5/group	Ammonia-functionalized MWCNTs with 0.2 mmol of NH_3+ per gram of material (8.1% elemental impurities [Fe and Ni]), size distributions following functionalization not reported	Tail-vein i.v. injection with 200 μg of functionalized MWCNTs	Urine production (pooled), water consumption, serum biochemistry (for 200 μg group only), organ weights, gross and microscopic pathology of kidneys, liver, spleen, heart, and lungs evaluated at 24 hrs post treatment	No effect on behavior; decreased total protein levels; no significant changes in organ weights, gross pathology, or histopathology.	Not found in the lung but accumulations of small clusters were observed in the liver and spleen.	Lacerda et al., 2008b, c
Same as above	Ammonia-functionalized MWCNTs with 0.9 mmol NH_3+ per gram of material (8.1% elemental impurities [Fe and Ni]), size distributions following functionalization not reported	Tail-vein i.v. injection with 200 μg of functionalized MWNTs	Urine production (pooled), water consumption, serum biochemistry (for 200 μg group only), organ weights, gross and microscopic pathology of kidneys, liver, spleen, heart, and lungs evaluated at 24 hrs post treatment	No effect on behavior or serum chemistry; no significant changes in organ weights, gross pathology, or histopathology.	Excreted in urine, no sign of tissue accumulation. Higher degree of ammonia functionalization prevented accumulations in liver and spleen.	Lacerda et al., 2008b
Same as above	Diethylentriaminepentaacetic-functionalized MWCNTs (MWCNT-DPA) (8.1% elemental impurities [Fe and Ni]), size distributions following functionalization not reported	Tail-vein i.v. injection with 400 μg of functionalized MWNTs	Urine production (pooled), water consumption, serum biochemistry (for 200 μg group only), organ weights, gross and microscopic pathology of kidneys, liver, spleen, heart, and lungs evaluated at 24 hrs post treatment	No effect on behavior, organ weights, gross pathology, or histopathology.	No sign of tissue accumulation.	Lacerda et al., 2008b

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Mice, gender, strain, and group size not specified	Pluronic F127-coated MWCNTs (97% pure, 2.9% unspecified metal particles, <1% carbon soot); 10–30 nm diameter, 2 μm length	Microinjection of 700 nL (350 nL at 700 μm and another 350 nL at 400 μm below the cortical surface) of PF127-MWCNTs (concentration in the range of 30–150 μg/mL) in to the cerebral cortex; concentration of PF127 <0.1%	Damage to brain cells	No damage to overall brain organization; tissue surrounding the lesion site was unaffected after 3 d; local gliosis engulfing the nanotubes without disruption of the surrounding tissue structure observed 18 d following injection; no increase to the damage induced by mechanical insult of microsyringe.	Corresponding in vitro study investigating the mechanism of action.	Bardi et al., 2009
Female Kunming mice (5 wks old, 22–25 g), 6/group	Pristine MWCNTs functionalized with taurine (95% pure, <0.1% Ni, Fe, Co), 12.6 nm average diameter, 269 nm average length	i.v. injection of 0, 60, or 100 mg/kg MWCNTs on d 0 and 4 hrs after the first injection	Behavior and clinical signs, body weight, spleen weight, and spleen biochemistry and histopathology at 1, 7, 15, 30, or 60 d post-exposure	Initial body weight decreases and spleen weight increases that recovered to normal levels with time, no significant changes to biomarkers of antioxidant defenses, no significant pathological lesions.	Accumulation of MWCNTs in spleen at all doses, accumulation increased over 60 d.	Deng et al., 2009

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Male Kunming mice (8 wks old, 25 g), 20/group	Acid-oxidized MWCNTs and Tween-80-dispersed MWCNTs, (98% pure, 0.86% Ni, 0.06% Fe, 0.04% Co, average length of 356 ± 185 nm) both created using pristine MWCNTs 10–20 nm diameter)	i.v. injection of functionalized MWCNTs (at 0, 10, or 60 mg/kg	Body and liver weight, serum biochemistry, liver pathology, and gene expression evaluated at 15 or 60 d post treatment	Decreased activity, decreased weight gain (Tween-80-dispersed MWCNTs at 60 mg/kg only), significant decreases in GSH level and superoxide dismutase activity in the high-dose Tween-80-dispersed MWCNT group at 15 d, but not at 60 d, elevated AST and total bilirubin levels, no histological changes in liver at 10 mg/kg. Tween-80-dispersed MWCNT induced severe inflammatory cell infiltration in the portal region, cellular necrosis, and focal necrosis at 60 mg/kg at both 15 and 60 d, whereas acid-oxidized MWCNT only induced slight inflammatory cell infiltration at 60 d. Gene expression was more affected by acid-oxidized MWCNTs than Tween-80-dispersed MWCNTs.	Accumulation of MWCNTs in the liver at 60 mg/kg at 60 d indicated by discoloration.	Ji et al., 2009
Male and female nude mice (8–12 wks old), 4/group (2/sex)	Non-covalently polyethylene glycolated SWCNTs, (1–5 nm diameter, 100–300 μm length) Oxidized, covalently functionalized nanotubes, (50–200 nm length)	i.v. injected with 0, 47 (non-covalently polyethylene glycolated SWCNTs), or 151 mg (oxidized, covalently functionalized nanotubes) (reported as the total mass of the injected compound)	Body weights, behavior, blood pressure, hematology, liver and spleen weights, immunohistochemistry, gross necropsy, and detailed histopathology evaluated up to 4 mo post treatment	No significant effects on behavior, blood pressure, body weight, hematology, organ weights, or pathology.	No aggregation was observed in solution; SWCNTs detected in liver and liver macrophages and to a lesser extent in spleen.	Schipper et al., 2008

Table 5.6. Summary of injection studies with SWCNTs or MWCNTs

Animal (gender, strain, group size)	Nanotube description	Exposure	Endpoints examined	Results	Notes	Reference
Male Kunming mice (5 wks old, 22–25 g), 20/group	Polyethylene-glycol functionalized MWCNTs and non-polyethylene-glycol functionalized MWCNTs prepared from pristine MWCNTs (>95% pure, <0.05% Ni, Fe, and Zn, 10–20 nm diameter, <1 μm length)	Tail-vein injection of 0, 10, or 60 mg/kg CNT	Body weights, behavior and clinical signs, serum biochemistry, liver weights, and histopathology, oxidative damage of the liver, and genome expression in liver at 15 and 60 d post treatment	No significant effects on body weights, behavior or general appearance. Non-polyethylene-glycol functionalized MWCNT induced significant increases in AST levels and severe inflammatory infiltration in the portal region, cellular necrosis and focal necrosis at 60 mg/kg, while polyethylene-glycol functionalized MWCNT only induced slight inflammatory cell infiltration at the same dose. No signs of oxidative stress damage in liver or changes in TNF-α levels, but changes in gene expression in the TNF-α and NF-κB signaling pathways.	Polyethylene-glycol functionalized MWCNTs and non-polyethylene-glycol functionalized MWCNTs did not form aggregations in mouse serum; discoloration of liver with increasing dose, indicating that MWCNTs trapped in the liver.	Zhang et al., 2010

5.2.5. Acute Dermal Toxicity, Skin Irritation/Sensitization, Eye Irritation

Overview: Limited tests in animals and humans provide evidence that nonfunctionalized CNTs are not potent skin irritants. Significantly increased skin thickness, indicative of skin edema, was observed in mice dermally exposed daily for 5 days to SWCNTs at 9.4 mg/kg (0.160 mg/mouse), but not at 4.7 mg/kg (Murray et al., 2009). No erythema or edema was observed in the skin of rabbits exposed for 4 hours (under occluded conditions) to MWCNTs at a dose of 500 mg/mouse (Kishore et al., 2009). No tests of the dermal sensitization potential of CNTs were located. Minimal eye irritation was noted in one eye irritation study of rabbits exposed to nonfunctionalized MWCNTs (Kishore et al., 2009). No signs of dermal irritation were reported in forty human subjects exposed to fullerene soot (containing an unspecified amount of SWCNTs) for 96 hours in a patch test (Huczko and Lange, 2001). No tests of the dermal sensitization potential of CNTs were located. Minimal eye irritation was noted in one eye irritation study of rabbits exposed to nonfunctionalized MWCNTs (Kishore et al., 2009), but no eye irritation was noted in rabbits exposed to fullerene soot with a high content of SWCNTs (Huczko and Lange, 2001).

Murray et al. (2009) topically exposed immune-competent hairless SKH-1 mice (3–4 weeks old, 16–18 g, group size and gender not specified) to unpurified SWCNTs (30% iron, 80% dispersed particles and 20% aggregated forms, size characteristics not reported) in deionized water at 0, 40, 80, or 160 μg/mouse (approximately 0, 2.4, 4.7, or 9.4 mg/kg based on an average body weight of 17 g) daily for 5 days. Skin bi-fold thickness was measured daily and used as an indicator of edema and skin inflammation. Cell numbers within the epidermis were assessed prior to sacrifice. Twenty-four hours following the last exposure, mice were sacrificed, and the skin was removed for mast cell counts in the dermis, evaluation of collagen accumulation, and biochemical (myeloperoxidase [MPO] activity, GSH concentration, protein carbonyl levels, and cytokine activity) and histological (PMN accumulation, mast cell influx, and inflammation) analysis. Skin thickness increased with dose, and a significant ($p < 0.05$) increase in skin bi-fold thickness was observed at the highest dose at 5 days compared with controls (data shown graphically). No changes in skin thickness were observed at time-points prior to 5 days of exposure (data not shown). Mid- and high-dose mice exhibited significant ($p < 0.05$) increases in the number of cells within the epidermis compared to controls (25 and 58% increases over controls at 4.7 and 9.4 mg/kg, respectively). High-dose mice demonstrated significant ($p < 0.05$) increases in mast cell influx (90% increase over controls) and in collagen accumulation (12% increase over controls) compared with controls. Additionally, MPO activity was 21% higher and GSH levels were 11% lower in high-dose mice than in controls ($p < 0.05$). The lower doses did not appear to significantly affect mast cell accumulation in the dermis, MPO activity, GSH levels, or collagen accumulation (data not shown). Skin from mid- and high-dose mice exhibited significant ($p < 0.05$) increases in carbonyl content compared with controls (34 and 41% increase over controls at 4.7 and 9.4 mg/kg, respectively). Skin from low- and mid-dose mice showed no increase in cytokine release (data not shown), but skin from high-dose mice showed a significant ($p < 0.05$) release of IL-10 and IL-6 over controls (100 and 80% increase over controls, respectively). Histological evaluation of the skin from high-dose mice revealed PMN accumulation within the epidermis and dermis localized around or within the hair follicles. The authors postulated that this finding may suggest that some SWCNT penetrate the stratum corneum, but this study did not specifically evaluate whether topical exposure to

SWCNTs caused penetration and deposition within skin compartments. The skin from these mice also showed increased inflammation in adipose tissue and a few sebaceous glands, as well as increased mast cell activity and degranulation that was not observed in the skin of control mice. These results combined with the in vitro findings from this study (summarized under Section 5.6.1) indicate that topical exposure to unpurified SWCNT can cause acute skin inflammation associated with free radical generation and oxidative stress.

Kishore et al. (2009) conducted in vivo and in vitro studies on the dermal irritation of two different sized MWCNTs (MWCNT 1: 99.9% pure, <0.1% iron, 5–8 μm in length with 3–8 nm inside diameter and 140 ± 30 nm outside diameter; MWCNT 2: 99.9% pure, <0.1% iron, 1–10 μm in length with 2–6 nm inside diameter and 10–15 nm outside diameter). The in vivo study was conducted by applying 0.5 g MWCNT 1 or MWCNT 2 moistened with a minimum volume of distilled water to the clipped skin area on the left side of six female New Zealand white rabbits (3/group carried out in a sequential testing strategy) under semi-occluded conditions for 4 hours. The right untreated side of each animal served as the control area. At the end of the 4-hour exposure, the residual test substance was washed away and dermal reactions were observed at 60 minutes, 24, 48, and 72 hours post exposure. After the 72-hour observation period, the rabbits were sacrificed and the skin was collected for histopathology analysis. The MWCNTs formed compact aggregates under SEM, but rough estimates of average sizes were 166 and 100 nm for MWCNT 1 and MWCNT 2, respectively. Neither MWCNT 1 nor MWCNT 2 appeared to induce dermal irritation, as there were no signs of erythema or edema observed in any rabbit. Microscopic evaluation of skin did not reveal any lesions. These results were supported by the in vitro skin irritation test conducted in the same study. Therefore, the authors concluded that MWCNT 1 and MWCNT 2 were non-irritating to rabbit skin in this assay.

Huczko and Lange (2001) concluded that fullerene soot with a high content of SWCNTs (not specified) was not irritating to human skin following a patch test in forty human volunteers with various irritation and allergy susceptibilities following exposure to 0.2 ml of a water suspension of soot for up to 96 hours.

Kishore et al. (2009) conducted in vivo and in vitro studies on the ocular irritation of two different sized MWCNTs (see above for particle characteristics). The in vivo study was conducted by applying 18 mg (equivalent weight to 0.1 mL) MWCNT 1 or MWCNT 2 into the conjunctival sac of the left eye of six female New Zealand white rabbits (3/group carried out in a sequential testing strategy). The lids were held together for 1 second in order to prevent loss of the material. The right eye of each animal served as the controls. The eyes of the test animals were washed with distilled water at 24 hours post exposure and the conjunctiva, iris, and cornea of both treated and control eyes were evaluated and scored according to the Draize method at 1, 24, 48, 72, and 96 hours post exposure. At the end of the observation period, treated and control eyes were collected for histopathology analysis. Treated eyes exhibited conjunctival redness and discharge from 1 hour post exposure onwards. The maximum mean scores for ocular lesions observed were 4.0 and 4.7 for MWCNT 1 and MWCNT 2, respectively. All of the animals recovered from ocular lesions by 96 hours. Microscopic evaluation of eyes did not reveal any lesions. Although conjunctival redness and discharge was seen in vivo, these reactions were reversible and were not observed in an in vitro HE-chorioallantoic membrane (CAM) test whereby 0.3 mg of the test materials were applied to the CAM of White Leghorn chicken eggs

for 5 minutes. Kishore et al. (2009) postulated that the in vitro test could not adequately mimic all the systemic mechanisms (cytotoxicity, inflammation, and penetration/permeability) in vivo. Therefore, the authors concluded that MWCNT 1 and MWCNT 2 were minimally irritating to rabbit eyes in this assay.

