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DOSSIER ON FULLERENES
- PART 2 -

Series on the Safety of Manufactured Nanomaterials
No. 48

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Series on the Safety of Manufactured Nanomaterials

No. 48

DOSSIER ON FULLERENES
- PART 2 -

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris, 2015

Dossiers also published in the Series on the Safety of Manufactured Nanomaterials:

No. 44, *Dossier on Gold nanoparticles (2015)*

No. 45, *Dossier on Cerium oxide (2015)*

No. 46, *Dossier on Dendrimers (2015)*

No. 47, *Dossier on Nanoclays (2015)*

No. 49, *Dossier on Multiwalled Carbon Nanotubes (MWCNTs) (2015)*

No. 50, *Dossier on Single-Walled Carbon Nanotubes (SWCNTs) (2015)*

No. 51, *Dossier on Silicon dioxide (2015)*

No. 52, *Dossier on Zinc oxide (2015)*

No. 53, *Dossier on Silver nanoparticles (2015)*

No. 54, *Dossier on Titanium dioxide (2015)*

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This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

The Inter-Organisation Programme for the Sound Management of Chemicals (IOMC) was established in 1995 following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. The Participating Organisations are FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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PREAMBLE

In November 2007, OECD's Working Party on Manufactured Nanomaterials (WPMN) launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The outcomes of the Testing Programme were intended to assess the applicability of the existing ***test guidelines***[1] to nanomaterials, as well as to provide useful information on any intrinsic properties of MNs, which are different from the same bulk material with greater external dimensions. Understanding the properties of NMs is crucial to choose appropriate strategies for hazard identification, risk assessment or risk management measures. The Testing Programme involved delegations from OECD member countries, some non-member economies and other stakeholders. The broad international representation, from a range of delegations enabled the programme to pool expertise and resources without which this programme would not have been possible.

Before launching the Testing Programme, the WPMN first identified a broad list of possible nanomaterials, and the list was later adjusted to a final selection of eleven MNs for testing[2]. This list comprised: i) fullerenes (C60); ii) single-walled carbon nanotubes (SWCNTs); iii) multi-walled carbon nanotubes (MWCNTs); iv) silver nanoparticles; v) titanium dioxide; vi) cerium oxide; vii) zinc oxide; viii) silicon dioxide; ix) dendrimers; x) nanoclays; and xi) gold nanoparticles. One fundamental criterion for selecting these materials was that they should be either in commercial use at the time or expected to be in the near future. At the same time, other considerations were also given attention, such as the production volume of the materials, the likely availability of such materials for testing and the existing information that would readily be available on the materials.

It was also agreed that 59 endpoints would be addressed[3] for each material corresponding to the following categories: i) nanomaterial information/ identification; ii) physical-chemical properties and material characterisation; iii) environmental fate; iv) toxicological and eco-toxicological effects; v) environmental toxicology; vi) mammalian toxicology; and vii) material safety. These endpoints were judged to be most important based largely on the general experience of testing chemicals, while taking into account the potentially different or new properties of nanomaterials. It is worth noticing that it was not expected that testing for all of the listed endpoints would be necessary for each of the selected MNs.

To assist with the Testing Programme, the WPMN developed two documents: i) a *Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials* [ENV/JM/MONO(2009)21]; and ii) *Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme* (Guidance Manual) in 2009, which was subsequently updated in 2010

[1] The OECD Test Guidelines are a collection of internationally agreed test methods used by government, industry and independent laboratories. They are used to determine the safety of chemicals.

http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

[2] Originally Iron nanoparticles, Aluminium, Carbon black, and Polystyrene were suggested but later withdrawn and replaced by gold nanoparticles.

[3] As specified in the Guidance Manual, "address" includes the term "completed" which provides that all dossiers will contain the identified endpoint information. Note that for some endpoints (for example, solubility) it is specified that the endpoint must be "completed". In such instances "completed" means that all Dossiers will be providing this endpoint information.

[ENV/JM/MONO(2009)20/REV][4]. The objective of this *Guidance Manual* was to guide sponsors[5] in the testing of the materials while ensuring that the information collected was reliable, accurate, consistent and therefore also comparable. The *Guidance Manual* addressed a whole range of issues including the organisation of the work.

The *Guidance Manual* contains detailed information on the selected endpoints for testing and recommendations on sample preparation and dosimetry.

The *Guidance Manual* also described the development of *Dossier Development Plans* (DDPs). These plans were prepared by Lead sponsors, Co-sponsors together with contributors to describe the specific plan for the testing of each nanomaterial including when and where the testing will be undertaken and by whom. The DDPs also included information on the materials to be tested as well as information on issues such as sample preparation and dosimetry. Each of the DDPs was prepared and reviewed by the WPMN before testing work began.

Based on the lessons learned during the Testing Programme, the WPMN also developed *Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials* [ENV/JM/MONO(2012)40]. This latter document is an update of an earlier text first published in 2010.

The work on OECD's Testing Programme was completed by the end of 2013. In June 2014 the WPMN agreed that for each nanomaterial the dataset would be published in IUCLID printed format[6] [7]. The document will include the protocols and methods to allow their wider use (regulators and researchers).

The dataset in this document has been declassified and made publicly available and it is expected regulators and researchers will wish to use it. Due to a broad dissemination of the data and the exploratory setting in which they were developed there are a number of limitations in using the data of which potential users should be aware. The programme focused on answering scientific questions in the field of the OECD test guidelines but not to provide conclusions on the hazard or risk of the materials selected. The absence of data for some endpoints may be a gap for some endpoints but for other end points there may not if the data was not considered necessary. Although the programme ensured a broad participation of many stakeholders it was not intended to arrive at any pre-defined regulatory datasets requirements or risk assessment decisions. It was recognised from the beginning that the exploratory nature of the work would require subsequent follow-up work for example to review the specific needs that may arise when performing risk assessment of nanomaterials. In this context, the programme's ultimate goal, to add to the knowledge of the properties of nanomaterials, would form a cornerstone.

[4] It is worth noting that while the *Guidance Manual for Sponsors* was primarily intended as a guide to WPMN's Testing Programme, it is also expected that it will be of value to anyone involved in testing NMs.

[5] The Guidance Manual noted, for example, that there could be three levels of participation to the programme. Lead sponsors, who would assume responsibility for conducting or coordinating all of the testing, determined to be appropriate for each of the endpoints for a specific nanomaterial. In some cases, "joint lead" arrangements were developed. Co-sponsors conducted some of the testing determined to be appropriate and feasible to address the endpoints for a specific listed nanomaterial. Contributors provided test data, reference or testing materials or other relevant information to the lead and co-sponsors.

[6] IUCLID is a software programme for the administration of data on chemical substances. Although it was originally developed to fulfill requirements in the EU for the evaluation and control of the risks of existing chemical substances, it is used by many others.

[7] SIAR = SIDS Initial Assessment Report (SIDS = Screening Information Data Set)

FOREWORD

As part of its Programme on the Safety of Manufactured Nanomaterials, OECD launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The Testing Programme mainly aimed to assess the applicability of the existing test guidelines to nanomaterials, as well as to provide useful information on any intrinsic properties of MNs, which are different from the same bulk material with greater external dimensions.

This document presents the Dossier of the carbon nanomaterials: Single-Walled Carbon Nanotubes (SWCNT), Multiwalled Carbon Nanotubes (MWCNT) and Fullerenes (C60), which was prepared under the leadership of Japan and the United States. This nanomaterial has been tested for a number of endpoints for: i) Nanomaterials Information / Identification; ii) Physical-Chemical Properties; iii) Environmental Fate; iv) Environmental Toxicology; and v) Mammalian Toxicologyy. They have been analysed using OECD Guidelines for the Testing of Chemicals (TG)[8]. The data is presented in an IUCLID[9] style format and includes the protocols and methods used (see Preamble).

Japan and the United States led the Testing Programme on carbon nanomaterials. This included the determination of the tests that were appropriate for carbon nanomaterials, performing a number of tests, as well as coordinating tests performed and inputs provided by other participating country and stakeholder, from Korea and the Business and Industry Advisory Committee to the OECD (BIAC).

Due to the large amount of information generated throughout the OECD Testing Programme on carbon nanomaterials, each dossier has been split into several parts, as follows:

- Single-Walled Carbon Nanotubes (SWCNT): 2 parts
- Multiwalled Carbon Nanotubes (MWCNT): 3 parts
- Fullerenes (C60): 2 parts

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

[8] http://www.oecd.org/env/testguidelines

[9] IUCLID is a software programme for the administration of data on chemical substances. It was originally developed to fulfil requirements in the EU for the evaluation and control of the risks of existing chemical substances. It is specifically relevant in the context of an international programme for the initial assessment of chemical substances.

ACKNOWLEDGMENTS

The OECD Secretariat and the WPMN is thankful to Japan and to the United Sates for leading the Testing Programme on Carbons, including Single-Walled Carbon Nanotubes (SWCNT), Multiwalled Carbon Nanotubes (MWCNT) and Fullerenes (C60). They are specifically grateful to Hiroyuki Hanawa from the Ministry of Economy, Trade and Industry of Japan, and to Philip Sayre from the Environment Protection Agency, USA. In addition, we appreciate the efforts made by other participating countries/ organisations, and in particular to those that coordinated the efforts within their respective delegations: Dr. Kyunghee CHOI from the Korean Ministry of Environment, as well as Dr. Daniel Bernard (Arkema, France) and Dr. Jacques Ragot (Bayer material Science, Germany) from the Business & Industry Advisory Committee to OECD (BIAC).

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PART 1

PART 2

7. TOXICOLOGICAL INFORMATION

7.1 Toxicokinetics, metabolism and distribution

7.1.1 Basic toxicokinetics

Endpoint study record: Basic toxicokinetics.001

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 2 (reliable with restrictions)

Rationale for reliability incl. deficiencies Acceptable, well-documented publication which meets basic scientific principles

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Shinohara, N. et al.	2010	Clearance kinetics of fullerene C60 nanoparticles from rat lungs after intratracheal C60 instillation and inhalation C60 exposure.	Toxicological Sciences 118 (2), 564-573					

Data access

data published

Materials and methods

Type of method

in vivo

Objective of study

distribution

excretion

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
yes

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom Purple
common name	Fullerene C60 nanoparticles

Test material form
nanomaterial

Radiolabelling
no

Details on test material
- Name of test material (as cited in study report): C60 nanoparticles
- Analytical purity: > 99.5 %
- Other: Supplier: Frontier Carbon Co., Ltd.

Test animals

Species
rat

Strain
Wistar

Sex
male

Details on test animals and environmental conditions
TEST ANIMALS
- Age at study initiation: 9 weeks-old

Administration / exposure

Route of administration
other: intratracheal or inhalation

Vehicle
other: Tween 80

Details on exposure
(Intratracheal instillation): C60 nanoparticles suspended in 0.1% Tween 80 solutions were intratrachally instilled at the doses of 0.1, 0.2 and 1 mg/rat.(Inhalation exposure): C60 nanoparticles suspended in 0.1% Tween 80 solutions were exposed to the rats at a concentration of 0.12 mg/m3 for 6 h/day and 5 days/week for 4 weeks.

Duration and frequency of treatment / exposure
(Intratracheal instillation) : single treatment(Inhalation exposure) : 6 hr/day and 5 days/week for 4 weeks The fullerenes concentrations in lungs, brain and liver are measured after a single intratracheal instillation up to 180 days after dosing.