Huczko and Lange (2001) reported that fullerene soot with a high content of SWCNTs (not specified) was not irritating to rabbit eyes following a modified Draize test in 4 albino rabbits whereby 0.2 ml of a water suspension of soot was instilled in to one eye of each rabbit without washing for up to 72 hours

5.3. REPEATED-DOSE TOXICITY

5.3.1. Oral Studies

No studies were located on the possible toxicity of repeated exposure to CNTs by the oral route.

5.3.2. Inhalation Studies

Pauluhn (2010a) exposed groups of 60 young adult Wistar rats (50 males and 10 females per group) nose-only to dispersed micronized pristine MWCNTs (0.53% cobalt) at target concentrations of 0.1, 0.4, 1.5, or 6 mg/m^3 for 6 hours/day, 5 days/week for 13 weeks. "Satellite" or subgroups comprising only male rats were examined during a postexposure period of up to 6 months. The MWCNTs used in this study are characterized by a thin and relatively narrow diameter in the range of 10 nm with median tube lengths in the range of 200–300 nm. The study authors characterized the exposure atmosphere using several particle size analyzers. The reader is referred to Pauluhn (2010a) for these details. MMAD measurements ranged from 2.74 ± 0.2 to 3.42 ± 0.2 μm (mean ± standard deviation) across the 0.4, 1.5, and 6.0 mg/m^3 groups when analyzed using a critical orifice cascade impactor, and from 1.67 ± 0.1 to 2.19 ± 0.13 μm (mean ± standard deviation) across all exposure groups when analyzed using a TSI APS 3321 laser velocimeter. Subgroups (6 males/group) were sacrificed intermittently during weeks 8, 13, 17, 26, and 39 for evaluation of pulmonary toxicity by BAL (total cell count, ALP, soluble collagen, LDH, total protein, γ-GT, and β-NAG) and histopathology. "Main group animals" (10/sex/group) were subjected to detailed evaluations including clinical observations before and after each exposure; twice weekly body weight measurements; weekly food and water consumption measurements, rectal temperature, and reflexes; ophthalmology prior to start and at the end of the exposure period; and general hematology, clinical chemistry, and urinalysis at the end of the exposure period. Following sacrifice, these animals were subjected to gross pathological examination including organ weights (adrenal glands, brain, heart, kidneys, liver, lung, LALNs, ovaries, spleen, testes, and thymus), and detailed histopathology.

There were no treatment-related clinical signs or mortality reported and no significant changes in reflexes, ophthalmology, body temperature, body weights, or food and water consumption following the 13-week exposure to inhaled MWCNTs in this study (Pauluhn, 2010a). Hematology, clinical pathology, and urinalysis were comparable between treated and control rats (data not shown). In general, BAL cell counts were significantly elevated over

controls at 1.5 and 6 mg/m^3 at all time points (data shown graphically). PMNs were also significantly elevated over controls at 0.4 mg/m^3. Lung and LALN weights were significantly ($p < 0.01$) elevated relative to controls in the 1.5 and 6 mg/m^3 groups at the end of the 13-week exposure period (data shown graphically). By the end of the 6-month recovery period, lung weights were still significantly elevated over controls in these groups, but LALN weights were only significantly elevated over controls at the highest exposure concentration. There were no other significant changes in organ weights reported. Histopathology revealed treatment-related lesions in the nasal passages, upper respiratory tract, lung, and LALNs of rats related to inflammatory responses at the site of initial deposition and retention of MWCNT structures at ≥0.4 mg/m^3 (severity increased with concentration). Pauluhn (2010a) did not report incidence, but provided a table of severity scores. The principal exposure-related lesions in the upper respiratory tract were characterized by goblet cell hyper- and/or metaplasia, eosinophilic globules, and focal turbinate remodeling. Lung lesions were characterized by thickening of epithelial cell layers and influx of inflammatory cells at 1.5 mg/m^3 and especially 6 mg/m^3 with gradually less prominent changes at 0.4 mg/m^3. In high-concentration rats, slight to moderate focal inflammation with granulomatous appearance was observed, and a time-dependent increase of a multifocal bronchoalveolar hyperplasia was evident. Additionally, increased insterstitial collagen staining occurred at ≥0.4 mg/m^3 in areas adjacent to sites of increased particle deposition and inflammatory infiltrates. Increased septal collagen staining was indicative of interstitial fibrosis. Significant increases in bronchus-associated lymphoid tissue containing black particle-laden macrophages were observed at ≥0.4 mg/m^3. All endpoints examined were unremarkable at 0.1 mg/m^3; therefore, this concentration (the lowest concentration tested in this study) is designated as a no-observed-adverse-effect level (NOAEL), and 0.4 mg/m^3 is identified as a lowest-observed-adverse-effect level (LOAEL) for changes in neutrophil counts and histological changes in the nasal passages, upper respiratory tract, lung, and LALNs.

Ma-Hock et al. (2009) head/nose-exposed groups of 20 Wistar rats (10/sex, 8–9 weeks old) to aerosol dust atmospheres of MWCNTs (90% pure, 10% metal oxide of which 9.6% was aluminum oxide with traces of iron and cobalt) at target concentrations of 0.1, 0.5, and 2 mg/m^3 for 6 hours/day, 5 days/week for 13 weeks (65 total exposures). Actual measured concentrations were 0.1 ± 0.03, 0.5 ± 0.12, and 2.5 ± 0.45 mg/m^3. The MWCNTs used in this experiment had diameters of 5–15 nm, lengths of 0.1–10 μm, and specific surface areas of 250–300 m^2/g. Particle size distributions were evaluated by cascade impactor, light-scattering spectrometer, and scanning mobility particle sizer. MMADs ranged between 0.7 and 2.0 μm across the different exposure groups. The authors reported an estimated 10% pulmonary deposition of particles in this aerodynamic size range in the rat following inhalation. The calculated mass fractions of particles <3 μm aerodynamic size ranged between 66.2 and 90.4%, indicating that the aerosol particles generated in this study were within the range of respirable particles for rats, with a high proportion in the size range capable of reaching the alveolar region of the lung. The MWCNTs were also examined morphologically by TEM and tested for the presence of ROS to address concerns that the brush generator might alter the test material. However, these analyses found no sign of ROS generation or damage to the tube walls during the dust generation process.

The rats were monitored at least once daily (3 times on exposure days) for clinical signs (Ma-Hock et al., 2009). Food consumption and body weights were measured weekly. Ophthalmological examination was conducted on control and high-exposure rats before and at

the end of the exposure period. A functional observational battery (FOB) was conducted on 10 rats (5/sex) on day 84 that included home-cage and open field observations, sensorimotor and reflex tests, and automated motor activity measurements. Blood samples for hematology (white blood cell count and differential, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentrations, platelets, and prothrombin time), and clinical chemistry (ALT, AST, ALP, gamma-glutamyltransferase, sodium, potassium, chloride, phosphate, calcium, magnesium, urea, creatinine, glucose, total bilirubin, total protein, albumin, globulins, triglycerides and cholesterol levels) were collected on the morning after the last day of exposure. All animals were subjected to necropsy following exposure (although not specifically noted, it is assumed that animals were sacrificed immediately following the collection of blood samples) and all control and high-concentration rats were subjected to full histopathological examinations. Only the respiratory tract was examined histologically in the other exposure groups.

Inhalation exposure to MWCNTs in this study did not cause any premature mortalities and had no effects on clinical signs, body weights, food consumption, ophthalmoscopy, FOB, or motor activity (Ma-Hock et al., 2009). Slight systemic inflammation was indicated at the highest exposure concentration by a significant increase in total white blood cell counts that corresponded with raised absolute and relative neutrophil counts and decreased relative lymphocyte counts. All other hematological changes were reported to be within the range of historical controls (data not reported). There were no treatment-related changes reported based on clinical chemistry. A concentration-dependent increase in lung weights was observed, as summarized in Table 5.7. The lungs of all mid- and high-exposure rats had diffuse grey discoloring, presumed due to the deposition and accumulation of MWCNTs. Additionally, discoloration of the mediastinal lymph nodes was observed in all high-concentration females and one high-concentration male, as well as in one rat of each sex at the mid concentration. Macroscopic lung foci were observed at the high concentration among six rats (3/sex) and were attributed to macrophage accumulation and/or lipoproteinosis. Histology revealed the presence of black particles, presumed to be the MWCNT test material, in the cytoplasm of lung and lymph node macrophages in all treatment groups, in bronchial epithelial cells overlying the bronchoalveolar lymphatic tissue (BALT) or within BALT cells in all treated females and in mid- and high-concentration males, and in the epithelium or associated macrophages in the submucosa of the larynx and trachea in some mid- and high-concentration animals. Incidence data on the histological lesions observed in the lungs and lymph nodes of treated animals are summarized in Table 5.8. As shown, diffuse histiocytosis was reported among all exposure groups, increasing in severity with increasing concentration. Additionally, the incidence of granulomatous inflammation increased in incidence and severity with increasing exposure concentration. Neither of these lesions were reported in the lungs of any control rats. All mid- and high-exposure rats demonstrated diffuse lung inflammation, mainly represented by alveolar macrophages and neutrophils. Alveoli among the mid- and high-exposure rats were found to contain multifocal eosinophilic, granular material considered to represent lipoproteinosis, a condition that has been reported in humans with acute silicosis. Most high-concentration animals and one mid-concentration male demonstrated minimal to moderate lympho-reticulocellular hyperplasia of the lymph nodes. Small granulomas formed by particle-loaded macrophages were observed in the lymph nodes of most animals, suggesting the slow degredation by the macrophages of the MWCNTs in the lymph nodes.

Table 5.7. Relative lung weights of rats exposed to MWCNTs by inhalation for 13 weeks[a]

Gender	Exposure concentration (mg/m^3)			
	0	0.1	0.5	2.5
Male	100%	+1%	+23%[b]	+81%[c]
Female	100%	+4%	+34%[c]	+90%[c]

[a]Rats were exposed for 6 hours/day, 5 days/week for 13 weeks.
[b]Significantly different from controls, $p < 0.05$.
[c]Significantly different from controls, $p < 0.01$.

Source: Ma-Hock et al. (2009).

Table 5.8. Incidences of lung and mediastinal lymph node lesions in rats exposed to MWCNTs by inhalation for 13 weeks[a]

Histology[b]	Gender	Exposure concentration (mg/m^3)			
		0	0.1	0.5	2.5
Diffuse histiocytosis in lung	Male	0/10 (0/0/0/0)	8/10 (8/0/0/0)	10/10 (0/9/1/0)	10/10 (0/1/9/0)
	Female	0/10 (0/0/0/0	10/10 (10/0/0/0)	10/10 (0/10/0/0)	10/10 (0/0/8/2)
Granulomatous inflammation in lung	Male	0/10 (0/0/0/0)	1/10 (1/0/0/0)	10/10 (0/10/0/0)	10/10 (0/0/10/0)
	Female	0/10 (0/0/0/0)	4/10 (4/0/0/0)	10/10 (3/7/0/0)	10/10 (0/1/9/0)
Intraalvolar lipoproteinosis in lung	Male	0/10 (0/0/0/0)	0/10 (0/0/0/0)	7/10 (4/3/0/0)	10/10 (0/7/3/0)
	Female	0/10 (0/0/0/0)	0/10 (0/0/0/0)	10/10 (1/8/1/0)	10/10 (0/0/7/3)
Diffuse neutrophilic inflammation in lung	Male	0/10 (0/0/0/0)	0/10 (0/0/0/0)	10/10 (10/0/0/0)	10/10 (10/0/0/0)
	Female	0/10 (0/0/0/0)	0/10 (0/0/0/0)	10/10 (10/0/0/0)	10/10 (10/0/0/0)
Lymphoreticular hyperplasia in lymph node	Male	0/10 (0/0/0/0)	0/10 (0/0/0/0)	2/10 (1/1/0/0)	8/10 (2/6/0/0)
	Female	0/10 (0/0/0/0)	0/10 (0/0/0/0)	0/10 (0/0/0/0)	10/10 (1/6/3/0)
Granulomatous inflammation in lymph node	Male	0/10 (0/0/0/0)	2/10 (2/0/0/0)	5/10 (1/4/0/0)	10/10 (6/4/0/0)
	Female	0/10 (0/0/0/0)	5/10 (4/1/0/0)	9/10 (1/8/0/0)	10/10 (0/10/0/0)
Particles in macrophages in lymph node	Male	0/10	5/10	9/10	10/10
	Female	0/10	9/10	10/10	10/10

[a]Rats were exposed for 6 hours/day, 5 days/week for 13 weeks and sacrificed 1 day after the last exposure.
[b]Numbers of rats showing each severity grade are indicated in parentheses. Severity grades: 1 = minimal, 2 = slight, 3 = moderate, 4 = marked.

Source: Ma-Hock et al. (2009).

The only other histopathological changes reported in this study were associated with nonspecific adaptive responses in the upper respiratory tract (nasal cavity and larynx) (Ma-Hock et al., 2009). Concentration-dependent multifocal hyperplasia of the mucous-producing goblet cells and increased intraepithelial eosinophilic inclusions were observed in the nasal cavity of mid- and high-concentration animals, and minimal epithelial alteration at the base of the epiglottis was observed in the larynx (authors indicated that this effect was seen "in treated groups"). However, no corresponding cytotoxicity was observed. No histopathological changes were noted in any other organ or tissue. A stand-alone LOAEL of 0.1 mg/m^3 (the lowest concentration tested) is identified for this study based on the concentration-related increases in incidence and severity of diffuse histiocytosis and granulomatous inflammation in lung tissue. A NOAEL was not identified in this study.

Studies conducted by Li et al. (2009b, 2007a) investigated the pulmonary toxicity induced by repeated inhalation of aerosolized MWCNTs in mice. These studies exposed groups of female Kunming mice (30 g, 10 weeks of age) to pristine MWCNTs (>95% pure, ash [lanthanum, nickel] <0.2%, amorphous carbon <3%) with an average external diameter of 50 nm, a mean length of 10 μm, and a special surface area of 280 m^2/g. In the longer duration studies reported by Li et al. (2009b), groups of nine mice were exposed to MWCNTs in an inhalation chamber every other day for 30 or 60 days. In addition, nine control mice were evaluated in this study (exposure conditions not described in detail). In the shorter duration studies reported by Li et al. (2007a), groups of six mice were exposed to MWCNTs in an inhalation chamber intermittently for 8, 16, or 24 days as follows: mice in the 8-day group were exposed on days 1, 2, 4, 6, and 7; mice in the 16-day exposure group were exposed on the same days as the 8-day exposure group as well as on days 9, 10, 12, 14, and 15; and mice in the 24-day exposure group were exposed on the same days as the 8- and 16-day exposure groups as well as on days 17, 18, 20, 22, and 23. There was no mention of a corresponding control group for the inhalation exposures in the Li et al. (2007a) study. The concentration of aerosolized MWCNTs in the inhalation chamber decreased regularly from about 80 to 13 mg/m^3 per 90 minutes. Similar exposures were continually repeated 4 times in an exposure day for a daily cumulative exposure time of 6 hours/day. The authors calculated the weighted mean concentration in a 90-minute exposure to be 32.61 mg/m^3, and the intra-lung deposition doses to be roughly 0.07, 0.14, 0.21, 0.21 (again), and 0.42 for the 8-, 16-, 24-, 30-, and 60-day exposure groups, respectively (assuming a fractional deposition ratio of aerosolized CNTs into lung of about 4% and a breathing rate of 30 mL/minute). The reader is referred to Li et al. (2009b, 2007a) for detailed calculations of these values. In both studies, silicon slices were placed horizontally on the bottom of the inhalation chamber for 90 minutes in order to evaluate the aspect of MWCNTs using SEM. The authors reported that the SEM micrograph showed small size clusters on the silicon slice formed from the aggregation of various numbers of nanotubes that were predominantly in respirable sizes.

Li et al. (2009b) evaluated mice exposed for up to 30 or 60 days for changes in biochemical indices and pathology. Following exposure, six mice were randomly chosen from each group for evaluation of changes in biochemical parameters (total protein, ALP, ACP, and LDH) in BALF. The remaining three mice in each group were sacrificed following exposure and the lungs of each mouse were removed and evaluated by light microscopy for pathological changes. The mice exposed by Li et al. (2007a) were also evaluated for pathological changes in

the lungs using light microscopy. Li et al. (2009b) observed increasing severity in biochemical and pathological changes with increased exposure. All four biochemical parameters were increased over controls at both 30 and 60 days, althought statistical significance was only achieved at 60 days (Table 5.9). Similarly, aggregations of MWCNTs were observed deposited in the bronchi and alveoli resulting in thickening of the alveolar wall at 30 and 60 days, with larger aggregations and more obvious thickening of the alveolar wall at 60 days. In both groups, the aggregations in the alveolar wall were far smaller than those adhered to the bronchial wall. In the shorter duration studies, Li et al. (2007a) observed aggregations of MWCNTs adhered to the lining wall of the bronchi with no accumulation of inflammatory cells, and smaller aggregations of MWCNTs entered into the alveolar walls resulting in moderate proliferation and thickening. The only concentration tested in this study, 32.6 mg/m^3, was identified as a LOAEL for pulmonary toxicity (increased LDH, ALP and ACP activities in BALF and increased alveolar wall thickening at 60 days).

Table 5.9. Changes of biochemical indices in BALF in mice exposed to MWCNTs by inhalation for up to 60 days

BALF parameter	Control	30-d group	60-d group
Total protein (g/L)	0.14 ± 0.04[a]	0.16 ± 0.04	0.24 ± 0.06[b]
ALP (U/L)	1.36 ± 0.44	1.56 ± 0.19	2.91 ± 0.92[b]
ACP (U/L)	2.13 ± 0.43	2.36 ± 0.84	4.96 ± 0.36[c]
LDH (U/L)	582.04 ± 82.49	667.09 ± 40.59	1,248.74 ± 28.05[c]

[a]Values presented are assumed to be mean ± standard deviation, although this is not specified in the report.
[b]Significantly different from controls, $p < 0.05$.
[c]Significantly different from controls, $p < 0.01$.

Source: Li et al. (2009b)

5.3.3. Dermal Studies

No studies were located on the possible toxicity of repeated exposure to CNTs by the dermal route.

5.4. CHRONIC TOXICITY/CARCINOGENICITY

5.4.1. Oral Studies

No oral studies were located on the possible carcinogenicity of repeated exposure to CNTs.

5.4.2. Inhalation Studies

No inhalation studies were located on the possible carcinogenicity of repeated exposure to CNTs.

Based on the similarities in physical properties between CNTs and asbestos fibers (e.g., elongated shape and high aspect ratio), and because inhalation, intratracheal instillation, and pharyngeal aspiration studies have reported lung effects in rodents following exposure to CNTs

(see Sections 5.2.2 and 5.2.3), there is concern that inhalation of CNTs may cause similar lung pathologies as asbestos fibers including mesothelioma, a cancer of the mesothelial lining. As described in Section 5.2.2, Ryman-Rasmussen et al. (2009a) showed that MWCNTs reach the subpleura, increase the accumulation of mononuclear cell aggregates on the pleural surface, and induce subpleural fibrosis in mice after a single 6-hour inhalation exposure to 30 mg/m^3 MWCNTs. MWCNTs were also observed within subpleural mesenchymal cells and the collagen matrix of the subpleura 1 day after inhalation. Some of the inhaled nanotubes remained in the subpleural wall for at least 14 weeks, although most appeared to be cleared by this time (data not shown). However, this study did not include quantitative measures of clearance dynamics, and was not designed to evaluate whether MWCNTs cause mesothelioma (mice were allowed to live only to 14 weeks after exposure).

5.4.3. Other Studies

A recent aspiration study provides evidence that CNTs are capable of reaching and persisting within the lung pleura, which supports the concern that CNTs may induce mesothelioma. In this study, groups of 7–8 male C57BL/6J mice (7 weeks old) were aspirated with suspensions of MWCNTs (0.46% sodium, 0.32% iron, 49 nm mean diameter, 3.86 μm median length) at 0, 10, 20, 40, or 80 μg and observed by SEM for lung distribution of MWCNTs at 1, 7, 28, and 56 days post-exposure (Mercer et al., 2010). The authors reported rapid, frequent, and persistent MWCNT penetrations of alveolar macrophages, alveolar epithelium, and pleura. At day 1, the majority of lung burden was deposited in the alveolar region, with alveolar macrophages receiving 62% of the total dose. The airways, alveolar regions, and visceral pleura region (subpleural tissue and intrapleural space) accounted for 18, 81, and 0.6% of the MWCNT lung burden, respectively. Intrapleural levels of MWCNTs decreased from 1 to 7 days post aspiration, indicating clearance of MWCNT from the subpleural tissue, likely due to macrophage and subpleural lymphatic activity. However, intrapleural levels of MWCNTs rose again at 28 days and remained constant through 56 days post-aspiration. Mercer et al. (2010) postulated that the lung burden of MWCNTs acts as a reservoir to replenish MWCNTs in the intrapleural space. Mercer et al. (2010) indicated that the National Institute for Occupational Safety and Health (NIOSH) is currently conducting a 12-day inhalation study for MWCNT with an evaluation period of up to 1 year to attempt to elucidate issues concerning long-term kinetics of penetration versus clearance of MWCNTs from the intrapleural space.

Several injection studies have attempted to evaluate whether CNTs cause mesothelioma in rodent models.

Poland et al. (2008) reported that abdominal injection of long MWCNTs (average of 13–56 μm length), but not short MWCNTs (1–20 μm length), induced inflammation and granulomatous lesions on the abdominal side of the diaphragm of female C57B1/6 mice 1 week post exposure at a concentration of 50 μg. The granulomas observed in this study comprised aggregates of cells containing fibers and foreign body giant cells with MWCNTs and associated deposition of collagen within the lesions. The mesothelial lining on the pleural side of the diaphragm was normal. However, the short observation time of this study (7 days) limits the ability to observe changes in the mesothelium. This study did not address whether the MWCNT-exposed mice that developed inflammatory and granulomatous changes would go on

to develop mesotheliomas.

Takagi et al. (2008) i.p. injected 19 p53-heterozygous male mice (9–11 weeks old) with a single injection of 1×10^9 MWCNTs (0.35% iron, average width of 100 nm, 27.5% of the particles were longer than 5 μm, aggregates measured 50–200 μm) particles in a 1-mL suspension. The authors reported that this suspension corresponded to a 3 mg/kg dose. Three other groups of 19 p53 mice each received single i.p. injections of 1-mL vehicle solution (negative control) or 3 mg/kg of either fullerene or crocidolite asbestos (positive control). Satellite groups of six wild-type C57BL/6 male mice each were similarly treated and sacrificed at day 10 for the observation of early peritoneal responses. Mice were monitored until one of the groups reached 100% mortality. The study was terminated at week 25 (day 180). Liver, kidney, spleen, lung, digestive tract, and macroscopic tumors were examined microscopically. At 10 days postexposure, the wild-type mice exposed to MWCNTs showed slight fibrinous adhesion with a trace amount of ascites with scattered black spots of MWCNT aggregates and edematous and hypotonic intestinal loops. Mice exposed to crocidolite showed similar responses at 10 days postexposure but to a lesser extent. The highest lethality in the p53 mice groups was seen in the MWCNT group (data not shown). The major cause of death to MWCNT-exposed mice was reported to be from large/invasive mesotheliomas. Among those moribund/dead or terminated at week 25, there were three mice with incidental mesotheliomas where the cause of death was determined to be constriction ileus.

At day 84, the overall incidence of mesothelioma was 14/16 in the MWCNT group (87.5%, 11 found as cause of death, 3 as incidental) (Takagi et al., 2008). This is compared to the positive control group where the overall incidence of mesothelioma was 14/18 (77.8%, 8 found as cause of death, 6 as incidental). No tumors were reported in control mice or mice exposed to fullerenes. Mice exposed to MWCNTs showed moderate to severe fibrous peritoneal adhesion with slight ascites, fibrous peritoneal thickening with occasional black-colored depositions, and a high incidence of macroscopic peritoneal tumors. This peritoneal adhesion and fibrous thickening was due to the formation of fibrous scars and foreign body granulomas with phagocytic cells including multinucleated giant cells. A spectrum of peritoneal mesothelial lesions was observed adjacent to the fibrogranulomatous lesions. These lesions ranged from accumulations of atypical mesothelial cells, typical epithelial mesotheliomas with occasional hobnail appearance, and mild to moderate fibrovascular stem formation, to large tumors composed of anaplasic cells with high mitotic rates and occasional central necrosis compatible with the diagnosis of high-grade malignant mesothelioma. The large tumors were found to invade the abdominal wall, diaphragm, liver parenchyma, and pancreas, and in some cases involve the thoracic cavity, but no distant metastasis was observed. Takagi et al. (2008) concluded that MWCNTs possess carcinogenic potential in p53 mice.

The Takagi et al. (2008) study was criticized for inherent flaws in the study design including using the p53 mouse model, using a dose much higher than the maximum tolerated dose, and poor characterization of the MWCNTs (Donaldson et al., 2008; Ichihara et al., 2008). In regard to these criticisms, Takagi et al. (2008) indicated that the goal of the 2008 study was to identify whether or not MWCNTs could induce mesothelioma and not to provide information for further dose-response studies if tumors were induced. Therefore, Takagi et al. (2008) argued that the high dose and the p53 mouse system used in their study were valid for hazard identification

purposes. In the response to Donaldson et al. (2008), Takagi et al. (2008) indicated that they immediately started a follow-up dose-response study with MWCNT doses 10, 100, and 1,000 times lower than the previous study. Their response indicated that after >350 days of observation, macroscopic findings of moribund/dead animals preliminarily indicated that mesotheliomas were induced in all dosage groups. However, a report of this study is not yet available.

Sakamoto et al. (2009) attempted to address some of the concerns raised with the Takagi et al. (2008) study by injecting male Fischer 344 rats with 0 or 1.0 mg/kg MWCNTs (same as those used by Takagi et al., 2008; 3,500 ppm iron, maximum width of 90 nm, maximum length of 2 μm). The authors reported that this dose corresponded to 0.24 mg/rat. In this study, seven rats were exposed to MWCNTs by a single intrascrotal injection. Rats were observed for up to 52 weeks. Rats that died or became moribund during the observation period were immediately autopsied. At the end of the 52 weeks, all surviving animals were sacrificed and macroscopically examined, and all major organs and tissues including tumor tissues were examined histologically. In a carboxymethyl cellulose suspension, MWCNTs appeared as agglomerates and dispersed multi-sized rod-shaped or fibrous particles using TEM. Only one of the seven rats treated with MWCNTs survived until the end of the 52-week observation period. Severe anemia and abdomen enlargement due to the accumulation of ascites were commonly observed in the rats that died during weeks 37–40 (four rats) or became moribund at the ends of weeks 40 and 50 (two rats). Macroscopic evaluations of MWCNT-exposed rats revealed hemorrhagic ascites; severe fibrous adhesions in the diaphragm, liver, stomach, pancreas, spleen, and omentum; deformed livers; and tumor nodules in the peritoneal cavity, and to a lesser extent in the thoracic cavity. The majority of tumor nodules were small, although large tumors were observed, mostly around the diaphragm and involving the liver, stomach, pancreas, spleen, and their surrounding stroma, in four of the seven exposed rats. Metastatic lesions were detected on the peri- and epicardium of four dead animals. Histology revealed mesothelial hyperplasias and mesotheliomas in 100% and 85% of MWCNT-treated rats, respectively. The overall incidence of mesothelioma in MWCNT-treated rats was 85%, which was significantly ($p < 0.05$) higher than both the vehicle controls (0%) and the positive control rats exposed to crocidolite (0%). The authors postulated that the negative findings based on crocidolite exposure in this study were likely due to the administered dose. Sakamoto et al. (2009) set the dose of crocidolite at 2 mg/kg to make it in an equivalent range on a weight basis with that of the MWCNT dosage. However, the authors note that previous studies showing induction of mesotheliomas with crocidolite following i.p. or intrathoracic administration were generally performed with higher doses and/or longer periods of exposure than used in this study. Granulomas were found scattered in the submesothelial layer of the fibrously thickened parietal and visceral peritoneum of both MWCNT-treated and crocidolite-treated rats. The cellularity of these granulomas was much less in crociodilte-treated rats compared with MWCNT-treated rats.