Doses / concentrations
(Intratracheal instillation): 0.1, 0.2 and 1.0 mg/rat(Inhalation exposure): 0.12 ± 0.03 mg/m3

No. of animals per sex per dose
6 rats / dose / observation time point

Control animals
other: (Intratrachal instillation): 0.1% Tween 80; (Inhalation exposure): air only

Positive control
Not applicable

Details on dosing and sampling
PHARMACOKINETIC STUDY (distribution, excretion)
- Tissues sampled: lung (left), liver and brain
- Time and frequency of sampling: (Intratrachal instillation): collected at 18 h (except for the 0.1 mg group), 3 days (only in the case of the 1 mg group), 1week, 1month, 3 months and 6 months after the instillation; (Inhalation exposure): collected at 3 days and 1 month after the 4-week exposure period.
- Other:
- Analysis: The left lung, liver, and brain of five rats in each group were dissected, weighed and cut into small pieces with scissors. The pieces were homogenized with three times their weight of saline in an electric homogenizer (Polytron RT3100) at 10krpm and stored at -80°C until analysis. SDS (0.01M, 0.5 ml), acetic acid (0.1M, 0.5 mL), and toluene (HPLC grade, 5 ml) were added to 200 mg of homogenized tissue samples. The resultant mixture was shaken for 5 h in a shaker and sonicated for 15 min in ultrasonic bath. The mixture was then centrifuged at 200 X g for 10 min to separate the toluene solution, and the supernatant was collected in a test tube. The residue was shaken for 2 min with 2 ml of toluene, sonicated for 15 min, and separated by centrifugation; the supernatant was collected in a test tube. Then 1 ml of toluene was gently added to the residue, and the supernatant toluene was collected in a test tube twice. Approximately 14 ml of the extracted toluene solutions (supernatant) in the test tube were filtered with 0.2-μm filter to prevent the HPLC column from clogging by pieces of rat tissue, which are present at the water-toluene interface and concentrated to 0.20 ml with 0.4ml/min of N2 gas at 40°C by using a nitrogen concentration system (EVAN-SPE). The extracted and concentrated C60 and fullerene oxide (C60O) were analyzed using HPLC system (Shimadzu LC-10A system) equipped with a UV detector (wavelength, 333

nm). The mobile phase was 70% (v/v) toluene and 30% acetonitrile (v/v) with a flow rate of0.425 ml/min. The analyte (9.0 µl) was injected into a reverse phase triacontylsilyl silica (C30) packed column (particle size, 5 µm; internal diameter, 3.0 X 150 mm; Develosil RP Fullerene), which was maintained at 30°C.
- Observation by TEM:Lung tissues of the rats were observed through an energy-filtering TEM (EM922; Carl Zeiss SMT) equipped with an OMEGA energy filter. The specimen for TEM was prepared as follows. Lung tissues were fixed in an glutaraldehyde and osmium tetroxide solution, dehydrated in ethanol, and embedded in epoxy resin. Ultrathin specimens were cut using a diamond knife microtome. Some of the specimens were stained with 2% uranyl acetate solution and 0.5% lead citrate solution at room temperature.

Any other information on materials and methods incl. tables

Calculation for deposition fraction and clearance of C60:
The clearance of C60 particles from the lung was assumed to resemble a 2 -compartment model. Inhaled airborne particles are deposited on the alveolar surface, depending on their size. The amount of deposited particles is equivalent to the pulmonary burden in compartment 1. Two clearance pathways, namely, direct clearance from the lung (rapid clearance) and clearance via compartment 2 (slow clearance), were assumed to operate in this model. Particles deposited on the alveolar surface are mainly phagocytized by macrophages, transferred to the tracheobronchial region, and cleared via the sputum. Phagocytosis by alveolar macrophages is responsible for rapid clearance. Some of the deposited particles penetrate the epithelial cell barrier and slowly cleared from the lung via the lymph nodes. These pathways are responsible for slow clearance via compartment 2.

Results and discussions

Bioaccessibility

Any other information on results incl. tables

In the instillation experiment, TEM image of the alveolar macrophages in the lungs at one week after the instillation of 0.2 mg showed phagosome in alveolar macrophage, in which many black particles resembling fullerene were seen. These particles were identified as C60 particles because high-resolution images showed lattice plane of crystalline fullerene. From TEM image of the alveolar macrophage at 6 months after the instillation of 0.2 mg, it was difficult to see the fullerene particles in the alveolar macrophage, suggesting that most of fullerene particles were exhausted. In the inhalation exposure experiment, phagosome in alveolar macrophage including fullerene particles was also confirmed in the lungs of rats at 3 days after exposure in TEM observation.

Overall remarks, attachments

Remarks on results including tables and figures

After intratracheal instillation or inhalation exposure of each dose, lung burdens of C60 were examined. The results were as follows.

Table 1 Lung burdens of C60 after intratracheal instillation (µg per lung)

Dose	Time after instillation					
	18 hours	3 days	1 week	1 month	3 months	6 months
0.1 mg	-	-	70.8±14	26.5±15	5.22±0.5	2.86±2.0
0.2 mg	210±9.0	-	-	62.3±30	17.3±5.0	14.9±3.1
1.0 mg	1000±170	1000±160	1180±180	481±79	91.4±25	22.0±6.3

Values represent mean ± S.D.
-: Not measured
In this experiment, C60 was not detected in the liver and brain after the instillation (detection limit: 0.0089 µg / g tissue).

Table 2 Lung burdens of C60 after inhalation exposure (μg)

Dose	Time after last exposure (day)	
	3.0	30
0.12 mg/m3 exposure	9.92 ±2.2	5.36±1.2

Values represent mean ± S.D.

In this experiment, C60 was not detected in the liver and brain after the inhalation experiment (detection limit: 0.0089 μg / g tissue).

Table 3-Half life and 90% decay period calculated in the instillation experiment

Dose	0.1 mg	0.2 mg	1.0 mg
Half life (days)	15	17	28
90% decay period (days)	58	77	87

The deposition fraction of the C60 nanoparticles (diameter, 55 and 96 nm) were estimated to be 0.18 and 0.14, respectively.

Applicant's summary and conclusion

Conclusions

The C60 burden in the lungs, liver and brain of rats was determined after intratracheal instillation and inhalation. Pulmonary C60 burden decreased with time and depend on the C60 concentration administered. The concentration of C60 in the liver and brain was below the detection limit: 8.9 ng/g tissue. The half-life in the lung of intratracheally instilled C60 was 15-28 days. Mode evaluation revealed that most instilled particles could be eliminated by the fast clearance pathway

Endpoint study record: Basic toxicokinetics.002

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 2 (reliable with restrictions)

Rationale for reliability incl. deficiencies Acceptable, well-documented publication which meets basic scientific principles

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Kubota, R. et al.	2011	Time-dependent variation in the biodistribution of C60 in rats determined by liquid chromatography-tandem mass spectrometry	Toxicol. Lett., vol. 206, 172-177 (2011)					

Data access

data published

Materials and methods

Type of method
in vivo

Objective of study
distribution

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
yes

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom Purple
common name	C60

Test material form
nanomaterial

Radiolabelling
no

Details on test material
- Name of test material (as cited in study report): C60- Analytical purity: > 99.9 %- Other: Supplier: Frontier Carbon Co., Ltd.

Test animals

Species
rat

Strain
Wistar

Sex
male

Details on test animals and environmental conditions
TEST ANIMALS
- Source: Japan SLC, Inc.
- Age at study initiation: 6 weeks-old
- Diet (e.g. ad libitum): CRF-1 (Oriental Yeast Co., Ltd.), ad libitum
- Water (e.g. ad libitum): tap water, ad libitum
- Acclimation period: not stated.

ENVIRONMENTAL CONDITIONS
- Temperature (°C): not stated
- Humidity (%): not stated
- Air changes (per hr): not stated
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Administration / exposure

Route of administration
intravenous

Vehicle
other: liposome

Details on exposure
PREPARATION OF DOSING SOLUTIONS:
A dosing solution was prepared as follows. L-α-Phosphatidyl-choline (PC) and 3-sn-phosphatidyl L-lserine (PS) were dissolved in chloroform as 25 mg/mL stock solutions and were stored at -80°Cuntil use. C60 was dissolved in toluene as a 2.5 mg/mL stock solution and was stored at 4°C until use. A PC and PS mixture was prepared such that each compound ha a concentration of 0.5 mg/mL (PC: PS=1:1 (w/w)) in chloroform. 1 mg/mL C60 solution (mixture of toluene and chloroform) was prepared by diluting the 2.5 mg/mL C60 stock solution with a stream of nitrogen gas. After volatilization, 1 x PBS buffer (pH 7.4), an amount equivalent to the mixture of toluene and chloroform, was added, and the mixture was vortexed for a few seconds. The liposomes containing C60 were sonicated using a bath sonicator for 10-15 min at 60°C and were centrifuged at 1000 rpm for 10s, and the supernatant (at room temperature) was used for tail vein administration. The supernatant was given to rats immediately and the solution was sonicated using a bath sonicator before each treatment to rats.

VEHICLE
- Justification for use and choice of vehicle (if other than water): suitable to obtain a suspension for intravenous administration.
- Amount of vehicle (if gavage): 5 mL/kg

Duration and frequency of treatment / exposure
Tail vein injections. Once per day for four consecutive days.

Doses / concentrations

5 mg/kg bw/injection x 4 times. Total amount of C60 injected: ca. 929.1 µg

No. of animals per sex per dose

5 rats per each time point

Control animals

yes, concurrent vehicle

Positive control

Not applicable.

Details on dosing and sampling

PHARMACOKINETIC STUDY (Absorption, distribution, excretion)
- Tissues and body fluids sampled: Brain, kidneys, liver, lungs, spleen, and blood (taken from the heart). Collected tissues and blood were stored at -80 °C until analyzed.
- Time and frequency of sampling: on days 1, 7, 14 and 28 after completion of injections.
- Other:
- Sample preparation: Freshly harvested whole tissues were weighed and placed in polypropylene copolymer (PPCO) centrifuge tubes. Tissues were frozen at -80°C, and frozen tissues were freeze-dried over night. Each freeze-dried tissue was weighed and completely homogenized. In the case of small tissues (<0.5 g dry wt., brain, kidneys, spleen, and lungs), 0.2M SDS solution (1 mL) and acetic acid (1 mL) were added to the centrifuge tubes, and the centrifuge tubes were vortexed and sonicated using a bath sonicator. An internal standard solution (C70 toluene solution, 0.5 mL) and 3.5 mL toluene were added to the centrifuge tubes, and they were shaken for 5 h at room temperature in the dark. After shaking, the centrifuge tubes were centrifuged for 30 min at 3500 rpm. 1 mL of supernatant was removed and placed in glass vials to be used for analysis. In the case of blood samples, untreated whole blood (2 mL, taken from the heart) was used for the extraction. Because the dry tissue weight of the liver (ca. 3 g dry wt.) was heavier than other tissues, a six-fold amount of each solution was used for the liver extraction. The limit of quantification (LOQ) in analytical solution was determined by analyzing the lowest level standard at least 5 times. The LOQ was calculated as 10-fold the standard deviation of these determinations. The LOQ for each tissue was 0.026 µg/g wet wt. for liver, 0.026 µg/g wet wt. for kidneys, 0.09 µg/g wet wt. for spleen, 0.046 µg/g wet wt. for lungs, and 0.023 µg/g wet wt. for brain, respectively.
-Analysis: The extracted tissues and concentrated C60 and fullerene oxide (C60O) were analyzed using Waters Alliance 2695 HPLC system interfaced to to a Waters Micromass Quattro Micro API triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface. The mobile phase was 70% (v/v) toluene and 30% acetonitrile (v/v) with a flow rate of 1 ml/min. Fullerenes were separated using a Develosil RPFULLERENE column (5 µm; 4.6 mm X 250 mm) at 30°C (column oven temperature). The autosampler was kept at 10°C and the injection volume was 20 µL. The mass spectrometry was operated in the ACI negative ion mode with multiple reaction monitoring (MRM).

Statistics

Statistical analyses were performed using the program Excel Statistics. Kolmogorov-Smirnov's test showed that some variables were not normally distributed. Therefore, non-parametric test was used for statistical analysis. Kruskal-Wallis test was used for validation of difference in the C60 concentrations in four tissues among treated groups. Where appropriate, Mann-Whitney's U-test, Scheffe's test, or Student's t-test was conducted to verify the difference in the C60 concentration in four tissues among treated group.

Results and discussions

Pharmacokinetic studies

Details on distribution in tissues

C60 concentrations in tissues and blood of rats after tail vein injection were shown in Table 1.The highest C60 concentration was detected in the lungs, followed by spleen, liver, and kidneys. On the other hand, although C60 was detected in all of the brains from the Day 1 and Day 7 groups, C60 was detected in only one brain specimen from the Day 14 group and none of the specimens from Day 28 group. Moreover, no C60 was observed in blood samples from any of the groups.

Table 1 Concentration of C60 in five tissues and blood of Wistar rats (n=5)----------Concentration (μg/g wet wt.)Group ---------- Lungs Spleen Liver Kidneys Brain Blood----------Day 1 254±114 53.0±18.8 25.5±5.56 1.30±0.68 0.08±0.01 <0.020----------Day 7 199±88.4 45.3±10.7 22.6±9.63 0.42±0.11 0.04±0.01 <0.020---------- Day 14 109±51.5 59.0±14.4 21.9±4.94 0.20±0.02 0.04 (n=1)* <0.020---------- Day 28 133±47.5 70.6±32.0 14.8±3.00 0.16±0.04 <0.023 <0.020----------Values represent mean ± S.D. of 5 samples except for brain on day 14.*: The values in the remaining 4 samples were all <0.023.The time-dependent variation in the biodistribution of C60 in rats was examined from the four rat groups (Days 1, 7, 14, and 28). Although the number of samples for each group was small (n=5), a time-dependent decrease in C60 concentrations was observed in all tissues, except the spleen. A significant decrease in C60 concentration was found in the kidneys and brain. In the case of the kidneys, significant difference in C60 concentration from the four treated groups was found (Kruskal-Wallis, p=0.0007). C60 concentrations from the Day 14 group were significantly lower than those from the Day 1 group (Scheffe's test, p=0.0396). Moreover, C60 concentrations from the Day 28 group were also significantly lower than those of the Day 1 group (Scheffe's test, p=0.0024). In the case of the brain, C60 concentrations from the Day 7 group were significantly lower than those from the Day 1 group (Student's t-test, p=0.0002). Furthermore, C60 concentrations of the Day 14 and Day 28 groups were also lower than those of the Day 7 group. On the other hand, in the lungs and liver, the decreasing trend in C60 concentration was slower as compared with the trend in the kidneys and brain. In the case of the lungs, significant difference in C60 concentration from the four treated groups was found (Kruskal-Wallis test, p=0.0493) and C60 concentrations from the Day 14 group were significantly lower than those from the Day 1 group (Mann-Whitney's U-test, p=0.0163). In the case of the liver, significant difference in C60 concentration from the four treated groups was found (Kruskal-Wallis test, p=0.0251) and C60 concentrations from the Day 28 group were significantly lower than those from the Day 1 group (Scheffe's test, p=0.0298).

Bioaccessibility

Any other information on results incl. tables

The current study demonstrated that C60 after tail vein administration was widely distributed between various tissues, such as brain, kidneys, liver, lungs, and spleen of rats. Moreover, the large variability in C60 concentrations among tissues was found and the highest C60 concentration was observed in the lungs, followed by spleen, liver, kidneys and brain. These results suggested that C60 injected in the tail vein could be filtered by lung capillary vessels and accumulate in the lungs prior to being distributed to other tissues. Furthermore, C60 not being detected in the blood indicated that clearance of C60 from the blood by filtration might effectively occur in the lungs. A time-dependent decrease in C60 concentrations was observed in all tissues, except spleen. Moreover, a decreasing trend of C60 levels differed among tissues, which could be due to differences in accumulation. These result suggested that unmodified C60 and/or C60

metabolites by metabolic enzymes could be excreted into feces and/or urine.

Applicant's summary and conclusion

Conclusions

Biodistribution of C60 in rats after tail vein administration was examined using LC-MS/MS. C60 was detected in various tissues, such as brain, kidneys, liver, lungs, and spleen of rats. On the other hand, no C60 was found in blood. The highest C60 concentration was observed in the lungs, followed by spleen, liver, kidneys and brain. These results suggested that C60 injected in the tail vein could be filtered by lung capillary vessels and accumulate in the lungs prior to being distributed to other tissues. Furthermore, C60 not being detected in the blood indicated that clearance of C60 from the blood by filtration might effectively occur in the lungs. The time-dependent variation in the biodistribution of C60 was evaluated. A time-dependent decrease in C60 concentrations was observed in all tissues, except spleen. Moreover, a decreasing trend of C60 levels differed among tissues, which could be due to differences in accumulation. These result suggested that unmodified C60 and/or C60 metabolites by metabolic enzymes could be excreted into feces and/or urine.

Endpoint study record: Basic toxicokinetics.003

Administrative Data

Purpose flag supporting study

Study result type experimental result

Reliability 3 (not reliable)

Rationale for reliability incl. deficiencies Documentation insufficient for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
		2008		National Institute of Health Sciences					

Materials and methods

Type of method

in vivo

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test animals

Species
rat

Strain
other: Crl:CD(SD)

Sex
male

Administration / exposure

Route of administration
intratracheal

Duration and frequency of treatment / exposure
C60 dispersed with phospholipids at the concentration of 0.25, 0.5, 1.0 mg/ml were injected intratracheally. After administration with 0.2 ml/rat of the C60 solution, organs (lung, liver, spleen, kidney, brain and blood) were collected for analysis at days of 3, 7, 14 and 28

Doses / concentrations
0.2 ml/rat of the C60 solution/0.25, 0.5, 1.0 mg/ml

No. of animals per sex per dose
5 animals/dose

Details on dosing and sampling
PHARMACOKINETIC STUDY (Absorption, distribution, excretion)
- Tissues and body fluids sampled (delete / add / specify): lung, liver, spleen, kidney, brain and blood)
- Time and frequency of sampling: at days of 3, 7, 14 and 28 after completion of injections.

Results and discussions

Pharmacokinetic studies

Details on absorption
The concentrations of C60 in all organs except lung were below detection limit (0.5 μg/g (wet) tissue). The depositions of C60 in lungs were dose-dependent, and the concentrations were gradually decreased until day 28. In days 14 and 28 after injection, the concentrations in high dose group were significantly decreased.

Bioaccessibility

Any other information on results incl. tables

Table Concentrations of C60 fullerene in ling of SD rats

Dose	concentration (μg/g wet wt.)		
0.25 mg/ml	Day 0	Day 7	Day 28
	0.174	N.D.	N.D.
	N.D.	N.D.	N.D.
	N.D.	N.D.	N.D.
	N.D.	N.D.	N.D.
	0.741	0.345	N.D.
0.5 mg/ml	2.04	1.01	N.D.
	0.846	1.35	N.D.
	0.803	1.32	0.107
	0.992	1.63	0.59
	0.496	0.921	0.232
1 mg/ml	2.55	1.71	N.D.
	5.35	0.528	1.47
	3.07	4.25	1.47
	1.71	2.75	0.726
	2.04	4.65	0.541

N.D. :not dertermined.

7.1.2 Dermal absorption

7.2 Acute Toxicity

7.2.1 Acute toxicity: oral

Endpoint study record: Acute toxicity: oral.001

Administrative Data

Purpose flag supporting study

Study result type experimental result **Study period** 2008

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Matsumoto, K. et al.	2008	Final Report of Micronucleus Test of Fullerene In Mice (Japanese)		The Institute of Environmental Toxicology	IET 07-0092	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2008-02-18
publication	Shinohara, N. et al.	2009	In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles	Toxicol. Lett., vol. 191, 289-296 (2009)					

Data access

data published

Materials and methods

Test type

other:

Test guideline

Qualifier	Guideline	Deviations
according to	other guideline: OECD Guideline 474	

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom Purple
common name	Fullerene C60 nanoparticles

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): C60 nanoparticles
- Analytical purity: > 99.5%
- Storage condition of test material: stored in cold and dark place.
- Other: Supplier: Frontier Carbon Co., Ltd., Japan.

Test animals

Species

mouse

Strain

ICR

Sex

male

Details on test animals and environmental conditions

TEST ANIMALS
- Source: Charles River, Japan.
- Age at study initiation: 8 weeks-old (definitive study)
- Weight at study initiation: 31.7-41.4 g (definitive study)
- Assigned to test groups randomly: yes, under following basis: body weight
- Fasting period before study: fasted for 3 hours each before and after administration.
- Housing: The mice were housed in groups of 5 animals per cage in aluminum cages (215W x 330D x 180H mm) with wire-mesh floor.
- Diet (e.g. ad libitum): MF (Oriental Yeast Co.), ad libitum
- Water (e.g. ad libitum): tap water, ad libitum
- Acclimation period: 14 days (definitive study)

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 21.5-22.5 °C
- Humidity (%): 46.7-62.8 %
- Air changes (per hr): at least ten air changes per hour
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Administration / exposure

Route of administration

oral: gavage

Vehicle

other: Tween 80

Details on oral exposure

VEHICLE
- Concentration in vehicle: 0.1%

- Amount of vehicle (if gavage): 20 mL/kg
- Justification for choice of vehicle: To obtain a homogenous suspension since the test material was insoluble in water.
MAXIMUM DOSE VOLUME APPLIED:
20 mL/kg in each group

DOSAGE PREPARATION (if unusual):
The available highest concentration of C60 in 0.1% Tween 80 (vehicle) was 4.41 mg/mL. This suspension was used for the dosing solution for the high dose group. And the suspension was diluted with vehicle to give the dosing solutions for the middle and low dose groups.

Doses
22, 45 and 88 mg/kg bw; once a day for two consecutive days

No. of animals per sex per dose
5 animals per group

Control animals
yes

Results and discussions

Mortality
No deaths occurred in any dose group.

Clinical signs
No clinical signs of toxicity was observed in any dose group.

Overall remarks, attachments

Attached full study report

Applicant's summary and conclusion

Conclusions
It was concluded that twice oral administration up to 88 mg/kg of C60 nanoparticles resulted in no deaths or no abnormalities in male mice.

7.2.2 Acute toxicity: inhalation

7.2.3 Acute toxicity: dermal

7.2.4 Acute toxicity: other routes

7.3 Irritation / corrosion

7.3.1 Skin irritation / corrosion

Endpoint study record: Skin irritation / corrosion.001

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Matsuda, A. et al.	2010	Final report of the primary skin irritation test of fullerene in rabbits (Japanese)		Ina Research Inc.	ZT10140	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2010-08-24

Data access

data submitter is data owner

Materials and methods

Type of method

in vivo

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 404 (Acute Dermal Irritation / Corrosion)	

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
yes

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom Purple

Test material form
nanomaterial

Details on test material
- Name of test material (as cited in study report): fullerene C60- Physical state: black, powder, odorless- Analytical purity: >96%- Lot/batch No.: 8A0027-A- Storage condition of test material: stored in refrigerator

Test animals

Species
rabbit

Strain
New Zealand White

Details on test animals and environmental conditions
TEST ANIMALS
-source: Kitayama Labes Co., Ltd.
- Age at study initiation: 17weeks
- Weight at study initiation: 2.98-3.62 kg
- Housing: housed individually in aluminum cages(81Wx50Dx35H cm)
- Diet : ad libitum
- Water : ad libitum
- Acclimation period: 1 week

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 22.0-24.5°C
- Humidity (%): 43.4-62.3%
- Air changes (per hr): 17-23 times per hr
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Test system

Type of coverage
occlusive

Preparation of test site
shaved

Vehicle
other: olive oil

Amount/concentration applied
TEST MATERIAL
- Amount(s) applied (volume or weight with unit): 0.5g

VEHICLE
- Amount(s) applied (volume or weight with unit): 0.5 g
- Lot/batch no. (if required): 012W2225

Duration of treatment / exposure
The test substance was occlusively applied for 4 hours at the skin of the back with patch.

Observation period
At 1, 24, 48 and 72 hrs after removal of the patch, application sites were observed.

Number of animals
Three male rabbits

Control animals
no data

Details on study design
TEST SITE
- Area of exposure: patch (2.5 X 2.5 cm)
- Type of wrap if used: The test patch was covered with the gauze, overwrapped and fixed with bandage.