Two other rat studies did not find mesothelioma in animals exposed to CNTs (Varga and Szendi, 2010; Muller et al., 2009).

Muller et al. (2009) exposed groups of 50 specific pathogen free male Wistar rats (9–12 weeks old) to MWCNTs via a single i.p. injection. Two types of MWCNTs were used in this study, MWCNTs with structural defects (MWCNT+; 2% aluminum, 0.5% iron, 0.5% cobalt,

11.3 nm diameter, 0.7 μm length) and MWCNTs without structural defects (MWCNT-; 0.37% aluminum, <0.01% iron or cobalt, 11.3 nm diameter, 0.7 μm length). Rats were injected with 2 or 20 mg of MWCNT+ or 20 mg of MWCNT-. Additional groups of 26 rats each were injected with an equal volume of PBS (negative controls) or 2 mg crocidolite asbestos (positive controls). Rats were observed for up to 24 months. Survival and body weight were monitored over the first 3 months of the experiment. Rats that died or became moribund prior to study termination were subjected to full gross necropsy. Following the 24-month observation period, animals were examined macroscopically and external lesions and tumors and major peritoneal organs and tissues (including tumors) were examined microscopically. This study also included a preliminary test that verified the contrasting inflammatory response at 24 hours post-injection to MWCNT+ and MWCNT-. No significant effects on body weight or survival were reported during the first 3 months of the experiment. A slight inflammatory reaction with adherences to the liver was sometimes noted on the peritoneal aspect of the diaphragm of animals treated with the highest dose of MWCNT+ or MWCNT-. Granulomas with very limited inflammatory reactions were also observed in these rats. One vehicle control animal presented with mesothelioma. Four percent (2/50) of rats exposed to the low-dose of MWCNT+ had mesotheliomas, whereas none of the high-dose MWCNT+ rats had mesotheliomas. Six percent (3/50) of rats exposed to MWCNT- had mesotheliomas. Incidence of mesotheliomas among the positive controls was 34.6% (9/26). Three rats exposed to the high-dose of MWCNT+ did present with non-mesothelial tumors including lipoma, angiosarcoma, and liposarcoma. Lipomas were also observed in three rats exposed to MWCNT-. Statistical analysis conducted by the authors did not show any significant differences in the incidence of total peritoneal tumors ($p = 0.623$), mesothelioma ($p = 0.427$), or other tumors ($p = 0.569$) in exposed versus control rats.

Varga and Szendi (2010) evaluated the mesothelioma-inducing effects of both SWCNTs (90% pure, <2 nm diameter, 4–15 μm length) and MWCNTs (95–98% pure, 10–30 nm diameter, 1–25 μm length) in groups of six F344 rats (sex not specified, 400 g) by implanting CNT-filled gelatin capsules into the peritoneal envelope lined with mesothelial cells. The capsules were filled with 0 or 25 mg/kg (corresponding to 10 mg/animal) of SWCNTs or MWCNTs. Rats were autopsied and organs/tissues (not specified) were examined histologically at 12 months post-instillation. No mortality occurred prior to study termination. The authors did not observe any significant signs of mesothelioma macroscopically or microscopically. Macroscopic findings included dispersed bulks of carbon on the adjacent organs to the point of instillation, partial expansion of the gastric wall, and residual carbon on the peritoneal envelope of the liver. Histological examination showed granulomas with multinucleated giant cells on the livers of treated rats (findings were comparable between SWCNT- and MWCNT-exposed rats).

The translocation potential of CNTs to the pleura, combined with the bio-persistent nature and high aspect ratio of CNTs, supports concern that exposure to CNTs could provoke persistent latent interactions with mesothelial cells. Although Takagi et al. (2008) reported that i.p. injection of MWCNTs results in mesotheliomas in p53 +/- mice, issues raised with this study limit its usefulness for risk assessment purposes. Sakamoto et al. (2009) reported that intrascrotal injection of MWCNTs resulted in mesotheliomas in Fischer 344 rats, but no mesotheliomas were induced by the positive asbestos control in this study, raising questions about the methodology used. Muller et al. (2009) reported that i.p. injection of MWCNTs did

not induce mesotheliomas in Wistar rats. However, the length of MWCNTs used in this study was short (<1 μm on average), and the study by Poland et al. (2008) would have predicted minimal effect from exposure to such short nanotubes. Varga and Szendi (2010) also reported negative findings of mesothelioma following an implantation study in F344 rats with CNTs measuring up to 15–25 μm in length, but this study was limited by small group sizes. Therefore, based on these inconsistent findings and the differences between studies that limit comparative analyses, further data are need to fully elucidate the possible carcinogenic effects of CNTs on mesothelial cells in vivo.

5.5. REPRODUCTIVE/DEVELOPMENTAL TOXICITY

No standard mammalian tests of reproductive or developmental toxicity were located for CNTs.

5.6. MECHANISTIC DATA ON CNTs

5.6.1. Cytotoxicity

Overview: There have been a large number of in vitro studies conducted with mammalian cell lines to elucidate the cytotoxicity of various types of CNTs. Most of these studies have evaluated CNT effects on lung or skin cells as a means of assessing pulmonary and dermal toxicity. Several recent reports have attempted to summarize these cytotoxicity data (Cui et al., 2010; Firme et al., 2010; Jaurand et al., 2009; Shvedova et al., 2009). Cytotoxicity results across studies are somewhat contradictory, with the majority of studies demonstrating a cytotoxic effect of CNTs (Kisin et al., 2007; Zhang et al., 2007; Bottini et al., 2006; Kagan et al., 2006; Sayes et al., 2006; Tian et al., 2006; Cui et al., 2005; Ding et al., 2005; Jia et al., 2005; Manna et al., 2005; Monteiro-Riviere et al., 2005a, b; Shvedova et al., 2003) and others demonstrating their biocompatibility (Mutlu et al., 2010; Yamashita et al., 2010; Chen et al., 2006; Dumortier et al., 2006; Flauhaut et al., 2006; Garibaldi et al., 2006). Issues with comparing across in vitro studies include differences in administration and evaluation methodologies, differences in the CNT particles themselves (e.g., size, surface charge), and incomplete characterization of CNTs following purification and/or functionalization. The effects of various factors, such as length, diameter, surface charge, metal content, presence or absence of functional groups, and degree of agglomeration or dispersibility in aqueous media, on cytotoxicity have been investigated, indicating that the cytotoxicity of CNTs is influenced by these factors in an as yet undetermined fashion (Pulskamp et al., 2007; Zhang et al., 2007; Dumortier et al., 2006; Kagan et al., 2006; Cui et al., 2005).

5.6.1.1. Cellular Uptake

The cellular uptake of CNTs is controversial, as studies have reported both significant uptake and absence of cellular internalization. Cellular uptake is likely dependent on a variety of factors associated with cellular receptors and cell surface functions, as well as CNT surface reactivity. Studies with human alveolar type II cancer cells (Davoren et al., 2007; Herzog et al., 2007), human bronchial epithelial cells (Herzog et al., 2007), mouse peritoneal macrophage cells (Shvedova et al., 2005), human mesothelial cells (Tabet et al., 2009), and human umbilical vein

endothelial cells (Flahaut et al., 2006) reported that CNTs are not taken up in vitro. Mercer et al. (2008) reported that alveolar macrophages took up dispersed SWCNTs to a limited degree following aspiration in a mice, and those escaping phagocytosis migrated into the interstitium of the alveolar septa. In contrast, significant in vitro uptake of CNTs by rat and guinea pig alveolar macrophages has also been reported (Dutta et al., 2007; Pulskamp et al., 2007; Jia et al., 2005). Dutta et al. (2007) showed an important role for adsorbed proteins in modulating cellular uptake.

Pantarotto et al. (2004) reported that functionalized SWCNTs conjugated with a peptide responsible for G protein function entered the nucleus of human 3T6 fibroblasts and keratinocytes and murine 3T3 cells. This study also reported that fluorescein isothiocyanate-conjugated functionalized SWCNTs were able to enter these cells in <1 hour. Kang et al. (2008) reported a concentration-dependent uptake of SWCNTs functionalized with folate acid within human hepatocellular carcinoma cells (Hep G2 cells) that was mediated via a folate receptor pathway. This study found that these functionalized SWCNTs were only present in the cytoplasm of Hep G2 cells, and were not observed in the nuclei. TEM analysis showed SWCNTs in lysosomes and within the extracellular space after incubation for 5 hours. Kostarelos et al. (2007) demonstrated that functionalized SWCNTs and MWCNTs with a wide variety of functional groups exhibit a capacity to be taken up by a wide range of mammalian cells, some of which exhibited deficient phagocytosis (fibroblasts), even under endocytosis-inhibiting conditions. This study also reported intracellular trafficking of functionalized CNTs through cellular barriers toward the perinuclear region. Several other studies have also reported that CNTs can penetrate the mammalian cell membrane without any requirement of external transporter systems (Zhang et al., 2007; Monteiro-Riviere et al., 2005a, b; Cherukuri et al., 2004). However, Dumortier et al. (2006) suggested that an active uptake mechanism is likely involved in the entry of functionalized CNTs into macrophages after observing big bundles inside isolated mouse macrophages. Becker et al. (2007) demonstrated that cellular uptake may be a function of CNT size. This study demonstrated a length-selective cellular uptake of DNA-wrapped SWCNTs by fetal lung tissue cells, whereby only SWCNTs shorter than 189 nm were found localized within the cellular cytoplasm.

5.6.1.2. Human Cell In Vitro Cytotoxicity

Decreased cell viability has been reported in a number of human cell lines following CNT exposure (Patlolla et al., 2010a; Belluci et al., 2009; Lindberg et al., 2009; Tabet et al., 2009; Bottini et al., 2006; Cui et al., 2005; Monteiro-Riviere et al., 2005a, b).

Cui et al. (2005) reported a dose-dependent and time-dependent loss of cell number, viability and adhesive ability of SWCNT-treated human embryo kidney (HEK293) cells at concentrations ranging from 0.8 to 200 μg/mL. Western blot analysis showed that SWCNTs downregulate expression of adhesion-associated proteins such as laminin, fibronectin, cadherin, FAK, and collagen IV. Moreover, Cui et al. (2005) reported morphological changes characteristic of apoptosis and cell cycle arrest in the G1 phase at concentrations ≥25 μg/mL. Biochip analysis showed downregulation of cell cycle genes and signal transduction-associated genes and upregulation of certain cell cycle-associated genes.

Kang et al. (2008) reported a concentration-dependent apoptosis in human hepatocellular

carcinoma cells (Hep G2 cells) incubated with functionalized SWCNTs at concentrations ranging from 50 to 500 μg/mL. No obvious cell death was observed at concentrations <50 μg/mL. Similar to Cui et al. (2005), Ding et al. (2005) observed cell cycle arrest and increases in apoptosis and necrosis in human skin fibroblast cells exposed to MWCNTs at concentrations of 0.06 and 0.6 mg/L. However, antibody and DNA staining in this study suggested that the cell cycle was arrested in the G2 phase in this study. Expression array analysis indicated that multiple cellular pathways were affected by exposure to MWCNTs including cellular transport, metabolism, cell cycle regulation and stress response. Moreover, microarray results demonstrated that interferon and p38/ERK-MAPK cascades are critical pathway components in the induced signal transduction observed following exposure to MWCNTs.

Bottini et al. (2006) found that MWCNTs induced a loss of cell viability through programmed cell death in Jurkat T leukemia cells and reported differences in toxicities between pristine and oxidized MWCNTs. As reported by the authors, these two types of MWCNTs differ in hydrophobicity (pristine CNTs are hydrophobic), size and shape (the oxidized CNTs were shorter and straighter than the non-treated pristine CNTs), and dispersion in aqueous solution (oxidized CNTs are better dispersed in aqueous solution and therefore reach higher concentrations of free CNTs at similar weight per volume values). At 400 μg/mL, oxidized MWCNTs caused a loss of >80% of the cells within 5 days, while pristine MWCNTs killed less than half. A smaller effect was observed at a 10-fold lower concentration (40 μg/mL), but again, the oxidized MWCNTs reduced cell growth more than pristine MWCNTs.

In a study by Tabet et al. (2009), MWCNT exposure formed agglomerates on top of human epithelial cells (A549 cell line) that varied in size and number dependent on dispersion media. Significant decreases in cell viability were observed with 10 and 100 μg/mL, reaching 60% of control values for 100 μg/mL incubation at 48 hours post-exposure. Agglomerates were significantly larger and more numerous when MWCNTs were dispersed in PBS than in ethanol or dipalmitoyl lecithin. However, cell viability was similar for the different dispersion media; incubation with 100 μg/mL MWCNT induced a similar decrease in metabolic activity without changing cell membrane permeability or apoptosis. Neither MWCNT cellular internalization nor oxidative stress was observed in this study.

Zhang et al. (2007) reported differences in cytotoxicity related to various dispersion methods. This study evaluated the effects of dimethylsulfoxide and a 1% anionic surfactant Pluronic F127 on the cytotoxicity of 6-aminohexanoic acid derivatized-SWCNTs (6-aminohexanoic acid derivatized-SWCNTs) in human epidermal keratinocytes. Surfactant treatment resulted in the increased dispersion of 6-aminohexanoic acid derivatized-SWCNTs in the culture medium and less cytotoxicity. Furthermore, the findings by Monteiro-Riviere et al. (2005b) suggest that surfactant-CNT interactions are more complex than simple dispersion alone. In this study, MWCNTs were found to be cytotoxic to human epidermal keratinocytes independent of surfactant exposure.

The functionalization of CNTs has been shown to lead to a dramatic reduction in toxic effects in human cancer and fibroblast cells (Chen et al., 2006; Sayes et al., 2006). Using human dermal fibroblasts, Sayes et al. (2006) showed that as the degree of sidewall functionalization of

SWCNTs increased, cytoxicity decreased. Chen et al. (2006) constructed glycopolymer-coated CNTs that were not toxic to Jurkat T leukemia cells at a concentration of 100 μg/mL. Cells cultured with unmodified CNTs did not multiply during the course of the 3-day experiment. Chen et al. (2006) speculated that the unmodified CNTs either inhibited cell growth or induced cell death at a rate comparable to the proliferation rate. Either way, it appeared that the glycopolymer coating rendered the CNTs nontoxic.