REMOVAL OF TEST SUBSTANCE
- Washing (if done): The test site was rinsed with lukewarm water.
- Time after start of exposure: 4 hours

SCORING SYSTEM:
Evaluations of skin reactions were scored as follows [OECD TG 404 (2002)]
- Erythema and Eschar Formation; 0 = No erythema, 1 = Very slight erythema (barely perceptible), 2 = Well defined erythema, 3 = Moderate to severe erythema, 4 = Severe erythema (beef redness) to eschar formation preventing grading of erythema- Oedema Formation; 0 = No oedema, 1 = Very slight oedema (barely perceptible), 2 = Slight oedema (edges of area well defined by definite raising), 3 = Moderate oedema (raised approximately 1 mm), 4 = Severe oedema (raised more than 1 mm and extending beyond

area of exposure)

Results and discussions

Irritation / corrosion results

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
overall irritation score	mean	24, 48 and 72 hrs after removal of patches	0	0		

Irritant/corrosive response data

No indicative of skin irritancy was observed for all test sites in all rabbits through the observation period.Primary irritation index (PII) was calculated as 0.0.

Any other information on results incl. tables

Primary skin irritation study of Fullerene in rabbits
Observation of skin

Animal No.	Skin signs	Score of skin reaction[a)]						
		First test			After removal of patches			
		3 min	1 h	4 h	60 min	24 h	48 h	72 h
TT1M01	Erythema and eschar formation	0	0	0	0	0	0	0
	Oedema formation	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
TT1M02	Erythema and eschar formation	-	-	-	0	0	0	0
	Oedema formation	-	-	-	0	0	0	0
	Total				0	0	0	0
TT1M03	Erythema and eschar formation	-	-	-	0	0	0	0
	Oedema formation	-	-	-	0	0	0	0
	Total				0	0	0	0
Mean					0.0	0.0	0. 0	0.0
P.I.I.[b)]						0. 0		

-: Not examined

a) Evaluations of skin reactions were scored as follows [OECD TG 404 (2002)]

Erythema and Eschar Formation;

0 = No erythema, 1 = Very slight erythema (barely perceptible), 2 = Well defined erythema,

3 = Moderate to severe erythema, 4 = Severe erythema (beef redness) to eschar formation preventing grading of erythema

Oedema Formation;

0 = No oedema, 1 = Very slight oedema (barely perceptible), 2 = Slight oedema (edges of area well defined by definite raising), 3 = Moderate oedema (raised approximately 1 mm), 4 = Severe oedema (raised more than 1 mm and extending beyond area of exposure)

b) P.I.I. (Primary Irritation Index) = (24 h mean scores + 48 h mean scores + 72 h mean scores) / 3

Criteria for primary skin irritation:

0 < P.I.I.≤2 = Mildly irritating, 2 < P.I.I.≤5 = Moderately irritating, 5 < P.I.I. = Severely irritating

When no skin reactions were observed in any application site during the observation period, the test formulation was judged to be "Not irritating".

Applicant's summary and conclusion

Interpretation of results

not irritating

Criteria used for interpretation of results
expert judgment

Conclusions
No indicative of skin irritancy was observed for all test sites in all rabbits through the observation period.Since PII was 0.0, it was concluded that fullerene was classified as not irritating.

Endpoint study record: Skin irritation / corrosion.002

Administrative Data
Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Kawabe, M. et al.	2011	Final report of skin irritation study of fullerene in rabbits (Japanese)		DIMS Institute of Medical Science, Inc.	11104	National Institute of Health Sciences (NIHS), Japan		2011-03-21

Data access
data submitter is data owner

Materials and methods

Type of method
in vitro

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 404 (Acute Dermal Irritation / Corrosion)	

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
yes

Test material identity

Identifier	Identity
CAS number	99685-96-8
common name	Fullerene nanom purple SU

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): Fullerene
- Physical state: black powder
- Analytical purity: 99.9%
- Lot/batch No.: 10B 0130-A
- Storage condition of test material: stored under room temperature and dark.
- Other: Supplier: Frontier Carbon Co.

Test animals

Species

rabbit

Strain

other: JW

Details on test animals and environmental conditions

TEST ANIMALS
- Source: Kitayama Labes Co., Ltd.
- Age at study initiation: 10 weeks old.
- Weight at study initiation: 2.058-2.112 kg
- Housing: housed individually in metal cage (W350xD500xH350 mm)
- Diet (e.g. ad libitum): ad libitum
- Water (e.g. ad libitum): ad libitum
- Acclimation period: 6 days

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 21.0-23.0 °C
- Humidity (%): 48-58 %
- Air changes (per hr): more than ten times per hr
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Test system

Type of coverage

occlusive

Preparation of test site
shaved

Vehicle
other: 5% gum arabic solution

Amount/concentration applied
TEST MATERIAL
- Amount(s) applied (volume or weight with unit): 0.02 g with 0.2 ml of vehicle (direct application) or 0.5 ml of 20% suspension
- Concentration (if solution): 20% (suspension)

VEHICLE
- Amount(s) applied (volume or weight with unit): 0.2 ml (direct application)
- Concentration (if solution): 5%

Duration of treatment / exposure
The test substance was occlusively applied for 4 hours at the back skin of the animals.Three application sites assigned in each animal included the patches as follows: control (vehicle only), direct application and suspension of fullerene.

Observation period
At 1, 24, 48 and 72 hrs after removal of the patch, application sites were observed.

Number of animals
Three male animals

Control animals
yes, concurrent vehicle

Details on study design
TEST SITE
- Area of exposure: patch (ca. 2.5x2.5 cm2)
- Type of wrap if used: The test patch was covered with the gauze, overwrapped and fixed with bandage.

REMOVAL OF TEST SUBSTANCE
- Washing (if done): The remaining substance was cleaned by absorbent cotton moistened with 5% gum arabic solution.
- Time after start of exposure: 4 hrs after start of exposure

SCORING SYSTEM:
Evaluations of skin reactions were scored as follows [OECD TG 404 (2002)]
- Erythema and Eschar Formation; 0 = No erythema, 1 = Very slight erythema (barely perceptible), 2 = Well defined erythema, 3 = Moderate to severe erythema, 4 = Severe erythema (beef redness) to eschar formation preventing grading of erythema- Oedema Formation; 0 = No oedema, 1 = Very slight oedema (barely perceptible), 2 = Slight oedema (edges of area well defined by definite raising), 3 = Moderate oedema (raised approximately 1 mm), 4 = Severe oedema (raised more than 1 mm and extending beyond

area of exposure)

Results and discussions

Irritation / corrosion results

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
overall irritation score	mean	1, 24. 48 and 72 hrs after exposure	0	0		

Irritant/corrosive response data

In either exposure method of direct application or suspension, fullerene did not cause both erythema and oedema during 72 hours after exposure. Primary irritation index (PII) was, thus, calculates as 0.0.

Any other information on results incl. tables

Table 1 Skin irritation study of fullerene in rabbits (The test site observation data)

TEST CHEMICAL	ANIMAL	Findings	Time after removal				Totalscore	Averagescore	Irritationscore	IrritationIndex	
			1 h	24 h	48 h	72 h					
5% Gum arabic	11104001	Er.[a]	0	0	0	0	0	0.0	0.0	0.0	
		Ed.[b]	0	0	0	0					
	11104002	Er.	0	0	0	0	0	0.0			
		Ed.	0	0	0	0					
	11104003	Er.	0	0	0	0	0	0.0			
		Ed.	0	0	0	0					
0.02g Fullerene	11104001	Er.	0	0	0	0	0	0.0	0.0	0.0	

		Ed.	0	0	0	0					
	11104002	Er.	0	0	0	0	0	0.0			
		Ed.	0	0	0	0					
	11104003	Er.	0	0	0	0	0	0.0			
		Ed.	0	0	0	0					
20% Fullerene	11104001	Er.	0	0	0	0	0	0.0	0.0	0.0	
		Ed.	0	0	0	0					
	11104002	Er.	0	0	0	0	0	0.0			
		Ed.	0	0	0	0					
	11104003	Er.	0	0	0	0	0	0.0			
		Ed.	0	0	0	0					

a)Erythema and Eschar Formation;

0 = No erythema, 1 = Very slight erythema (barely perceptible), 2 = Well defined erythema,

3 = Moderate to severe erythema, 4 = Severe erythema (beef redness) to eschar formation preventing grading of erythema

b)Oedema Formation;

0 = No oedema, 1 = Very slight oedema (barely perceptible), 2 = Slight oedema (edges of area well defined by definite raising), 3 = Moderate oedema (raised approximately 1 mm), 4 = Severe oedema (raised more than 1 mm and extending beyond area of exposure)

Applicant's summary and conclusion

Interpretation of results
not irritating

Criteria used for interpretation of results
expert judgment

Conclusions
In either exposure method of direct application or suspension, fullerene did not cause both erythema and oedema in rabbits during 72 hours after exposure. Primary irritation index (PII) was, thus, calculates as 0.0. It was concluded that fullerene was classified as not irritating.

7.3.2 Eye irritation

Endpoint study record: Eye irritation.001

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Matsuda, A. et al.	2010	Final report of the eye irritation test of fullerene in rabbits (Japanese)		Ina Research Inc.	ZT10141	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2010-08-24

Data access
data submitter is data owner

Materials and methods

Type of method
in vivo

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 405 (Acute Eye Irritation / Corrosion)	

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom Purple

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): fullerene C60
- Physical state: black, powder, odorless
- Analytical purity: >96%
- Lot/batch No.: 8A0027-A
- Storage condition of test material: stored in refrigerator

Test animals

Species

rabbit

Strain

New Zealand White

Details on test animals and environmental conditions

TEST ANIMALS
- Source: Kitayama Labes Co., Ltd.
- Age at study initiation: 11 weeks-old
- Weight at study initiation: 2.31-2.51 kg
- Housing: housed individually in aluminum cages (81Wx50Dx35H cm)
- Diet: ad libitum
- Water: ad libitum
- Acclimation period: 1 week

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 22.0-24.5°C
- Humidity (%): 43.4-62.3%
- Air changes (per hr): 17-23 times per hour
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Test system

Vehicle
unchanged (no vehicle)

Amount/concentration applied
TEST MATERIAL
- Amount(s) applied (volume or weight with unit): 0.1 g of the test material

Duration of treatment / exposure
0.1 g of the test material was applied in conjunctiva sac of the left eye and the eyelid was gently closed for one second. The right eye was untreated and served as a control.

Observation period
At 1, 24, 48 and 72 hours after treatment, the irritating reactions of the eyes were observed.

Number of animals
3 male rabbits

Control animals
other: Untreated right eye of each animal

Details on study design
REMOVAL OF TEST SUBSTANCE
- Washing (if done): rinsed the eye with lukewarm saline for around one minute.
- Time after start of exposure: 1 hour after exposure

SCORING SYSTEM:
Evaluations of ocular reactions were scored as follows:
- Cornea: Opacity: degree of density; 0 = No ulceration or opacity, 1 = Scattered or diffuse areas of opacity (other than slight dulling of normal lustre); details of iris clearly visible,2 = Easily discernible translucent area; details of iris slightly obscured, 3 = Nacrous area; no details of iris visible; size of pupil barely discernible,4 = Opaque cornea; iris not discernible through the opacity
- Conjunctivae: Redness; 0 = Normal, 1 = Some blood vessels hyperaemic (injected), 2 = Diffuse, crimson colour; individual vessels not easily discernible, 3 = Diffuse beefy red- Iris: 0 = Normal, 1 = Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia; or injection; iris reactive to light (a sluggish reaction is considered to be an effect), 2 = Hemorrhage, gross destruction, or no reaction to light- Chemosis: Swelling; 0 = Normal, 1 = Some swelling above norma, 2 = Obvious swelling, with partial eversion of lids, 3 = Swelling, with lids about half closed, 4 = Swelling, with lids more than half closed

TOOL USED TO ASSESS SCORE:
hand-slit lamp / fluorescein

Results and discussions

Overall irritation / corrosion results

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
conjunctivae score	mean	1 hr after exposure	1	1	fully reversible within: 24 hrs	

Irritant/corrosive response data

At 1 hr after exposure, redness of conjunctivae (score = 1) was observed in the treated eyes of all three rabbits, but recovered within 24 hrs. Besides, no abnormality was found in each animal throughout the observation period.