Several studies have investigated the effect of length on CNT toxicity. Becker et al. (2007) reported decreased cellular viability in human alveolar basal epithelial and human fibroblast cells following treatment with DNA-wrapped SWCNTs shorter than 189 nm. SWCNTs longer than this did not affect the viability of the cells. Sato et al. (2005) observed similar cytotoxic effects in vitro in a human acute monocytic leukemia cell line following treatment with MWCNTs of differing lengths, even though differences in inflammatory responses were reported in vivo.

Several studies have reported cytotoxic effects on mesothelial cells (Tabet et al., 2009; Kaiser et al., 2008; Pacurari et al., 2008; Wick et al., 2007). Pacurari et al. (2008) detected minimal ROS activity following exposure of normal and malignant human mesothelial cells to purified MWCNTs containing low levels of iron (99.5% pure, 0.26–0.34% iron, 81 nm diameter, 8.19 μm average length) despite observed cell toxicity manifested through decreased cell viability, release of LDH, and increased apoptosis. MWCNT (2.4% aluminum, 2% iron, 12 nm diameter, 0.1–13 μm length) exposure at 100 μg/mL for 48 hours formed agglomerates on top of large TSV40-transformed mesothelial cells (MeT5A human cell line) and resulted in a dose-dependent decrease in cell viability attributable to altered mitochondrial metabolism with no apparent effect on cell membrane permeability (Tabet et al., 2009). No particle internalization or oxidative stress was observed in MeT5A cells, despite cytotoxicity. Kaiser et al. (2008) and Wick et al. (2007) reported differences in cytotoxicity on human lung mesothelial cells (MSTO-21 1H cell line) dependent on the degree of dispersion of purified SWCNTs (metal contaminants were under the detection limit by ICP-OES). The toxicity of well-dispersed SWCNTs (well-dispersed material with a bundle diameter of around 20 nm) was less than that of rope-like agglomerates (rope diameter in the micron-range) at the same concentrations (Wick et al., 2007). Kaiser et al. (2008) further reported that the agglomerated SWCNTs affected cytoskeleton organization, decreased cell adherence and increased apoptosis/necrosis to a greater extent than the well-dispersed bundles of SWCNTs, while the well-dispersed SWCNTs appeared to have a greater effect on cell proliferation and cell activity than the agglomerated SWCNTs.

Signs of inflammation have been observed following in vitro exposure of human skin cells or tissues to CNTs (Murray et al., 2009; Monteiro-Riviere et al., 2005a; Shvedova et al., 2003). A dose-dependent increase in free radical formation and accumulation of peroxidative products and a decrease in total sulfhydrils and vitamin E content was observed in human epidermal keratinocytes following treatment with SWCNTs at 0.06, 0.12, or 0.24 mg/mL for 18 hours (Shvedova et al., 2003). Shvedova et al. (2003) attributed these oxidative effects to the iron content of the SWCNTs (30% iron), since some iron compounds are known to induce oxidation or peroxidation in cells. Similarly, in vitro exposure of engineered human skin tissue (EpiDermFT) to unpurified SWCNTs containing 30% iron caused increased epidermal thickness and accumulation and activation of dermal fibroblasts, associated with increased collagen and

release of pro-inflammatory cytokines (Murray et al., 2009). However, the inflammatory response may not be attributed only to the metal content of CNTs. For example, Monteiro-Riviere et al. (2005a) reported a slight decrease in cell viability and a time- and dose-dependent release of the proinflammatory cytokine IL-8 from human epidermal keratinocytes treated with MWCNTs with no detectable iron content. Likewise, although in vitro exposure of mouse JB6P+ epidermal cells to SWCNTs containing 30% iron, but not purified SWCNTs containing 0.23% iron, caused a dose-dependent activation of activator protein 1 (AP-1, a regulator of differentiation, proliferation, and apoptosis), both types of SWCNTs activated NFκB, a protein complex that controls DNA transcription in response to stress, cytokines, and other stimuli (Murray et al., 2009).

5.6.1.3. Animal Cell In Vitro Cytotoxicity

Kisin et al. (2007) reported a loss of cell viability in a concentration- and time-dependent manner after exposure of Chinese hamster lung fibroblast cells to purified SWCNTs (99.7% pure) at 24, 48, or 96 μg/cm^2 for up to 24 hours.

Jia et al. (2005) reported relative differences in cytotoxicities of SWCNTs and MWCNTs in alveolar macrophages isolated from guinea pigs at doses ranging from 1.4 to 226 μg/cm^2. Although both types of CNTs significantly reduced cell viability (assayed as enzymatic ability to reduce MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), in a dose-dependent manner, SWCNTs induced greater viability reductions than MWCNTs at similar concentrations (Jia et al., 2005). Similarly, SWCNTs impaired phagocytosis at a concentration of 0.38 μg/cm^2, while MWCNTs only achieved this effect at 3.06 μg/cm^2. At these concentrations, alveolar macrophages displayed characteristic features of necrosis and degeneration.

Chen et al. (2006) constructed glycopolymer-coated CNTs that were not toxic to Chinese hamster ovary cells at a concentration of 100 μg/mL. Cells cultured with unmodified CNTs were unable to grow during the course of the 3-day experiment. The idea that functionalization of CNTs increases biocompatibility was supported by the findings of Dumortier et al. (2006) who reported cellular uptake of functionalized CNTs by B and T lymphocytes isolated from BALB/c mice without effects on cell viability or lymphocyte functionality. Dumortier et al. (2006) also found that differences in functionalization can result in different effects on macrophage behavior. Ammonium functionalized SWCNTs, which were highly water soluble, did not appear to stimulate macrophage activity, whereas oxidized SWCNTs derivatized with poly(ethylene glycol) (PEG) chains at the carboxylic functions (which were not water soluble, but formed homogeneous suspensions) provoked the secretion of proinflammatory cytokines by macrophages at concentrations of 10 or 50 μg/mL. PEG-only treatment did not stimulate cytokine secretion, indicating that the effects observed by the PEG-functionalized SWCNTs are likely due to the presence of aggregates in the culture medium, because the material is not fully soluble.

Kagan et al. (2006) investigated the role of iron in the toxicity of SWCNTs using a mouse leukemic monocyte macrophage cell line (RAW 264.7 macrophages). RAW264.7 macrophages were incubated with either iron-rich (non-purified) SWCNTs (26% iron) or iron-

stripped (purified) SWCNTs (0.23% iron) at concentrations of 0.12–0.5 mg/mL for 1–2 hours. SEM analysis revealed that both types of SWCNTs were well-dispersed and separated in the incubation medium. SEM also confirmed the differences in iron content and corresponding redox activity. The production and conversion of superoxide radicals was investigated with co-incubations of zymozan-activated RAW 264.7 macrophages with non-purified or purified SWCNTs. A 15% enhancement in the generation of hydroxyl radicals was detected with the non-purified SWCNTs relative to the purified SWCNTs. Hydroxyl radicals were not detected in co-incubations with non-activated macrophages. Kagan et al. (2006) speculated that the lack of detectable superoxide radical adducts by non-activated macrophages was due to a relatively low steady-state concentration of superoxide radicals. To verify this, Kagan et al. (2006) generated superoxide radicals by an enzymatic system in the presence of RAW 264.7 macrophages. Using a spin trap, two types of spin adducts were detected, superoxide-5,5-dimethyl-1-pyrroline-N-oxide adducts and hydroxyl radical adducts. When non-purified SWCNTs were added to the incubation system, efficient conversion of superoxide radical adducts into hydroxyl radical adducts occurred. Purified SWCNTs induced a markedly less effective conversion. Non-purified SWCNTs caused a significant loss of intracellular low molecular weight thiols (GSH) and accumulation of lipid hydroperoxides in stimulated RAW 264.7 macrophages. Catalase was able to partially protect macrophages against SWCNT-induced elevation of biomarkers of oxidative stress (enhancement of lipid peroxidation and GSH depletion). These findings indicate that the iron content of SWCNTs used in this study was important for enhanced catalysis of extracellular oxidative stress as well as for intracellular depletion of antioxidant reserves and accumulation of lipid peroxidation products in macrophages.

Becker et al. (2007) reported decreased cell viability in clonal murine calvarial cells and embryonic rat thoracic aorta medial layer myoblast cells following treatment with DNA-wrapped SWCNTs shorter than 189 nm. SWCNTs longer than this did not affect the viability of the cells.

Murray et al. (2009) used mouse epidermal JB6 P+ cells to study the effects of SWCNT exposure on the AP-1 and NF-κB pathways involved in mediating inflammatory responses by exposing FBS treated cells (FBS treatment minimizes basal AP-1 and NF-κB activity) to unpurified (30% iron) or partially purified (0.23% iron) SWCNTs at concentrations of 0, 0.06, 0.12, or 0.24 mg/mL for 24 hours. This study also evaluated free radical formation and cell viability in JB6 P+ cells following a 5-minute incubation with 0.12 mg/mL unpurified SWCNT. Exposure of JB6 P+ cells to unpurified SWCNTs resulted in the formation of hydroxyl radicals. Due to the high iron content of the unpurified SWCNTs, Murray et al. (2009) also incubated JB6 P+ cells with SWCNTs in the presence of deferoxamine, a known iron chelator, and observed a reduction in radical formation. The authors suggested that the observed ROS formation was induced via a metal-dependent Fenton reaction. Exposure to unpurified SWCNTs caused a significant ($p < 0.05$) dose-dependent induction of both AP-1 (15, 50, and 92% increase over controls with 0.06, 0.12, and 0.24 mg/mL, respectively) and NF-κB (44, 50, and 76% increase over controls with 0.06, 0.12, and 0.24 mg/mL, respectively). Partially purified SWCNTs did not significantly induce activation of AP-1, but did induce a similar activation of NF-κB as the unpurified SWCNTs (80% with 0.24 mg/mL, data only shown graphically for low and mid-exposure concentrations).

5.6.2. Genotoxicity

Overview: Results from in vitro studies indicate that, although MWCNTs and SWCNTs have not induced mutations in most in vitro tests of mutagenicity, both types of CNTs induced DNA damage, DNA repair mechanisms, and micronuclei in a number of cell types (see Section 5.6.2.1 and Table 5.10 for references). The ability of CNTs to produce genotoxic effects has been demonstrated in animals in vivo following acute exposures to SWCNTs or MWCNTs by several routes of exposure (see Section 5.6.2.2 for more details). Observed genotoxic effects in in vivo studies include: mitochondrial DNA damage in aortic tissue of mice following pharyngeal aspiration of acid-purified SWCNTs (Li et al., 2007b); increased K-*ras* mutations in lungs of mice exposed by inhalation to non-purified SWCNTs (Shvedova et al., 2008a); oxidative DNA damage in liver and lungs of rats after gavage administration of non-purified SWCNTs (Folkmann et al., 2009); and increased frequencies of chromosome aberrations and micronuclei in bone marrow cells and Comet-assay-detected DNA damage in lymphocytes in Swiss-Webster mice following i.p. injections of nonfunctionalized MWCNTs or acid-purified, carboxylated MWCNTs (Patlolla et al., 2010b).

It is expected that that the DNA damaging action of CNTs is a function of a number of factors, such as length, diameter, surface charge, metal content, presence or absence of functional groups, and degree of agglomeration or dispersibility in aqueous media. For example, Muller et al. (2008a, b—see Table 5.10) noted that ground MWCNTs (with short lengths and oxygenated structural defects produced by grinding) induced micronuclei in cultured rat epithelial lung cells, but "annealed" ground MWCNTs did not (i.e., treated at 2,400°C to anneal structural defects and remove oxygen and metal residuals). Another example of variability in genotoxic action is the report that two types of MWCNTs (with diameters ranging from 20 to 100 nm) induced DNA damage in a human epithelial cell line, but samples of MWCNT or SWCNTs with diameters <10 nm did not at the tested concentration (Yamashita et al., 2010—see Table 5.10). In addition, acid-purified, carboxylated MWCNTs appeared to be more potent than nonfunctionalized MWCNTs in inducing genotoxic effect in mice following i.p. administration (Patlolla et al., 2010b). However, available data are inadequate to determine the relative importance of these factors or characterize their interactions. The molecular modes of action by which CNTs induce DNA damage are uncertain, although the involvement of ROS from biological inflammatory responses (see Pacurari et al., 2010 for review) and direct interaction with DNA (see Lindberg et al., 2009 for review) have been proposed.

5.6.2.1. In Vitro Studies

Table 5.10 summarizes studies of in vitro genotoxicity of CNTs. MWCNTs did not induce reverse mutations in several *Salmonella typhimurium* strains or *Escherichia coli* WP2*uvr*A (Di Sotto et al., 2009; Wirnitzer et al., 2009) or mutations at the hgprt locus in Chinese hamster lung cells (Asakura et al., 2010), but did induce mutations at the Aprt locus in mouse embryonic stem cells (Zhu et al., 2007). SWCNTs did not induce reverse mutations in *S. typhimurium* (Kisin et al., 2007) or mutations at the *cII* locus in mouse lung epithelial cells (Jacobsen et al., 2008). MWCNTs did not induce chromosomal aberrations (Asakura et al., 2010; Wirnitzer et al., 2009), but did induce polyploidy (Asakura et al., 2010) in Chinese hamster ovary cells. DNA damage, assayed by the Comet assay, was consistently observed

across several cell types exposed to several types of CNTs including Chinese hamster lung fibroblasts exposed to SWCNTs (Kisin et al., 2007); a mouse macrophage cell line exposed to SWCNTs or MWCNTs (Migliore et al., 2010); human bronchial epithelial cells exposed to a mixture of CNTs (Lindberg et al., 2009); human dermal fibroblasts exposed to carboxylated MWCNTs (Patlolla et al., 2010a); human mesothelial cells exposed to MWCNTs (Pacurari et al., 2008); and human alveolar carcinoma epithelial cells exposed to two types of MWCNTs (Yamashita et al., 2010). No DNA damage was detected in mouse lung epithelial cells exposed to SWCNTs for 3 hours, a duration shorter than that used in most of the in vitro Comet assays reporting positive results (Jacobsen et al., 2009). The weight of evidence indicating that CNTs can induce DNA damage in vitro is supported by several reports (see Table 5.10) of the induction of DNA repair mechanisms, including p53 tumor suppressor protein (Zhu et al., 2007); phosphorylated γH2AX, a histone involved in repair of double-strand DNA breaks (Cveticanin et al., 2010; Pacurari et al., 2008; Zhu et al., 2007); double-strand break repair protein Rad 51 and x-ray cross-complementation group 4 (XRCC4) (Zhu et al., 2007); and 8-oxoguanine-DNA glycolase 1, a base excision enzyme that repairs 8-oxo-guanine-DNA adducts produced by ROS (Zhu et al., 2007). Positive or equivocal evidence for micronuclei induction has been reported in several cell types exposed to MWCNTs (Asakura et al., 2010; Cveticanin et al., 2010; Migliore et al., 2010; Muller et al., 2008a, b) or SWCNTs (Cveticanin et al., 2010; Migliore et al., 2010; Lindberg et al., 2009; Kisin et al., 2007).