Any other information on results incl. tables

Table 1 A Primary Eye Irritation Study of Fullerene in Rabbits
Observation of Eyes
Gross observation - Scores of ocular irritation, Grading of ocular lesions (OECD TG 405 (2002))

Animal No.	Parameter	At animal selection		Post-dosing								
				1 h		24 h		48 h		72 h		
		R	L	R	L	R	L	R	L	R	L	
UT1M01	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0	
	Conjunctivae, Redness	0	0	0	1	0	0	0	0	0	0	
	Iris	0	0	0	0	0	0	0	0	0	0	
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0	
UT1M02	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0	
	Conjunctivae Redness	0	0	0	1	0	0	0	0	0	0	
	Iris	0	0	0	0	0	0	0	0	0	0	
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0	
UT1M03	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0	
	Conjunctivae Redness	0	0	0	1	0	0	0	0	0	0	
	Iris	0	0	0	0	0	0	0	0	0	0	
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0	
Mean	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0	
	Conjunctivae Redness	0	0	0	1	0	0	0	0	0	0	
	Iris	0	0	0	0	0	0	0	0	0	0	
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0	

R: Right eye (untreated) L: Left eye (Fullerene)
Evaluations of ocular reactions were scored as follows:
Cornea:
Opacity: degree of density; 0 = No ulceration or opacity, 1 = Scattered or diffuse areas of opacity (other than slight dulling of normal lustre); details of iris clearly visible,2 = Easily discernible translucent area; details of iris slightly obscured, 3 = Nacrous area; no details of iris visible; size of pupil barely discernible,4 = Opaque cornea; iris not discernible through the opacity
Conjunctivae:
Redness; 0 =, 1 = Some blood vessels hyperaemic (injected), 2 = Diffuse, crimson colour; individual vessels not easily discernible, 3 = Diffuse beefy red

Iris: 0 =, 1 = Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia; or injection; iris reactive to light (a sluggish reaction is considered to be an effect),
2 = Hemorrhage, gross destruction, or no reaction to light
Chemosis:
Swelling; 0 =, 1 = Some swelling above norma, 2 = Obvious swelling, with partial eversion of lids,
3 = Swelling, with lids about half closed, 4 = Swelling, with lids more than half closed

Table 2 A Primary Eye Irritation Study of Fullerene in Rabbits
Observation of Eyes
Corneal staining - Scores of corneal epithelium damage, McDonald-Shadduck scoring system -

Animal No.	Parameter	At animal selection		Approximately 72 hours post-dosing	
		R	L	R	L
UT1M01	Fluorescein staining	0	0	0	0
UT1M02	Fluorescein staining	0	0	0	0
UT1M03	Fluorescein staining	0	0	0	0
Mean	Fluorescein staining	0	0	0	0

R: Right eye (untreated) L: Left eye (Fullerene)

Applicant's summary and conclusion

Interpretation of results
slightly irritating

Criteria used for interpretation of results
expert judgment

Conclusions
At 1 hr after exposure, redness of conjunctivae (score = 1) was observed in the treated eyes of all three rabbits, but recovered within 24 hrs. No other changes were observed in any animals during the observation period. As a result, it was concluded that irritability of fullerene to the rabbit's eye is very weak.

7.4 Sensitisation

7.4.1 Skin sensitisation

Endpoint study record: Skin sensitisation.001

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Matsuda, A. et al.	2010	Final report of the skin sensitization test of fullerene in guinea pig (Japanese)		Ina Research Inc.	ZT10142	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2010-08-24

Data access

data submitter is data owner

Materials and methods

Type of method

in vivo

Type of study

Buehler test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 406 (Skin Sensitisation)	

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom Purple

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): fullerene C60
- Physical state: black, powder, odorless
- Analytical purity: >96%
- Lot/batch No.: 8A0027-A

- Storage condition of test material: stored in refrigerator

Test animals

Species
guinea pig

Strain
Hartley

Sex
male

Details on test animals and environmental conditions
TEST ANIMALS
- Source: Japan SLC, Inc.
- Age at study initiation: 5-6 weeks-old (main test)
- Weight at study initiation: 324-418 g (main test)
- Housing: housed individually in a stainless wire-meshed cage (61Wx22Dx21H cm)
- Diet: ad libitum
- Water: ad libitum
- Acclimation period: 1 week

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 22.7-23.2 °C
- Humidity (%): 47.9-65.1 %
- Air changes (per hr): 15-17 times per hour
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Test system

Traditional sensitisation test

Route of induction exposure
epicutaneous, occlusive

Route of challenge exposure
epicutaneous, occlusive

Vehicle
olive oil (challenge: vaseline)

Concentration
induction: 10%challenge: 10%

No. of animals per dose

10 for negative control, 20 for test substance-treated group and 10 for positive control

Details on study design (Traditional tests)

RANGE FINDING TESTS:
Concentration levels of 1, 5 and 10% of the test material in olive oil and vaseline were tested preliminarily. As a result, maximum applicable and non-irritating level of 10% was selected as concentrations of both induction and challenge in main study.

MAIN STUDY
A. INDUCTION EXPOSURE
- No. of exposures: 3(three times)
- Exposure period: 6 hours
- Test groups: 0.4 ml of 10% fullerene in olive oil
- Control group: 0.4 ml of saline
- Site: left flank of the animals
- Frequency of applications: 3 times for 14days (days 0, 7 and 14)
- Duration: 14days
- Concentrations: 10%

B. CHALLENGE EXPOSURE
- No. of exposures: 1(once)
- Day(s) of challenge: 2 weeks after the last induction (day 28)
- Exposure period: 6 hours
- Test groups: 0.2 g of vasseline (10% fullerene)
- Control group: 0.2 g of vasseline (10% fullerene)
- Site: left or right flank of the animals
- Concentrations: 10%
- Evaluation (hr after challenge): 24 and 48 hrs after challenge

OTHER:
Evaluation method: according to Magnusson and Kligman
Evaluations of skin reactions were scored as follows (Magnusson B. and Kligman A.M. system):
0 = No visible change,
1 = Discrete or patchy erythema,
2 = Moderate and confluent erythema,
3 = Intense erythema and swelling

Positive control substance(s)

yes (0.1% DNCB)

Results and discussion

Positive control results

In positive control group, at 24 and 48 hrs after the challenge by 0.1% DNCB, strong erythema and swelling (score=3) was observed in all ten animals. Mean score was 3.0 and incidence was 100% in each time point.

Traditional sensitisation test

Results of test (except LLNA)

Reading	Hours after challenge	Group	Dose level	No. with + reactions	Total no. in group	Clinical observations
1st reading	24	test group	10%	0	20	No abnormalities
2nd reading	48	test group	10%	0	20	No abnormalities
1st reading	24	positive control	0.1%	10	10	No abnormalities
2nd reading	48	positive control	0.1%	10	10	No abnormalities

LLNA

Any other information on results incl. tables

Table 1 A Skin Sensitization Study of Fullerenein Guinea Pigs (Buehler Test)
Skin Sensitization Reactions

Group	Substance for induction	Substance for challenge	Number ofanimals	Time after the end of challenge (h)	Mean of score	Positive ratio[a)]
Negative control	Physiological saline	Petrolatum	10	24	0.0	
				48	0.0	
		10%Fullerene[d)]	10	24	0.0	
				48	0.0	
Test article	10%Fullerene[b)]	Petrolatum	20	24	0.0	0/20
				48	0.0	0/20
		10%Fullerene[d)]	20	24	0.0	0/20
				48	0.0	0/20
Positive control	1% DNCB[c)]	Acetone	10	24	0.0	0/10
				48	0.0	0/10
		0.1% DNCB[e)]	10	24	3.0	10/10
				48	3.0	10/10

a) Number of animals with positive / Number of animals tested

b) Vehicle: Olive oil c) Vehicle: 70 vol% ethanol d) Vehicle: Petrolatum e) Vehicle: Acetone

Table 2 A Skin Sensitization Study of Fullerenein Guinea Pigs (Buehler Test)
Clinical Observations (From the first sensitization (Day 0) to the final observation (Day 30)

Group	Number of animals	Finding
Negative control	10	No abnormalities
Test articles	20	No abnormalities
Positive control	10	No abnormalities

Applicant's summary and conclusion

Interpretation of results

not sensitising

Criteria used for interpretation of results

expert judgment

Conclusions
Based on the result of this study, it was concluded that 10% fullerene did not elicit skin sensitization in guinea pigs.

Endpoint study record: Skin sensitisation.002

Administrative Data
Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Kawabe, M. et al.	2011	Final report of skin sensitization study of fullerene in guinea pigs (Buehler test) (Japanese)		DIMS Institute of Medical Science, Inc.	11105	National Institute of Health Sciences (NIHS), Japan		2011-02-27

Data access
data submitter is data owner

Materials and methods

Type of method
in vivo

Type of study
Buehler test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 406 (Skin Sensitisation)	

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	99685-96-8
common name	Fullerene nanom purple SU

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): Fullerene
- Physical state: black powder
- Analytical purity: 99.9%
- Impurities (identity and concentrations):
- Composition of test material, percentage of components:
- Isomers composition:
- Purity test date:
- Lot/batch No.: 10B 0130-A
- Storage condition of test material: stored under room temperature and dark.
- Other: Supplier: Frontier Carbon Co.

Test animals

Species

guinea pig

Strain

Hartley

Sex

male

Details on test animals and environmental conditions

TEST ANIMALS
- Source: Japan SLC, Inc.
- Age at study initiation: 7 weeks-old
- Weight at study initiation:
- Housing: housed in metal cage (W300×D450×H200 mm)
- Diet (e.g. ad libitum): ad libitum
- Water (e.g. ad libitum): ad libitum
- Acclimation period: 11 days

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 21.0-23.0 °C
- Humidity (%): 48-60 %
- Air changes (per hr): more than 10 times per hour
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Test system

Traditional sensitisation test

Route of induction exposure
epicutaneous, occlusive

Route of challenge exposure
epicutaneous, occlusive

Vehicle
other: 5% gum arabic

Concentration
induction: 20%challenge: 20%

No. of animals per dose
10 animals for negative control group; 20 animals for test group

Details on study design (Traditional tests)
RANGE FINDING TESTS:
Concentration levels of 2.5, 5, 10 and 20% of the test material in 5% gum arabic were tested preliminarily. As a result, maximum applicable and non-irritating level of 20% was selected as concentrations of both induction and challenge in the main study.

MAIN STUDY
A. INDUCTION EXPOSURE
- No. of exposures: 3 (three times)
- Exposure period: 6 hours
- Test groups: 0.2 ml of 20% fullerene in 5% gum arabic solution
- Control group: 0.2 ml of 5% gum arabic solution- Site: left flank of the animals
- Frequency of applications: 3 times per 14 days (on day 0, 7 and 14)
- Duration: 14 days- Concentrations: 20%B.

CHALLENGE EXPOSURE
- No. of exposures: once- Day(s) of challenge: 14 days after the last induction exposure
- Exposure period: 6 hours- Test groups: 0.2 ml of 20% fullerene in 5% gum arabic solution
- Control group: 0.2 ml of 20% fullerene in 5% gum arabic solution
- Site: right flank of the animals
- Concentrations: 20%
- Evaluation (hr after challenge): At 24 hrs and 48 hrs after removal of the occlusive patches

OTHER:
- Evaluation score:
0= No visible change;
1= Discrete or patchy erythema;
2= Moderate and confluent erythema;
3= Intense erythema and swelling

Positive control substance(s)
yes 2,4-dinitrochlorobenze (DNCB)

Results and discussion

Positive control results
The positive control test was conducted separately from the main study of fullerene. In the positive control test, mean score and positive rate were 1.6 and 100% at 24 hrs, and 1.2 and 100% at 48 hrs after removal of the closed patch, respectively.

Traditional sensitisation test

Results of test (except LLNA)

Reading	Hours after challenge	Group	Dose level	No. with + reactions	Total no. in group	Clinical observations
1st reading	24	negative control	20% fullerene	0	10	
2nd reading	48	negative control	20% fullerene	0	10	
1st reading	24	test group	20% fullerene	0	20	
2nd reading	48	test group	20% fullerene	0	20	

LLNA

Any other information on results incl. tables

Table 1 The results of skin sensitization study of fullerene in guinea pigs

Group	Sensitization	Challenge	No. of animals	Challenge			
				Time after removal of the closed patch			
				24h		48h	
				Mean score	Positive rate (%)	Mean score	Positive rate (%)
Negative control	5% Gum arabic	20% Fullerene	10	0.0	0	0.0	0
Test materialsensitized	20% Fullerene	20% Fullerene	20	0.0	0	0.0	0

Mean score = Sum (score) / number of animals
Positive rate = Number of animals with skin reactions / total number of animals x 100

Applicant's summary and conclusion

Interpretation of results

not sensitising

Criteria used for interpretation of results

expert judgment

Conclusions

Based on the results of this study, it was concluded that the test substance is not sensitising.

7.4.2 Respiratory sensitisation

7.5 Repeated dose toxicity

7.5.1 Repeated dose toxicity: oral

Endpoint study record: Repeated dose toxicity: oral.001

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Doi, Y. et al.	2011	Final report of 28-day repeated dose toxicity study and recovery study of Fullerene in rats (Japanese)		DIMS Institute of Medical Science, Inc.	1029	National Institute of Health Sciences (NIHS), Japan		2011-03-22
publication	Takahashi, M. et al.	2012	Sub-acute oral toxicity study with fullerene C60 in rats.	J. Toxicol. Sci., 37(2), 353-361.					

Data access

data published

Materials and methods

Test type

subacute

Limit test

no

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 407 (Repeated Dose 28-Day Oral Toxicity in Rodents)	

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
yes

Test material identity

Identifier	Identity
CAS number	99685-96-8
common name	Fullerene C60 (Nanom Puple SU)

Test material form
nanomaterial

Details on test material
- Name of test material (as cited in study report): Fullerene C60
- Physical state: black powder
- Analytical purity: 99.9%
- Lot/batch No.: 10B 0098-A
- Storage condition of test material: stored under room temperature and dark.
- Other: Supplier: Frontier Carbon Co.

Test animals

Species
rat

Strain
other: Crl:CD(SD)

Sex
male/female

Details on test animals and environmental conditions
TEST ANIMALS
- Source: Charles River Japan, Inc.
- Age at study initiation: 5 weeks-old
- Weight at study initiation: male (151-169 g), female (112-128 g)
- Housing: The animals were housed one or two of the same sex per cage in plastic cages with stainless steel covers.
- Diet: MF (Oriental Yeast Co., Ltd.), ad libitum
- Water: tap water, ad libitum
- Acclimation period: 7 days

ENVIRONMENTAL CONDITIOMS
- Temperature (°C): 20.0-22.5 °C
- Humidity (%): 48-62 %
- Air changes (per hr): at least 10 air changes per hour
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Administration / exposure

Route of administration
oral: gavage

Vehicle
corn oil

Details on oral exposure
PREPARATION OF DOSING SOLUTIONS:
Fullerene C60 was weighed for each dosing level (0.1, 1, 10, 100 mg/mL) and the vehicle was added. Each dosing solution including that for the vehicle control was sonicated 3 times (for 5 min each) in a beaker cooled with ice. Sonication was performed at 5- to 10-min intervals after it was confirmed that the solution was sufficiently cool. The dosing solutions were prepared from 1 p.m. to 5 p.m. on the day before each administration day, and mixed using a stirrer at room temperature in a dark place until just before administration. For each dosing sample collected from the upper, central and lower parts of the glass container were observed and photographed under an optical microscope on the first day and last day of the administration period. All doses, even the lowest dose of 0.1 mg/mL, did not completely dissolve in corn oil, and included visible and invisible residues which could be seen at 40 X magnification, although the authors assumed the lowest dose as completely soluble. Photographs of samples of each dosage looked similar between the first day and the last day.

VEHICLE
- Amount of vehicle (if gavage): 10 mL/kg bw
- Lot/batch no. (if required): V0F3051, V0N4226

Analytical verification of doses or concentrations
no

Duration of treatment / exposure
Exposure period was 29 days by gavage.

Frequency of treatment
once daily

Doses/concentrations
0, 1, 10, 100, 1000 mg/kg bw/day

Basis actual ingested

No. of animals per sex per dose
10 animals/sex/dose for 0 and 1000 mg/kg bw/day (5 animals/sex/dose were used as the recovery group) 5

animals/sex/dose for 1, 10 and 100 mg/kg bw/day

Control animals

yes, concurrent vehicle

Details on study design

- Dose selection rationale: The dosage levels were determined based on the guideline and the maximum dose was 1000 mg/kg bw/day. The lowest dose was set at 1 mg/kg bw/day (concentration of the dosing solution: 0.1 mg/mL), at which concentration fullerene C60 was expected to be dissolved. The intermediate doses were selected as 100 and 10 mg/kg bw/day with a proportional factor of 10.
- Post-exposure recovery period: 14 days

Positive control

Not applicable.

Examinations

Observations and examinations performed and frequency

CAGE SIDE OBSERVATIONS: Yes
- Time schedule: Observed for all animals at least once daily for clinical signs of toxicity and twice daily for mortality.

DETAILED CLINICAL OBSERVATIONS: Yes
- Time schedule: Before administration and once a week during the administration period.
- Detailed clinical observations checked included as follows: general clinical signs, handling reactivity, and open field observations (exploration, gait, behaviour, posture, fur, twitch, convulsion, tremor, rearing, defecation, urination).

BODY WEIGHT:
- Time schedule for examinations: recoded on days 0, 7, 14, 21, and 28 of the administration period and on days 6 and 13 of recovery period.

FOOD CONSUMPTION AND COMPOUND INTAKE (if feeding study):
- Food consumption for each animal determined: Yes
- Time schedule for examinations: once a week during the administration and recovery periods.

FOOD EFFICIENCY: No

WATER CONSUMPTION AND COMPOUND INTAKE (if drinking water study): No

OPHTHALMOSCOPIC EXAMINATION: No

HAEMATOLOGY: Yes
- Time schedule for collection of blood: Prior to necropsy at the end of the administration and recovery periods, blood was collected from the abdominal aorta.
- Anaesthetic used for blood collection: Yes (ether anesthesia)
- Animals fasted: Yes
- How many animals: All animals
- Parameters examined: RBC count, Hemoglobin, Hematocrit, WBC count, Platelet count, Differential

leucocyte count, Prothrombin time(PT) and Activated partial thromboplastin time(APTT)

CLINICAL CHEMISTRY: Yes
- Time schedule for collection of blood: same as haematological examinations.
- Animals fasted: Yes-
- How many animals: All animals
- Parameters examined: Total protein, Albumin, Albumin-globulin (A/G) ratio, Glucose, Total cholesterol, Triglycerides, Total bilirubin, Urea nitrogen, Creatinine, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Gamma-glutamyl transpeptidase (GGTP), Lactate dehydrogenase (LDH), Phospholipid, Calcium, Inorganic phosphorus, Sodium, Potassium, Chlorine.

URINALYSIS: Yes
- Time schedule for collection of urine: One day in the fourth week of the administration period, urine was collected for 4 hours. Fresh urine was collected for urine pH on the same day.
- Metabolism cages used for collection of urine: No, but all the animals were housed in urine-collecting rack.
- Animals fasted: No
- Parameters checked in table [No.?] were examined.

NEUROBEHAVIOURAL EXAMINATION: Yes
- Time schedule for examinations: Once during the fourth week of treatment- Dose groups that were examined: All groups- Battery of functions tested: sensory activity (reactivity to sensory stimulation: visual, auditory, tactile, and nociceptive; cranial nerve reflexes: palpebral reflex, pinna reflex, and papillary reflex; spinal reflexes: flexor reflex and extensor thrust reflex; postural reaction: proprioceptive positioning reaction; righting reactions: surface righting reaction and aerial righting reaction; and landing foot splay)/ grip strength (fore/hind limb)/ motor activity.

SERUM HORMONE MEASUREMENT: Yes
- Time schedule for collection of blood: Conducted using the remaining serum for clinical chemistry.
- Animals fasted: Yes
- How many animals: All animals
- Parameters examined: Triiodothyronine (T3), Thyroxine (T4) and Thyroid stimulating hormone (TSH).

Sacrifice and pathology

GROSS PATHOLOGY: Yes.

ORGAN WEIGHT: Yes
- Organs examined: Pituitary, Thymus, Thyroids (including parathyroids), Heart, Liver, Spleen, Kidneys, Adrenals, Testes, Epididymides, Uterus, Ovaries (oviducts).

HISTOPATHOLOGY: Yes
- Dose groups that were examined: Control and the highest dose group for all organs/tissues described below. Other dose groups for only macroscopically abnormal site.
- Organs/tissues examined: The above organs weighed, Trachea, Lungs (including bronchus), Lymph nodes (mandibular, mesenteric, and axillary), Stomach, Duodenum, Jejunum, Ileum, Cecum, Colon, Rectum, Urinary bladder, Eyeballs, Mammary gland (male), Brain, Spinal cord (cervical, pectoral, and lumber), Sciatic nerve, Prostate, Bone marrow (femur) and other abnormal site.

Other examinations

Measurement of fullerene C60 in organs:
- Organs measured: Liver (median lobe), Kidneys, Spleen
- How many animals: All males in the control and the highest dose groups necropsied at the end of the administration and the recovery periods.
- Method: The organ samples obtained were weighed, frozen with liquid nitrogen, and stored in a deep freezer (-80 to -74°C) until use. The amount of fullerene C60 in the organ samples were treated properly and measured using LC-MS/MS according to the method described in the following paper (Kubota, R. et al.(2011) Tine-dependent variation in the biodistribution of C60 in rats determined by liquid chromatography-tandem mass spectrometry. Toxicol. Lett., vol. 206, 172-177.). The detection limits for each organ were 0.102 μg/g wet wt. (liver), 0.146 μg/g wet wt. (kidneys), and 0.587 μg/g wet wt. (spleen).

Statistics

Parametric data, such as neurobehavioral findings, body weight, food consumption, urinalysis (except for the results of qualitative analysis), hematology, clinical chemistry, serum hormone level, and organ weights, were analyzed by Bartlett's test for homogeneity of variance. If homogenous, Dunnett's test was conducted and, if not homogenous, Steel's multiple comparison test was conducted to compare control and individual treatment groups. For two groups, parametric data were analyzed by the F-test for homogeneity of variance. If homogenous, Student's t-test was conducted and, if not homogenous, Aspin-Welch's t-test was conducted for comparison. For significant differences in the incidence of neurobehavioral findings, urinalysis, and histopathology findings, Fischer's exact test was performed, and the grade of lesions was compared using Mann-Whiteney U-test. A 5% level of probability was used as criterion for significance.

Results and discussions

Effect levels

Endpoint	Effect level	Based on	Sex	Basis for effect level / Remarks
NOAEL	1000 mg/kg bw/day (actual dose received)	test mat.	male/female	After start of treatment, blackish feces were observed in male and female 1000 mg/kg bw/day group and at the necropsy, black content of stomach and large intestine were observed in the same groups. It was considered that a large amount of fullerene was contained in contents of intestine and feces. Treatment-related effects were observed at 1000 mg/kg bw/day in clinical chemistry and some organ weights, but these were slight and not accompanied with histopathological changes. Thus, NOAEL of this study was determined to be 1000 mg/kg bw/day.

Results of examinations

Clinical signs and mortality

yes

Body weight and weight gain

no effects

Food consumption and compound intake (if feeding study)

no effects

Food efficiency

not examined

Water consumption and compound intake (if drinking water study)
not examined

Ophthalmoscopic examination
not examined

Haematology
no effects

Clinical chemistry
yes

Urinalysis
no effects

Neurobehaviour
no effects

Organ weights
yes

Gross pathology
yes

Histopathology: non-neoplastic
no effects

Histopathology: neoplastic
no effects

Details on results
CLINICAL SIGNS AND MORTALITY:
No deaths or clinical signs of toxicity occurred in any group. In general appearance, blackish feces were observed in males and females at 1000 mg/kg bw/day from day 4 of administration through the administration period till day 1 or recovery.