Table 5.10. Summaries of in vitro genotoxicity studies of CNTs

Assay	Test System	Exposure	Particle data	Result	Comments	Reference
Reverse mutations	*S. typhimurium* TA98 and 100; *E. coli* WP2*uvr*A	0, 0.01–9.0 µg/plate, ± metabolic activation for 48 h	MWCNTs (90% pure, <0.1% metal impurities), 110–170 nm D, 5–9 µm L	Negative, ± metabolic activation		Di Sotto et al., 2009
Reverse mutations	*S. typhimurium* TA98,100, 102, 1535, and 1537	0, 50–5,000 µg/plate, ± metabolic activation for 48 h	Baytubes® - agglomerates of MWCNTs (95% pure), 0.2–1 µm L	Negative, ± metabolic activation		Wirnitzer et al., 2009
Reverse mutations	*S. typhimurium* YG1024 and YG1029	0, 60, 120, or 240 µg/plate for 48 h	SWCNTs (99.7% pure, 0.23% Fe), 0.4–1.2 nm D, 1–3 µm L	Negative	No metabolic activation provided	Kisin et al., 2007
Mutation at hgprt locus	Chinese hamster lung cells	0, 6.3–100 µg/mL for 48 h	MWCNT (4,400 ppm iron, 48 ppm Cr, and 17 ppm Ni)	Negative	MWCNTs had 5 µm L, 88 nm D	Asakura et al., 2010
Mutation at *cII* locus	FE1 Muta™ Mouse lung epithelial cells	0 or 100 µg/mL for 576 h	SWCNTs (95% carbon), 0.9–1.7 nm D, <1 µm L	Negative		Jacobsen et al., 2008
Mutation at Aprt locus	Mouse embryonic stem cells, Aprt+/- 3C4 Aprt deficient	0, 5, or 100 µg/mL for 2 or 4 h	MWCNTs purified to remove catalyst residues	Positive	Further properties of MWCNTs were not reported	Zhu et al., 2007
Chromosome aberrations	Chinese hamster lung fibroblast (V79) cells	0, 2.5, 5, or 10 µg/mL for 4 or 18 h	Baytubes® - agglomerates of MWCNTs (95% pure)	Negative, ± metabolic activation	MWCNTs had 0.2–1 µm L	Wirnitzer et al., 2009
Chromosome aberrations	Chinese hamster lung cells	0, 0.078–80 µg/mL for 24 or 48 h	MWCNT (4,400 ppm Fe, 48 ppm Cr, and 17 ppm Ni)	Negative	MWCNTs had 5 µm L, 88 nm D	Asakura et al., 2010
Polyploidy induction	Chinese hamster lung cells	0, 0.078–80 µg/mL for 24 or 48 h	MWCNT (see previous row)	Positive		Asakura et al., 2010
γH2AX foci induction (DS DNA break repair)	Human fibroblasts	0, 0.5–30 µL/mL for 24 h	SWCNTs (70% pure), amide-SWCNTs, and pristine MWCNTs (99% pure), 20–40 nm D, 1–5 µm L	Positive	All three CNTs induced γH2AX foci to similar magnitudes at similar doses	Cveticanin et al., 2010
γH2AX foci induction (DS DNA break repair)	Human mesothelial cells, normal and malignant	0, 12.5, 25, or 50 µg/mL for 24 h	Pristine MWCNTs (99.5% pure), 81 nm D, 8 µm L	Positive	Positive in both cell types	Pacurari et al., 2008
DS-DNA break repair induction	Mouse embryonic stem cells J11	0, 5, or 100 µg/mL for 2 or 4 h	MWCNTs purified to remove catalyst residues	Positive	Proteins induced: Rad 51, XRCC4; exposure also ↑ γH2AX foci	Zhu et al., 2007

Table 5.10. Summaries of in vitro genotoxicity studies of CNTs

Assay	Test System	Exposure	Particle data	Result	Comments	Reference
DNA repair induction	Mouse embryonic stem cells J11	0, 5, or 100 µg/mL for 2 or 4 h	MWCNTs purified to remove catalyst residues	Positive	8-oxoguanine-DNA glycoslase 1; and p53 were induced	Zhu et al., 2007
DNA damage, Comet assay	Chinese hamster lung fibroblast (V79) cells	0, 24, 48, or 96 µg/cm^2 for 3 or 24 h	SWCNTs (99.7% pure, 0.23% Fe), 0.4–1.2 nm D, 1–3 µm L	Positive		Kisin et al., 2007
DNA damage, Comet assay	RAW 264.7 cells: mouse macrophage cell line	0, 0.01–100 µg/mL for 2 or 24 h	SWCNTs (96.7% carbon), 0.7–1.2 nm D, 0.5–100 µm L; MWCNTs (>98% carbon), 110–170 nm D, 5–9 µm L	Positive	SWCNTs and MWCNTs positive at ≥10 and ≥1 µg/mL with 24-h exposure	Migliore et al., 2010
DNA damage, Comet assay	Human bronchial epithelial BEAS 2B cells	3.8–380 µg/mL for 24, 48, or 72 h	CNTs (>50% SWCNTs, ~40% other CNTs), 1.1 nm D, 0.5–100 µm L	Positive	Positive at all times tested	Lindberg et al., 2009
DNA damage, Comet assay	Human dermal fibroblast cells	0, 40, 200, or 400 µg/mL for 48 h	Functionalized MWCNTs (2–7% carboxyl groups), 15–30 nm D, 15–20 µm L	Positive		Patlolla et al., 2010a
DNA damage, Comet assay	Human mesothelial cells, normal or malignant	0, 25, or 50 µg/cm^2 for 24 h	Pristine MWCNTs (99.5% pure), 81 nm D, 8 µm L	Positive	Positive in both cell types	Pacurari et al., 2008
DNA damage, Comet assay	FE1 Muta™ Mouse lung epithelial cells	0 or 100 µg/mL for 3 h	SWCNTs (95% carbon), 0.9–1.7 nm diameter, <1 µm length	Negative	3-h exposure; other positive studies used 24 or 48 h	Jacobsen et al., 2008
DNA damage, Comet assay	Human alveolar carcinoma epithelial cells (A549)	0 or 50 µg/mL for 3 h	SWCNTs, <2 nm D and 5–15 µm L; 3 MWCNTs: M1: 20–60 nm D, 5–15 µm L M2: 60–100 nm D, 1–2 µm L; M3: <10 nm D, 1–2 µm L	Positive, M1 or M2 MWCNTs Negative, M3 MWCNTs, SWCNTs	Thicker diameters were associated with DNA damage at the tested concentration	Yamashita et al., 2010
Micronuclei induction	Chinese hamster lung fibroblast (V79) cells	0, 12, 24, 48, or 96 µg/cm^2 for 24 h	SWCNTs (99.7% pure, 0.23% iron), 0.4–1.2 nm D, 1–3 µm L	Equivocal	Micronuclei increased at 96 µg/cm^2, but not statistically significant	Kisin et al., 2007
Micronuclei induction	Chinese hamster lung cells	0, 0.02–5.0 µg/mL for 48 hrs	MWCNT (4,400 ppm Fe, 48 ppm Cr and 17 ppm Ni), 5 µm L, 88 nm D	Equivocal	Induction of bi- and multi-nucleated cells was clearly exposure-related	Asakura et al., 2010

Table 5.10. Summaries of in vitro genotoxicity studies of CNTs

Assay	Test System	Exposure	Particle data	Result	Comments	Reference
Micronuclei induction	Human lymphocyte cells	0, 0.25–150 µL per 5 mL of total cell culture volume	SWCNTs (70% pure) and functionalized SWCNTs (composition not reported); and pristine MWCNTs (99% pure), 20–40 nm D, 1–5 µm L	Positive	Pristine MWCNTs > functionalized SWCNTs > SWCNTs	Cveticanin et al., 2010
Micronuclei induction	Rat lung epithelial cells or Human breast cancer cells (MCF-7)	0, 10, 25, or 50 µg/mL for 12 h	Ground MWCNTs (98% pure), 11.3 nm D, 0.7 µm L	Positive	Positive in both cell types	Muller et al., 2008b
Micronuclei induction	Rat lung epithelial cells	0 or 25 µg/mL for 12 h	Ground MWCNTs, ground MWCNTs modified at 600°C, ground MWCNTs modified at 2,400°C; size dimensions not reported	Positive with ground MWCNT ± 600°C Negative with ground MWCNTs modified at 2,400°C	Modification at 600°C resulted in loss of oxygen and metal oxides; modification at 2,400°C resulted in elimination of metal clusters and ablation of carbon framework defects	Muller et al., 2008a
Micronuclei induction	RAW 264.7 cells	0, 0.01–100 µg/mL for 2 or 24 h	SWCNTs (96.7% carbon), 0.7–1.2 nm D, 0.5–100 µm L; MWCNTs (>98% carbon), 110–170 nm D, 5–9 µm L	Positive	SWCNTs and MWCNTs positive at ≥1 µg/mL	Migliore et al., 2010
Micronuclei induction	Human bronchial epithelial BEAS 2B cells	3.8–380 µg/mL for 24, 48, or 72 h	CNTs (>50% SWCNTs, ~40% other CNTs), 1.1 nm D, 0.5–100 µm L	Positive	Positive at 10 and 20 µg/cm^2 at 48 h, not at other time points	Lindberg et al., 2009

D = diameter; L = length; DS-DNA = double-stranded DNA

5.6.2.2. In Vivo Studies

The ability of CNTs to damage DNA has been demonstrated in animals following acute exposures to SWCNTs or MWCNTs by several different routes of exposure. Mitochondrial DNA damage was observed in aortic tissue of C57BL/6 mice at 7, 28, and 60 days following pharyngeal aspiration of 10 or 40 µg acid-purified SWCNTs, containing 0.23% iron (Li et al., 2007b). Elevated incidences of K-*ras* oncogene mutations in lung tissue were observed in C57BL/6 mice exposed by inhalation to aerosols of non-purified SWCNTs (containing 17% iron) at 5 mg/m^3, 5 hours/day for 4 days (10/16 exposed mice versus 4/15 controls; Shvedova et al., 2008a). Increased levels of oxidative DNA damage (levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine) were found in the liver and lung, but not in colon mucosa, of Fischer 344 rats after gavage administration of 0.064 or 0.64 mg/kg of SWCNTs, containing 2% iron and traces of cobalt, nickel, manganese, and polycyclic aromatic hydrocarbons (Folkmann et al.,

2009). A dose-dependent trend for increased frequency of micronuclei in type II pneumocytes was observed in Wistar rats following intratracheal instillation of 0.5 or 2 mg of purified, ground MWCNTs containing traces of iron and cobalt (Muller et al., 2008a). Increased frequencies of chromosome aberrations and micronuclei in bone marrow cells and Comet-assay-detected DNA damage in lymphocytes were observed in Swiss-Webster mice following four daily i.p. injections of nonfunctionalized MWCNTs (>95% pure, 15–30 nm outer diameter, up to 12 μm length) or acid-purified, carboxylated MWCNTs (2–7% carboxyl, 11.5 nm diameter, up to 12 μm length) at 0.25, 0.5, or 0.75 mg/kg (Patlolla et al., 2010b). In this study, the genotoxic responses were more pronounced from the carboxylated MWCNTs, compared with the nonfunctionalized MWCNTs; the authors proposed that the better dispersion of the carboxylated MWCNTs may have contributed to this difference in apparent potency (Patlolla et al., 2010b).

5.7. SUMMARY OF HAZARD AND DOSE-RESPONSE DATA

Inhalation Exposure: The toxicity of inhaled CNTs to the respiratory tract and dose-response relationships have been examined in two 13-week studies of Wistar rats exposed to aerosols of two different types of MWCNTs (Pauluhn, 2010a; Ma-Hock et al., 2009), two short-term companion studies of Wistar rats exposed for 6 hours, once or for 5 days, to MWCNTs and observed for up to 28 days (Ma-Hock et al., 2009) or 90 days (Ellinger-Ziegelbauer and Pauluhn, 2009) after exposure, and short-term studies of C57BL/6 mice exposed to MWCNTs for 6 hours and observed for up to 14 weeks after exposure (Ryman-Rasmussen et al., 2009), C57BL/6 mice exposed to MWCNTs for 6 hours/day for 7 or 14 days (Mitchell et al., 2007) and C57BL/6 mice exposed to SWCNTs for 5 hours/day for 5 days and observed for up to 28 days after exposure (Shvedova et al., 2008a). These studies are summarized in Table 5.11. No chronic-duration inhalation toxicity studies are available.