HAEMATOLOGY:
A decrease in the differential lymphocyte ratio and an increase in the differential eosinophil ratio were observed at 10 mg/kg bw/day in males at the end of the administration period. These changes were not dose-related and considered not treatment-related.

CLINICAL CHEMISTRY:
An increase in creatinine at 100 mg/kg bw/day in males, and a decrease in albumin at 1000 mg/kg bw/day in males were observed at the end of administration period, and an increase in total protein in females only at the recovery period. Of these, an increase of creatinine in males were not dose-related, and thus, considered to be not toxicologically significant.

URINALYSIS:
Only an increase in the number of positive incidences of ketone bodies was observed at 10 and 1000 mg/kg bw/day in males at the end of the administration period. This was not considered as toxicologically significant because of no dose-dependency.

ORGAN WEIGHTS:
An increase of relative thymus weight at 100 mg/kg bw/day in females and a decrease in relative kidney weight at 1000 mg/kg bw/day in males were observed at the end of administration period, but not at the end of recovery period. Increases in absolute and relative liver weights and absolute spleen weight were observed in males in the treatment group only at the end of recovery period. Of these, an increase of relative thymus weight in females was not dose-related, and was considered not toxicologically significant.

GROSS PATHOLOGY:
Black contents of the stomach and large intestine were observed in all animals at 1000 mg/kg bw/day at the end of administration period, but not at the end of recovery period.

HISTOPATHOLOGY:
No treatment-related changes were observed in any organs examined.

SERUM HORMONE MEASUREMENT:
No changes from controls were found serum levels of T3, T4 and TSH.

CONTENTS OF FULLERENE C60 IN THE ORGANS:
The contents of fullerene C60 were below the detection limit in all samples of the liver, kidneys, and spleen at the end of the administration period and at the end of recovery period.

Any other information on results incl. tables

Table 1: Principal blood biochemi cal values in male and female rats given fullerene C60 by gavage

		At the end of the administration period						At the end of the recovery period	
	Dose (mg/kg/day)	0	1	10	100	1000		0	1000
Male									
	No. of animals	5	5	5	5	5		5	5
	AST (U/L)	88.4 ± 20.2	92.8 ± 29.4	72.8 ± 13.8	80.6 ± 10.5	64.6 ± 11.6		127.8 ± 29.2	100.6 ± 34.9
	ALT (U/L)	33.8 ± 4.1	33.4 ± 4.2	33.4 ± 6.1	33 ± 8.4	32.6 ± 7.4		33.6 ± 5.9	31 ± 4.9
	ALP (U/L)	588.2 ± 114.1	634.8 ± 67	649.6 ± 100.3	564 ± 101.4	610.4 ± 134.2		423.4 ± 75.9	410 ± 43.7
	γ-GTP (U/L)	0.58 ± 0.28	0.42 ± 0.13	0.5 ± 0.19	0.6 ± 0.07	0.66 ± 0.34		0.58 ± 0.22	0.4 ± 0.34
	Lactate dehydrogenase (U/L)	127.6 ± 32.6	142.2 ± 34.4	121.8 ± 47.2	133.6 ± 50.2	113.2 ± 15.8		184.4 ± 40.4	161.4 ± 66.5
	Urea nitrogen (mg/dL)	9.44 ± 1.21	10.22 ± 1.14	9.78 ± 1.67	10.8 ± 2.16	9.6 ± 1.44		13.52 ± 1.32	12.84 ± 0.67
	Creatinine (mg/dL)	0.232 ± 0.044	0.282 ± 0.022	0.268 ± 0.035	0.32 ± 0.049 **	0.27 ± 0.019		0.286 ± 0.029	0.27 ± 0.016
	Glucose (mg/dL)	145.8 ± 21.4	150.6 ± 15.8	149.4 ± 15.9	155.8 ± 30.7	165.2 ± 10.7		138.6 ± 8	145 ± 27.3
	Total cholesterol (mg/dL)	50.8 ± 10.5	48.8 ± 11	55.4 ± 6.2	59.8 ± 7.6	55 ± 12.1		55.4 ± 10.7	65.8 ± 17
	Phospholipid (mg/dL)	93.2 ± 15.3	88 ± 13.8	101.2 ± 8.8	106 ± 7.6	101 ± 16.2		92 ± 13.4	106 ± 20.5
	Triglycerides (mg/dL)	59.4 ± 22.5	47.6 ± 9.2	54.2 ± 5.2	36.6 ± 9.3	66.4 ± 28.2		38.6 ± 16.4	64.6 ± 34.2

	Total protein (g/dL)	5.78 ± 0.08	5.72 ± 0.11	5.52 ± 0.13	5.76 ± 0.27	5.68 ± 0.13		5.94 ± 0.23	6.06 ± 0.17
	Albumin (g/dL)	2.52 ± 0.13	2.46 ± 0.05	2.38 ± 0.11	2.42 ± 0.11	2.34 ± 0.11 *		2.42 ± 0.08	2.44 ± 0.11
	A/G	0.774 ± 0.054	0.758 ± 0.036	0.76 ± 0.06	0.73 ± 0.064	0.704 ± 0.057		0.69 ± 0.023	0.674 ± 0.027
Female									
	No. of animals	5	5	5	5	5		5	5
	AST (U/L)	115.6 ± 35.4	119.4 ± 26.5	122 ± 28.6	108.6 ± 30.2	99.4 ± 40.2		109.8 ± 28.9	130.4 ± 58.9
	ALT (U/L)	31.6 ± 6.8	35.8 ± 6.3	34.8 ± 10.5	30.4 ± 9.4	33.4 ± 11		29 ± 4	35 ± 21.8
	ALP (U/L)	533.2 ± 238.9	399.4 ± 99	432.4 ± 86.1	343.4 ± 48.6	379.4 ± 45.9		270.8 ± 14	301 ± 42.3
	γ-GTP (U/L)	0.7 ± 0.32	0.78 ± 0.16	0.8 ± 0.14	0.64 ± 0.15	0.66 ± 0.25		0.74 ± 0.18	0.8 ± 0.48
	Lactate dehydrogenase (U/L)	177.6 ± 73	143.2 ± 41.4	169.6 ± 47.5	150.6 ± 15.3	168.6 ± 11.7		144.6 ± 31.7	129.2 ± 44.9
	Urea nitrogen (mg/dL)	10.88 ± 1.29	11.98 ± 2.08	11.32 ± 1.88	12.66 ± 1.27	12.84 ± 1.44		16.78 ± 2.47	17.9 ± 1.67
	Creatinine (mg/dL)	0.31 ± 0.029	0.322 ± 0.041	0.316 ± 0.029	0.326 ± 0.032	0.318 ± 0.029		0.352 ± 0.022	0.352 ± 0.013
	Glucose (mg/dL)	132.6 ± 13	124.6 ± 21.1	133.2 ± 16.1	139 ± 15.5	138.8 ± 18		124 ± 10.4	130.4 ± 5.9
	Total cholesterol (mg/dL)	56.4 ± 15.8	61.4 ± 6	56 ± 8.2	65.8 ± 12.4	60.4 ± 8.8		78 ± 8.2	75.6 ± 13.6
	Phospholipid (mg/dL)	107.8 ± 25.6	111.4 ± 11.1	104.6 ± 13.3	125.6 ± 18.6	116.2 ± 14.4		141.2 ± 15.1	142 ± 25.2
	Triglycerides (mg/dL)	25.8 ± 14.9	21.2 ± 12.6	19 ± 7	20.8 ± 6.9	16.2 ± 11.2		26 ± 8.4	35.6 ± 14.8
	Total protein (g/dL)	5.76 ± 0.21	5.82 ± 0.31	5.78 ± 0.24	5.94 ± 0.15	5.92 ± 0.11		6.06 ± 0.21	6.32 ± 0.11 *
	Albumin (g/dL)	2.56 ± 0.05	2.58 ± 0.18	2.54 ± 0.11	2.7 ± 0.19	2.72 ± 0.11		2.64 ± 0.09	2.72 ± 0.13
	A/G	0.802 ± 0.037	0.8 ± 0.089	0.784 ± 0.03	0.836 ± 0.081	0.85 ± 0.05		0.776 ± 0.081	0.756 ± 0.062

Table 2: Principal organ weights of male and female rats given fullerene C60 by gavage

		At the end of the administration period						At the end of the recovery period	
	Dose (mg/kg/day)	0	1	10	100	1000		0	1000
Male									
	No. of animals	5	5	5	5	5		5	5
	Body weight[a](g)	415.0 ± 39.0	426.6 ± 36.8	408.6 ± 32.8	424.4 ± 50.8	422.2 ± 39.4		454.4 ± 49.4	485.6 ± 19.3
	Thymus (g)	0.54 ± 0.16	0.45 ± 0.09	0.50 ± 0.10	0.51 ± 0.13	0.50 ± 0.13		0.46 ± 0.08	0.46 ± 0.11
		(0.130 ± 0.032)[b]	(0.104 ± 0.017)	(0.121 ± 0.024)	(0.119 ± 0.016)	(0.117 ± 0.025)		(0.100 ± 0.015)	(0.095 ± 0.026)
	Liver (g)	12.80 ± 1.93	13.33 ± 1.64	11.93 ± 0.71	13.96 ± 2.95	12.98 ± 1.39		12.17 ± 1.27	13.99 ± 1.15 *
		(3.076 ± 0.288)	(3.123 ± 0.253)	(2.926 ± 0.136)	(3.274 ± 0.385)	(3.073 ± 0.121)		(2.681 ± 0.113)	(2.878 ± 0.137 *)
	Kidneys (g)	2.84 ± 0.40	2.83 ± 0.26	2.71 ± 0.20	2.71 ± 0.17	2.59 ± 0.16		2.91 ± 0.20	3.15 ± 0.34
		(0.684 ± 0.046)	(0.663 ± 0.017)	(0.664 ± 0.035)	(0.643 ± 0.036)	(0.615 ± 0.031 *)		(0.644 ± 0.045)	(0.646 ± 0.047)
	Spleen (g)	0.56 ± 0.08	0.65 ± 0.08	0.61 ± 0.07	0.66 ± 0.15	0.61 ± 0.10		0.67 ± 0.09	0.82 ± 0.05 *

		(0.134 ± 0.009)	(0.153 ± 0.017)	(0.149 ± 0.005)	(0.153 ± 0.021)	(0.144 ± 0.019)		(0.148 ± 0.017)	(0.169 ± 0.015)
Female									
	No. of animals	5	5	5	5	5		5	5
	Body weight[a](g)	217.6 ± 20.5	222.4 ± 12.2	216.8 ± 12.8	211.6 ± 16.8	218.2 ± 7.4		235.0 ± 16.7	234.8 ± 22.8
	Thymus (g)	0.36 ± 0.07	0.45 ± 0.11	0.39 ± 0.05	0.47 ± 0.09	0.40 ± 0.09		0.41 ± 0.08	0.41 ± 0.11
		(0.163 ± 0.021)	(0.200 ± 0.042)	(0.180 ± 0.032)	(0.222 ± 0.032 *)	(0.185 ± 0.041)		(0.172 ± 0.026)	(0.176 ± 0.053)
	Liver (g)	6.43 ± 0.91	6.89 ± 0.57	6.66 ± 0.34	6.48 ± 0.54	6.85 ± 0.56		6.28 ± 0.42	6.19 ± 0.98
		(2.950 ± 0.203)	(3.097 ± 0.129)	(3.080 ± 0.252)	(3.066 ± 0.131)	(3.142 ± 0.236)		(2.675 ± 0.109)	(2.626 ± 0.214)
	Kidneys (g)	1.48 ± 0.12	1.41 ± 0.12	1.43 ± 0.07	1.51 ± 0.12	1.52 ± 0.17		1.67 ± 0.13	1.51 ± 0.28
		(0.682 ± 0.076)	(0.637 ± 0.045)	(0.662 ± 0.041)	(0.717 ± 0.050)	(0.697 ± 0.061)		(0.714 ± 0.048)	(0.640 ± 0.068)
	Spleen (g)	0.40 ± 0.04	0.46 ± 0.06	0.41 ± 0.04	0.43 ± 0.06	0.42 ± 0.05		0.45 ± 0.07	0.44 ± 0.07
		(0.185 ± 0.012)	(0.206 ± 0.027)	(0.187 ± 0.011)	(0.200 ± 0.018)	(0.195 ± 0.028)		(0.190 ± 0.020)	(0.189 ± 0.020)