Table 5.11. Summary of observed effects in animals exposed by inhalation to aerosols made from CNTs

Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Short-term exposure studies							
Wistar rats; 6 males/group; nose-only	0, 11, or 241 mg/m^3 for 6 h; pulmonary toxicity assessed on post-exposure d 7, 28, and 90. Acid-treated MWCNTs only assessed at 11 mg/m^3	Pristine and acid-treated MWCNTs, Baytubes®, containing 0.53 and 0.12% Co, respectively. 10–16 nm D, L not specified; 253 mg^2/g surface area; MMADs ranged from 1.9 to 2.9 µm, GSD 1.6–2.6. Aerosols were generated by a Wright-Dust Feeder.	Not determined	11	Increases ($p < 0.05$) in total cell and PMN counts, soluble collagen content, and LDH activity in BAL samples at 7 d after exposure at 11 mg/m^3, and at all time points after exposure to 241 mg/m^3. Increased lung weights at 7- and 90-d sacrifices in both exposure groups. Lung histology at 90 d after exposure to 11 mg/m^3 pristine MWCNTs: 6/6 with minimal enlarged foamy macrophages; Response to acid-treated MWCNTs was slightly more severe: 4/6 with minimal and 2/6 with slight/mild enlarged foamy macrophages, and 2/6 with minimal focal septal thickening.	Responses in BAL samples were greater in rats exposed to pristine MWCNTs, compared with acid-treated MWCNTs. In contrast, histological responses were more severe with acid-treated MWCNTS. Results suggest that metal contaminants may contribute to the initial (i.e., within 7 d) inflammatory response. Histological responses at 90 d to 241 mg/m^3 included: 6/6 with hypercellularity of bronchio-alveolar tissue (versus 0/6 in control); 6/6 with focal septal thickening (versus 1/6); and 6/6 with focal increase in septal collagen (versus 0/6), indicative of a fibrotic response.	Ellinger-Ziegelbauer and Pauluhn, 2009

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Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
C57BL/6 mice; 10 M/sacrifice per group; nose-only	0, 1, or 30 mg/m^3 for 6 h; lung tissue collected at 1 d, and 2, 6, 14 wks after exposure.	MWCNTs, 94% pure containing 5.5% Ni; 30–50 nm D, 0.3–50 µm L; reported MMADs were 164 and 183 nm for the low and high concentrations, respectively. Aerosols appeared to be agglomerated with individual tube lengths ranging from <100 nm to >10 µm.	Not determined	1	Lesions 1 d after exposure: increased incidence of pleural mononuclear cell aggregates with higher incidence after 30 mg/m^3 exposure. Subpleural fibrosis observed in 1/10, 1/10, and 3/10 mice at 2, 6, and 14 wks after exposure to 1 mg/m^3 (not found in controls at any time). After exposure to 30 mg/m^3, subpleural fibrosis found in 9/10, 6/10, and 1/10 mice at 2, 6, and 14 wks.	Subpleural fibrotic lesions progressed in initial time points after exposure, but appeared to resolve by 14 wks after exposure to the high concentration. Aerosols were generated from aqueous suspensions in 1% pluronic F-68.	Ryman-Rasmussen et al., 2009a
Wistar rats; 5 M/group; head-nose inhalation	0, 2, 8, or 30 mg/m^3, 6 h/d for 5 d. BAL samples collected 3 and 28 d after last exposure.	MWCNTs, 90% pure, 10% metal oxide (9.6% aluminum oxide with traces of Fe and Co); 5–15 nm D and 0.1–10 µm L; 250–300 mg^2/g surface area; MMADs between 0.5 and 1.3 µm; GSDs between 3.1 and 5.4; 77.4–86.3% mass of particles had aerodynamic size <3 µm	Not determined	2	Increases ($p < 0.05$) in total cell counts (principally PMNs), total protein content, and enzyme activities (LDH, β-NAG, ALP, and γ-GT) in BAL samples, 3 and 21 d after last exposure. Histology showed diffuse or focal histiocytosis, particle-laden macrophages, and brocheoalveolar hypertrophy and hyperplasia. Histological changes were not resolved in rats 3 wks after the last exposure.	Aerosols were generated by a brush generator. BAL fluid samples collected from 5 rats/group; histology of respiratory tract on 3 rats/group. Other effects at 8 mg/m^3: increased lung weight, small alveolar septal granulomas. Additional effects at 32 mg/m^3 included decreased body weight gain, increased upper respiratory tract irritation, minimal to mild diffuse pulmonary histiocytosis, and minimal infiltration with neutrophils.	Ma-Hock et al., 2009

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Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
C57BL/6 mice; 6 M/group; whole-body exposure	0, 0.3, 1, or 5 mg/m^3, 6 h/d for 7 or 14 d; BAL samples and lungs collected the day after the last exposure.	MWCNTs, >95% pure, 97.9% C, 2.1% oxidized C; 0.5% Ni and 0.5% Fe. 10–20 nm D and 5–15 µm L; 100 m^2/g surface area; MMADs were 0.7–1 µm (~2 GSD) for low and 1.8 µm (~2.5 GSD) for the highest concentration.	5	Not determined	Histology of lungs sampled within 24 hours after the last high-concentration (5 mg/m^3) exposure were reported to be nondistinguishable from control lungs. BAL samples collected the day after 14 d of exposure showed no exposure-related change ($p > 0.05$) in counts of macrophages, PMNs, or lymphocytes.	Aerosols were generated by mechanical agitation using a jet mill coupled to a dry chemical screw feeder. Lack of lung response may be due to relatively low tested concentrations or insufficient observation time to develop lung responses.	Mitchell et al., 2007
C57BL/6 mice; 12 F/sacrifice; whole-body exposure	0 or 5 mg/m^3 5 h/d for 5 d; pulmonary toxicity assessed at 1, 7, and 28 d after last exposure.	Unpurified SWCNTs: 82% pure,17.7% Fe, 0.16% Co, 0.049% Cr, and 0.046% Ni; 0.8–1.2 nm D and 0.1–1 µm L; mass mode aerodynamic diameter of aerosol = 4.2µm. Aerosols were generated by an acoustically fluidized powder feeder and a knife mill.	Not determined	5	Increases ($p < 0.05$) in PMN and macrophage counts, protein content, LDH activity in BAL samples at 1, 7, and 28 d after exposure. Increased proinflammatory and fibrogenic cytokines (TNF-α, IL-6, and TGF-β) in BAL samples at 1, 7, and 28 d after exposure. Alveolar wall collagen increased with increasing time after exposure.	Histology of respiratory tract showed pulmonary inflammation, bronchiolar epithelial cell hypertrophy, and "green-brown" foreign material in the lung interstitium, within macrophages, and free of lung tissue; most frequently aggregated near bronchoalveolar junctions.	Shvedova et al., 2008a

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Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Intermediate-duration exposure studies							
Wistar rats, 50 M and 10 F/ group; nose only	0, 0.1, 0.4, 1.5, or 6 mg/m^3 6 h/d, 5 d/wk for 13 wks. Pulmonary toxicity assessed 1 d, and 4, 13, and 26 wks after exposure.	Pristine MWCNTs, Baytubes®, containing 99.1% C, 0.8% O, and 0.53% Co; 10 nm D and 200–300 nm L; 257 m^2/g surface area; MMADs ranged from 2.74–3.05 µm (GSDs 1.98–2.14). Aerosols of micronized MWCNTs were generated by a Wright Dust Feeder.	0.1	0.4	BAL samples through post-exposure wks 3 or 13 showed increased ($p < 0.05$) PMN counts, soluble collagen and protein and, γ-GT activities. Lung ($p < 0.05$) lesions: particle laden macrophages at d 1 and wk 13; alveolar interstitial thickening or hypercellularity at bronchio-alveolar junction at all sampling dates after 13 wks of exposure; focal-widespread inflammatory cell influx at d 1 after exposure; focally increased collagen staining in terminal bronchiole at all sampling dates.	Severity scores for lung lesions with significantly increased incidences were reported. The number of lesions and their severity increased with increasing exposure. More pronounced and sustained changes in more BAL variables were observed at 1.5 and 6 mg/m^3 (e.g., increased total cell counts and activities of LDH and β-NAG. Additional lung lesions observed at 1.5 or 6 mg/m^3 at all sampling dates: pleural thickening (6 mg/m^3 only), increased collagen staining in terminal bronchioles. Upper respiratory tract lesions observed on d 1 after exposure included goblet cell hyperplasia and/or metaplasia, eosinophillic globules, and focal turbinate remodeling at 1.5 and 6 mg/m^3.	Pauluhn, 2010a

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Species and study type (n/sex/group)	Exposure (report concentrations, frequency, duration)	Particle characteristics	NOAEL (mg/m^3)	LOAEL (mg/m^3)	Responses at the LOAEL	Comments	Reference
Wistar rat, 10/sex/group, head – nose exposure	0, 0.1, 0.5, or 2.5 mg/m^3, 6 h/d, 5 d/wk for 90 d	MWCNTs, 90% pure, 10% metal oxide (9.6% aluminum oxide with traces of Fe and Co); 5–15 nm D and 0.1–10 µm L; 250–300 mg^2/g surface area; MMADs between 0.7 and 2.0 µm; GSDs between 2.1 and 4.1; 77.4–86.3% mass fractions of particles had aerodynamic size <3 µm	Not determined	0.1	Lung lesions: minimal diffuse histiocytosis (8/10 versus 0/10 in control); Mediastinal lymph node lesions: granulomatous inflammation (2/10 versus 0/10) Other lesions at 0.5 and 2.5 mg/m^3: granulomatous inflammation, diffuse neutrophillic inflammation, and intraalveolar lipoproteinosis in lung; lymphoreticular hyperplasia in lymph node; and particles in macrophages in lymph nodes.	Incidence and severity of lung and lymph node lesions increased with increasing concentration. No exposure-related changes in hematological or serum chemistry variables. Examination of a comprehensive set of other tissues and organs in control and high-concentration rats revealed no exposure-related increased incidence of lesions. Aerosols were generated by a brush generator.	Ma-Hock et al., 2009
Kunming mice; 9 F/30-d and 60-d exposure groups; whole-body exposure	0 or 32.61 mg/m^3 6 h/d for 5 d in an 8-d period, 10 d in a 16-d period, and 15 d in a 24-d period (Li et al., 2007a). Other groups exposed for 15 d in 30 d and 30 d in 60 d (i.e., every other day) (Li et al., 2009b).	MWCNTs, 95% pure, <0.2% La and Ni; surface area 280 m^2/g.	Not determined	32.61	In 24-, 30-, and 60-d mice, the principal lung pathology was reported to be proliferation and thickening of the alveolar walls. No incidence data were reported. In 60-d mice (but not 30-d mice), BAL samples showed increased ($p < 0.05$) activities of ALP, ACP, and LDH.	During 90-min exposure periods (four during exposure days), concentrations decreased from about 80 to 13 mg/m^3. The reported concentration is an average. Constant concentrations were apparently not attained. Histology data were only described qualitatively. Collected data are not useful for describing dose-response relationships.	Li et al., 2009b; 2007a

The short-term inhalation exposure studies clearly provide evidence for CNT-induced acute and persistent effects (e.g., inflammation, granulomas, or fibrosis) in bronchoalveolar, alveolar and subpleural regions of the respiratory tract that are similar to those described following acute intratracheal instillation or pharyngeal aspiration of nonfunctionalized or functionalized SWCNTs or MWCNTs (as discussed in Section 5.2.3). Combined results from these acute-duration exposure studies support the idea of a sequence of dose-related events starting with an acute inflammatory response that persists, followed by a fibrotic response culminating in increased collagen deposition, granulomas, and fibrotic tissue in lungs. For example, following exposure to 11 or 241 mg/m^3 MWCNTs for 6 hours, Wistar rats showed increased lung weights, and increased incidences of enlarged foamy macrophages and increased interstitial collagen (high-dose only) at 90 days after exposure (Ellinger-Ziegelbauer and Pauluhn, 2009—see Table 5.11). C57BL/6 mice exposed to 1 or 30 mg/m^3 for 6 hours showed increased incidence of supleural fibrosis at 2 and 6 weeks after exposure (Ryman-Rasmussen et al., 2009a—see Table 5.11). In addition, C57BL/6 mice exposed to 5 mg/m^3 SWCNTs showed increased indices of inflammation (e.g., LDH activities, levels of proinflammatory and fibrogenic cytokines) in BAL samples 1, 7, and 28 day after exposure, as well as increased amounts of alveolar wall collagen that increased with time after exposure (Shvedova et al., 2008a—see Table 5.11.).

The results from several short-term inhalation studies suggest that minimal responses occurred at MWCNT concentrations in the range of 1–11 mg/m^3 and increased in severity with increasing exposure concentrations (Ellinger-Ziegelberger and Pauluhn, 2009; Ma-Hock et al., 2009; Ryman-Rasmussen et al., 2009a). Variability in response in this concentration range is demonstrated by results from two other studies. Shvedova et al. (2008a) reported evidence for moderate inflammatory and fibrogenic responses in C57BL/6 mice exposed to 5 mg/m^3 SWCNTs containing 17.7% iron at 1, 7, and 28 days after exposure, and Mitchell et al. (2007) found no BAL or histological evidence for pulmonary inflammation or fibrosis in C57BL/6 mice exposed to up to 5 mg/m^3 MWCNTs containing 0.5% iron and 0.5% nickel for 6 hours/day for up to 14 days. The degree to which the variability in response across these short-term exposure studies at concentrations <11 mg/m^3 may be explained by variances in chemical and physical properties of the test materials (e.g., graphene framework, particle size dimensions, metal content, degree of functionalization), characteristics of the administered aerosols (e.g., degree of agglomeration), or time of observation (e.g., 1 day after exposure versus 2 weeks after exposure) is unknown. As discussed in Section 5.2.3, results from short-term intratracheal instillation and pharyngeal aspiration suggest that the acute inflammatory and persistent fibrotic lung responses to acute exposure to CNTs are a complex, as yet undefined, function of a number of factors including deposited dose, metal content, particle dimensions including length, degree of agglomeration of administered material, structural defects in the graphene framework, and presence and properties of functional groups added to the graphene framework.

Only two studies provide dose-response information for lung effects following intermediate-duration inhalation exposure to CNTs. Both studies administered respirable

aerosols of MWCNTs for 13 weeks to Wistar rats and found evidence for lung effects starting at concentrations of 0.1 mg/m^3 (increased incidence of minimal diffuse histiocytosis; Ma-Hock et al., 2009), and 0.4 mg/m^3 (e.g., increased PMN counts, soluble collagen and protein, and γ-GT activities in BAL samples, and minimal focally increased collagen staining in terminal bronchioles; Pauluhn, 2010a). Both studies demonstrated increased incidence, severity, and variety of lung responses with increasing exposure levels beyond these apparent LOAELs (see Table 5.1).

The lung appears to be the critical toxicity target of repeated exposure to inhaled CNTs as indicated by the finding of no exposure-related histological effects in a comprehensive set of nonrespiratory tract tissues and organs from Wistar rats exposed to 2.5 mg/m^3 for 13 weeks (Ma-Hock et al., 2009); the lung was the focus of the 13-week study by Pauluhn (2010a). As discussed in Section 5.2.3.4, results from acute pharyngeal aspiration studies of mice provide evidence that SWCNTs can increase markers of cardiovascular disease in aortic tissue (Li et al., 2007b), increase expression of genes involved in inflammation, oxidative stress, and thrombosis in aortic tissue (Erdely et al., 2009), and lower cardiac functional recovery following ischemia (Tong et al., 2009); however, no studies are available of similar cardiovascular endpoints following acute or intermediate inhalation exposure to CNTs. Other potential endpoints of CNT toxicity identified in short-term inhalation or aspiration studies include immunosuppression (Mitchell et al., 2007), neuroinflammation (Sriram et al., 2009), and degenerative liver lesions (Reddy et al., 2010), although supporting evidence is weak in each case.

Oral Exposure: No studies were located on the possible toxicity of repeated exposure to CNTs by the oral route. As discussed in Section 5.2.1, no evidence for toxic effects was found in mice after oral administration of single 1,000 mg/kg doses of three types of SWCNTs, differing in iron content and lengths (Kolosnjaj-Tabi et al., 2010) or in CD-1 mice within 30 days of administration of single oral doses up to 5 mg/kg of acid-functionalized MWCNTs (Carrero-Sanchez et al., 2006).

Dermal Exposure: No studies were located on the possible toxicity of repeated exposure to CNTs by the dermal route. As discussed in Section 5.2.5, limited tests in animals and humans provide evidence that nonfunctionalized CNTs are not potent skin irritants (Kishore et al., 2009; Murray et al., 2009; Huczko and Lange, 2001) or eye irritants (Kishore et al., 2009; Huczko and Lange, 2001). No tests of the dermal sensitization potential of CNTs were located.

5.8. DERIVATION OF POTENTIAL ACCEPTABLE DAILY INTAKE (ADI)

The two subchronic inhalation studies of CNT were considered together as the basis for derivation of a potential ADI for inhalation exposure. In both studies, male and female Wistar rats were exposed to aerosols of MWCNTs 6 hour/day, 5 days/week for 90 days. Pauluhn (2010a) identified a LOAEL of 0.4 mg/m^3 and a NOAEL of 0.1 mg/m^3 based on pulmonary effects (increased PMN counts, soluble collagen and protein, and γ-GT activities in BAL samples and minimal focally increased collagen staining in terminal bronchioles). Ma-Hock et al. (2009) also identified pulmonary effects as critical, but in

this study the 0.1 mg/m^3 exposure level was a LOAEL for increased incidence of minimal diffuse histiocytosis in the lungs, and a NOAEL was not identified.

The LOAEL of 0.1 mg/m^3 from the Ma-Hock et al. (2009) study is the most sensitive measure of effect in the database and could be chosen as the point of departure (POD) for derivation of a potential inhalation ADI for MWCNTs. Under this conservative approach, an inhalation ADI for MWCNTs of 0.2 $\mu g/m^3$ is derived by dividing the duration-adjusted LOAEL (0.1 mg/m^3 × 6 hour/24 hour × 5 day/7 day = 0.018 mg/m^3) by a total uncertainty factor (UF) of 100 (10 for extrapolation from animals to humans and 10 for protection of susceptible populations). Because the POD is a LOAEL, it may also be appropriate to apply an additional UF to extrapolate from the LOAEL to a NOAEL. The standard value of this UF would be 10, although a value of 3 might be applicable here due to the minimal nature of the effect at the LOAEL. Applying this additional UF would reduce the ADI to 0.07 $\mu g/m^3$ (UF=3) or 0.02 $\mu g/m^3$ (UF=10).

The NOAEL of 0.1 mg/m^3 from the Pauluhn (2010a) study may be a more appropriate POD for derivation of the inhalation ADI for MWCNTs, however, because the high aluminum oxide content (~10%) in the material used in the Ma-Hock (2009) study may have contributed to the observed pulmonary responses. Consistent results from acute pharyngeal aspiration, intratracheal instillation, and inhalation studies have demonstrated that metal contaminants in SWCNTs and MWCNTs can enhance pulmonary inflammatory responses (Porter et al., 2010; Ellinger-Ziegelbauer and Pauluhn, 2009; Shvedova et al 2008a, 2005; Lam et al., 2004). Baytube MWCNTs used in the Pauluhn study were >99% pure. Based on the NOAEL of 0.1 mg/m^3 from Pauluhn (2010a), an ADI of 0.2 $\mu g/m^3$ is calculated by adjusting to equivalent continuous exposure (0.1 mg/m^3 × 6 hour/24 hour × 5 day/7 day = 0.018 mg/m^3) and accounting for uncertainties in interspecies and intraspecies extrapolation (10 each).

In a draft assessment, NIOSH (2010) analyzed data from the Ma-Hock et al. (2009) and Pauluhn (2010a) studies and estimated that the lower 95% confidence limit on working lifetime exposure concentrations associated with a 10% extra risk of early-stage adverse lung effects was between 0.2 and 2 $\mu g/m^3$. NIOSH (2010) noted that current methods for reliably measuring airborne CNTs or carbon nanofibers (NIOSH Method 5040) have an upper estimate of the limit of quantitation of 7 $\mu g/m^3$ as an 8-hour time-weighted-average (TWA) concentration. Because of this quantitation limit, NIOSH (2010) proposed (for peer review purposes) a recommended exposure limit of 7 $\mu g/m^3$ as an 8-hour TWA concentration.

Pauluhn (2010b) proposed an occupational exposure limit for MWCNTs of 0.05 mg/m^3 (50 $\mu g/m^3$, 8-hour TWA). This value was derived by dividing the NOAEL of 0.1 mg/m^3 in the 13-week rat study reported by Pauluhn (2010a) by an adjustment factor of 2 for interspecies differences in retained lung dose ($AF_{R \rightarrow H\ retained\ dose}$) defined by the following equation:

$$AF_{R\rightarrow H\ retained\ dose} = [AF_{R\rightarrow H\ deposited\ dose}] \times [AF_{R\rightarrow H\ kinetics}] \times [AF_{R\rightarrow H\ AMvol}]^{-1},$$

$$= [1] \times [10] \times [5.75]^{-1} \approx 2$$

where;
$AF_{R\rightarrow H\ deposited\ dose}$ = 1 = [ventilation ratio] × [pulmonary deposition ratio];
Ventilation ratio = [0.014 m^3/kg-day human] / [0.29 m^3/kg-day rat];
Pulmonary deposition ratio = 0.118 human / 0.057 rat; ratio of the fractions of inhaled particles with MMAD = 3 µm deposited in the pulmonary region;
$AF_{R\rightarrow H\ kinetics}$ = 10 human/1 rat; this adjustment factor was based on a ~10-fold interspecies difference in lung burdens of rats exposed for 3 months and humans exposed long enough to attain steady state in lung burdens; and
$AF_{R\rightarrow H\ AMvol}$ = 5.75 = [5×10^{11} μm^3/kg bw, human] / [8.7×10^{10} μm^3/kg bw, rat]; ratio of alveolar macrophage volume per lung, normalized to body weight.

Pauluhn's derivation applied no duration adjustment to the exposure concentration and no uncertainty factor for protecting sensitive individuals.

CPSC recognizes Pauluhn's interspecies adjustment factor of 2 for inhaled carbon nanotubes as a scientifically defensible replacement for the standard interspecies UF of 10. Using the duration-adjusted NOAEL of 0.018 mg/m^3 from Pauluhn (2010a) as the POD, the interspecies adjustment factor derived by Pauluhn (2010b), and the standard UF of 10 to account for sensitivity in the human population, the following CPSC-recommended inhalation ADI is derived for highly purified MWCNTs:

$$ADI = 0.018\ mg/m^3 / (2 \times 10)$$
$$= 0.9\ \mu g/m^3.$$

ADIs for oral or dermal exposure to CNTs are not derived due to the lack of repeated-dose oral or dermal toxicity data for any types of CNTs.

5.9. CONCLUSIONS

The available repeated-exposure toxicity studies clearly identify pulmonary effects as the most sensitive endpoint in Wistar rats exposed to respirable aerosols of MWCNTs for 13 weeks and provide adequate data to demonstrate increased incidence, severity, and variety of adverse lung responses with increasing exposure levels (Pauluhn, 2010a; Ma-Hock et al., 2009). Ma-Hock et al. (2009) also conducted a comprehensive histological examination of *non-respiratory* tract tissues and organs and found no exposure-related lesions in rats following exposure to 2.5 mg/m^3 MWCNTS for 13 weeks. Supporting evidence for the lung as a sensitive toxicity target of inhaled CNTs comes from several short-term inhalation studies, which observed similar lung responses in rats (Ellinger-Ziegelberger and Pauluhn, 2009; Ma-Hock et al., 2009) and mice (Ryman-Rasmussen et al., 2009a) exposed to MWCNTs and in mice (Shvedova et al., 2008a) exposed to SWCNTs at concentrations in the range of 1–11 mg/m^3. Additional support comes from the observations of acute and persistent lung effects (pulmonary

inflammation, granulomas, and fibrosis) in many studies of rodents exposed to nonfunctionalized or functionalized SWCNTs or MWCNTs by intratracheal instillation or pharyngeal aspiration (see Tables 3.4 and 3.5 for references).

Although the available data are adequate to derive a recommended inhalation ADI for purified MWCNTs, several areas of uncertainty are associated with extrapolating the observed effects in rodents to possible human exposure scenarios and in using the recommended inhalation ADI for other types of CNTs, other than the purified MWCNTs used in the principal study.

(1) *Subchronic-to-Chronic Extrapolation:* The recommended inhalation ADI is based on an assumption that the dose-response relationships for adverse lung effects in rats exposed to aerosols of MWCNTS are similar for subchronic and chronic durations of exposure. No data are available, however, to describe exposure-response relationships for lung effects in animals chronically exposed to aerosols made from any type of CNTs. Thus, any use of the recommended inhalation ADI to estimate risks for lung effects from chronic exposure has an inherent uncertainty, because it is based on subchronic-duration data.

(2) *Extrapolation to Other Types of CNTs:* Results from short-term intratracheal instillation and pharyngeal aspiration suggest that the acute inflammatory and persistent fibrotic lung responses to acute exposure to CNTs are a complex, as yet undefined, function of a number of factors including deposited dose, metal content, particle dimensions including length, degree of agglomeration of administered material, structural defects in the graphene framework, and presence and properties (e.g., water solubility) of functional groups added to the graphene framework. The influence of some of these factors is better known than others. For example, consistent results from acute pharyngeal aspiration, intratracheal instillation, and inhalation studies have demonstrated that metal contaminants in SWCNTs and MWCNTs can enhance pulmonary inflammatory responses (Porter et al., 2010; Ellinger-Ziegelbauer and Pauluhn, 2009; Shvedova et al 2008a, 2005; Lam et al., 2004). On the other hand, the complexity of the influence of CNT length on pulmonary inflammatory responses is illustrated by observations that inflammatory responses in rats following intratracheal instillation were stronger with shorter, ground MWCNTs (0.7 μm), compared with longer, nonground MWCNTs (5.9 μm), whereas the fibrotic response to the longer MWCNTs was stronger than that to the shorter MWCNTs, which were more rapidly cleared from the lungs (Muller et al., 2005). Similarly, well-dispersed SWCNTs were less potent than agglomerated SWCNTS in inducing pulmonary inflammation in a study of mice following intratracheal instillation (Mutlu et al., 2010), whereas, in another study following pharyngeal aspiration, both agglomerated and well-dispersed SWCNTs produced pulmonary inflammation but with a different pattern (localized for agglomerated and widely distributed for well-dispersed) (Mercer et al., 2008). The available data indicate that intratracheal instillation or pharyngeal aspiration of CNTs functionalized with water-soluble functional groups cause pulmonary effects qualitatively similar to those induced by

nonfunctionalized CNTs (Kagan et al., 2010; Wang et al., 2007; Carrero-Sanchez et al., 2006), although limited comparative data indicate that addition of water-soluble functional groups may increase the potential of CNTs to cause acute pulmonary responses (on a mass basis); it is uncertain if the increased toxic potency is due to increased surface charge or increased dispersability and wider distribution in lung tissues (Tong et al., 2009; Saxena et al., 2007 as cited by Tong et al., 2009).

The recommended inhalation ADI of 0.9 μg/m^3 for purified MWCNTs is based on NOAEL and LOAEL values of 0.1 and 0.4 mg/m^3 for histological and BAL signs of pulmonary inflammation following 13 weeks of exposure to aerosols of purified MWCNTs (Pauluhn 2010a; see Table 5.1 for characteristics of the test materials and the administered aerosols). In the other 13-week study (Ma-Hock et al., 2009), 0.1 mg/m^3 was a LOAEL for lung lesions (minimal diffuse histiocytosis) in Wistar rats exposed to aerosols of a MWCNT containing 10% aluminum oxide. It is possible that the high metal oxide content in the administered material may account for the lesions at the 0.1 mg/m^3 concentration, which did not occur in the rats exposed to the purified MWCNTs in the Pauluhn (2010a) study. Although it is possible that inhalation exposure to respirable aerosols of other types of CNTs may induce similar lung effects, it is likely that dose-response relationships may vary from those described in the principal study, based on results from the short-term intratracheal instillation and pharyngeal aspiration studies comparing CNTs with different physical and chemical characteristics. Thus, any use of the recommended inhalation ADI to estimate risks for lung effects from exposure to other types of CNTs (including SWCNTs) has inherent uncertainty.

(3) *Lung as the Critical (i.e., Most Sensitive) Toxicity Target of Inhaled CNTs:* The lung is clearly identified as a sensitive target of CNTs (MWCNTs and SWCNTs) in mice or rats from short-term and subchronic inhalation exposure and short-term intratracheal instillation or pharyngeal aspiration exposure. There are a few reports, however, that CNTs may target other tissues. Of particular interest are reports from acute pharyngeal aspiration studies of mice that provide evidence that SWCNTs can increase markers of cardiovascular disease in aortic tissue (Li et al., 2007b), increase expression of genes involved in inflammation, oxidative stress, and thrombosis in aortic tissue (Erdely et al., 2009), and lower cardiac functional recovery following ischemia (Tong et al., 2009); a report of immunosuppresson in mice exposed for 14 days to aerosols of MWCNTs by inhalation (Mitchell et al., 2007); and a report of neuroinflammation and cellular stress in certain brain regions in mice exposed to MWCNTs by inhalation for 12 days or by acute pharyngeal aspiration (Sriram et al., 2009). None of these endpoints were evaluated in the subchronic inhalation studies used as the basis for the inhalation ADI derivation.

Potential ADIs are not derived for CNT by oral or dermal exposure due to the lack of repeated-dose oral or dermal toxicity data for any types of CNTs.

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