Table 3: Number of animals with histopathological findings in male and female rats given fullerene C60 by gavage

		Male		Female	
	Dose (mg/kg/day)	0	1000	0	1000
	No. of animals	5	5	5	5
At the end of the administration period					
Liver					
	Normal	2	1	1	0
	Granuloma, minimal	1	3	3	1
	Granuloma, slight	0	0	0	1
	Granuloma, moderate	0	0	0	1
	Tension lipidosis, slight	0	1	0	0
	Vacuolation, cytoplasmic, minimal	3	3	3	4
	Vacuolation, cytoplasmic, slight	0	0	1	1
Kidney					
	Normal	4	5	4	2
	Mineralization, minimal	0	0	1	3
	Scar, mininal	1	0	0	0
Prostate					
	Normal	4	5		
	Cellular infiltration, lymphocyte, minimal	1	0		
Uterus					
	Normal			3	5
	Dilatation, lumen, slight			2	0
At the end of the recovery period					
Liver					

	Normal	3	3		
	Granuloma, minimal	2	1		
	Vaculoation, cytoplasmic, minimal	0	1		
Spleen					
	Normal	5	5		

Overall remarks, attachments

Remarks on results including tables and figures

In males and females at 1000 mg/kg bw/day, blackish feces and black contents of the stomach and large intestine were considered to result from the administered fullerene C60 itself. In 1000 mg/kg bw/day group, a decrease in albumin in males (administration group), an increase of total protein in females (recovery group), a decrease in relative kidney weight in males (administration group), increases in absolute and relative liver weights and absolute spleen weight in males (recovery group) were observed, and these cannot be denied the possibility of treatment-related effects. However, there were no histopathological changes in these organs, and it was considered that these changes seen at 1000 mg/kg bw/day were no adverse effects.

Applicant's summary and conclusion

Conclusions

After start of treatment, blackish feces were observed in male and female 1000 mg/kg bw/day group and at the necropsy, black content of stomach and large intestine were observed in the same groups. It was considered that a large amount of fullerene was contained in contents of intestine and feces. Treatment-related effects were observed at 1000 mg/kg bw/day in clinical chemistry and some organ weights, but these were slight and not accompanied with histopathological changes. Thus, NOAEL of 28-day repeated oral dose of fullerene 60 was determined to be 1000 mg/kg bw/day.

7.5.2 Repeated dose toxicity: inhalation

Endpoint study record: Repeated dose toxicity: inhalation.001

Administrative Data

Purpose flag	key study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Acceptable, well-documented publication which meets basic scientific principles

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Morimoto, Y. et al.	2010	Inflammogenic effect of well-characterized fullerene in inhalation and intratracheal instillation studies	Part. Fibre Toxicol., vol. 7, 1-18 (2010)					
publication	Ogami, A. et al.	2011	Pathological features of rat lung following inhalation and intratracheal instillation of C60 fullerene	Inhal. Txicol., 23(7), 407-416 (2011)					
publication	Fujita, K. et al.	2009	Gene expression profiles in rat lung after inhalation exposure to C60 fullerene particles	Toxicol., 258, 47-55 (2009)					

Data access

data published

Materials and methods

Test type

subacute

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
common name	Fullerene C60 (nanom purple)

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): Fullerene or C60
- Analytical purity: > 99.5 %
- Other: Supplier: Frontier Carbon Co., Ltd.

Test animals

Species

rat

Strain

Wistar

Sex

male

Details on test animals and environmental conditions

TEST ANIMALS
- Source: Kyudo Co., Inc.
- Age at study initiation: 9 weeks-old (at purchase)
- Weight at study initiation: not stated.
- Housing: After exposure period, rats were housed within polycarbonate cages at a controlled temperature of 22°C.
- Diet (e.g. ad libitum): standard chow diet, ad libitum
- Water (e.g. ad libitum): tap water, ad libitum
- Acclimation period: not stated

Administration / exposure

Route of administration

inhalation: aerosol

Type of inhalation exposure

whole body

Vehicle

clean air

Details on inhalation exposure

GENERATION OF TEST ATMOSPHERE / CHAMBER DESCRIPTION
- Exposure apparatus: The whole-body exposure system used to expose rats to fullerene C60 aerosols (C60 in a Tween 80 suspension) consisted of a pressurized nebulizer and a mist dryer, connected to an exposure chamber (volume: 0.52 m3) at a controlled temperature of 21°C and 56% relative humidity.
- Method of holding animals in test chamber:
- Source and rate of air:
- Method of conditioning air:
- System of generating particulates/aerosols:

- Temperature, humidity, pressure in air chamber:
- Air flow rate:
- Air change rate:
- Method of particle size determination: The size and number concentrations of aerosol particles at the exit of the nebulizer and inside the exposure chamber were analyzed in-line using a particle spectrometer consisting of a differential mobility analyzer (DMA) and a condensation particle counter (CPC) (Model 1000XP WPS, MSP Corp.) throughout the exposure period.
- Treatment of exhaust air:

TEST ATMOSPHERE
- Brief description of analytical method used:
- Samples taken from breathing zone: yes/no

VEHICLE (if applicable)
- Justification for use and choice of vehicle:
- Composition of vehicle:
- Type and concentration of dispersant aid (if powder):
- Concentration of test material in vehicle:
- Lot/batch no. of vehicle (if required):
- Purity of vehicle:

Analytical verification of doses or concentrations

yes

Details on analytical verification of doses or concentrations

The aerosol was generated for 6h/day, 5days/week for 4 weeks, and its size distribution and number concentration in the chamber were measured during every day of exposure. The C60 aerosol in the chamber had an average mass concentration of 0.12±0.03 mg/m3 (0.5 ± 0.1 mg/m3, including Tween 80), a particle concentration (4.1 ± 0.4)x10^4 particles/cm3, and an average geometric diameter of 96 ±5 nm.

Duration of treatment / exposure

4 weeks

Frequency of treatment

6h/day, 5days/week

Doses/concentrations

0.12mg/m3 of fullerene C60

Basis analytical conc.

No. of animals per sex per dose

10 rats/group/observation time point. (Three groups: Control (clean air only), C60-treatment and NiO-treatment)The lungs of 5 rats/each group were used for bronco-alveolar lavage fluid (BALF) examinations etc., and the lungs of the remaining 5 rats/each group were used for DNA microarray analysis.

Control animals

yes, sham-exposed

Details on study design

- Post-exposure recovery period in satellite groups: After an exposure period of 4 weeks, the rats of control and treatment groups were sacrificed at 3 days, 1 month, and 3 months of recovery.

Positive control

NiO (99.8% purity) was used as a positive control. The rats were exposed to NiO nanoparticle aerosol to rats for 4 weeks and were sacrificed after exposure period as similar as the C60-treatment group. The NiO aerosol in the chamber had an average mass concentration of 0.2±0.1 mg/m3, a particle concentration of (9.2±4.9)x10^4 particles/cm3, and an average geometric diameter of 59 ±3 nm.

Examinations

Observations and examinations performed and frequency

CAGE SIDE OBSERVATIONS: No data
DETAILED CLINICAL OBSERVATIONS: No data
BODY WEIGHT: No data
FOOD CONSUMPTION: No
FOOD EFFICIENCY: No
WATER CONSUMPTION: No
OPHTHALMOSCOPIC EXAMINATION: No
HAEMATOLOGY: No data
CLINICAL CHEMISTRY: No data
URINALYSIS: No
NEUROBEHAVIOURAL EXAMINATION: No

Sacrifice and pathology

GROSS PATHOLOGY: Yes
- Organs weighed: lungs, liver, brain.

HISTOPATHOLOGY: Yes
- Organs examined: lungs, liver, kidney, spleen, cerebrum, cerebellum, testis, and nasal cavity.

Other examinations

- BALF (BRONCO-ALVEOLAR LAVAGE FLUID) EXAMINATIONS:
BALF was collected using physiological saline that was poured through a cannula inserted in the respiratory tract into the right lung, while the left lung was clamped. Three to 10 ml of physiological saline was infused per time and lavage fluid was collected up to 50 ml in total. Total cell and neutrophil in the BALF were counted and alkaline phosphatase (ALP) activity released in the BALF supernatant was measured using LabAssayTM ALP (Wako Pure Chemical Industries, Ltd.).

- CHEMOKINE MEASUREMENT OF LUNG TISSUE:
Chemokine such as cytokine-induced neutrophil chemoattractant (CINC) in the lung tissue was measured as follows. Lung tissue was homogenized with a T-PER tissue protein extraction reagent, and then centrifuged (1500xg for 10 min). The protein concentration of the supernatant was measured by the BCA Protein Assay Kit (PIERCE) using bovine serum albumin. Total protein concentration was adjusted with a final concentration of 500 μg/ml for CINC-1 and CINC-2αβ and 4000 μg/ml for CINC-3. Chemokine concentration was determined by Quantikine Rat CINC-1, CINC-2αβ, and CINC-3 (R&D Systems) and absorbance at 450 nm was measured by a microplate reader. CINC-1, CINC-2αβ, and CINC-3 in the lung tissue were determined.

- GENE EXPRESSION OF CINC MRNA IN THE LUNG:
RNA was extracted from the lung using RNeasy(R) Mini Kit (Quiagen). Single-strand cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNA levels of CINC-1, CINC-2αβ, and CINC-3 were measured according to RT-PCR method. RT-PCR was performed using the 7500 Real-Time PCR System (Applied Biosystems) with TaqMan Universal PCR Master Mix reagents.

- C60 FULLERENE AND NICKEL LUNG BURDEN:
The left lung tissues exposed to clean air, C60 fullerene or NiO were individually homogenized and digested via a digester (Multiwave 3000, Anton Paar GmbH). The concentration of C60 fullerene or nickel in the homogenized tissues was determined by liquid chromatography combined with UV absorptiometry, LC-UV (HP1100, Agilent Technologies) or an inductively coupled argon plasma mass spectrometer, ICP-MS (Agilent Technologies) respectively. The particle burdens were estimated using the left lung weights. - RNA extraction and DNA microarrayThe right lungs were homogenized using QIAzol lysis reagent with a Tissue Ruptor (Qiagen). Total RNA from the homogenates was extracted using the RNeasy Midi Kit (Qiagen) following the manufacturer's instructions. RNA quality and concentration were determined using an Agilent 2100 bioanalayzer (Agilent Technologies) and a NanoDrop ND-1000 (NanoDrop Technologies). cRNA labeled with fluorescent Cyanine 3-CTP was used for hybridization onto the Whole Rat Genome Oligo Multiplex Microarray slides (#G4131F, Agilent Technologies) counting approximately 41,000 oligonucleotide probes at 65°C for 17h. Hybridized microarray slides were washed according to the manufacture's instructions, and were scanned with Agilent DNA Microarray Scanner (#G2565BA, Agilent Technologies) at 5 μm resolution. The scanned images were analyzed numerically using the Agilent Feature Extraction Software version 9.5.3.1.

Statistics

- DATA FOR BALF EXAMINATIONS AND CHEMOKINE ASSAY IN LUNG TISSUES:
Statistical analysis was carried out using the Mann-Whitney test with differences of $p < 0.05$ considered to be statistically significant.- Data for C60 and nickel lung burden:Significant differences between time points within each group were assessed by one-way factorical ANOVA. Student's t-test was used to assess statistical siginificance at $p < 0.05$.

- MICROARRAY DATA ANALYSIS:
Normalized data were analyzed using GeneSpring GX version 7.3.1 software (Agilent Technologies). In each nanoparticle exposure experiment at the same post-exposure periods, genes with over a 2-fold or less than 0.5-fold intensity ratio compared with those of clean air exposure (negative control) were considered as up- or down-regulated genes, respectively. P-values by one sample Student's t-test were calculated for each sample in each of the experimental groups. Gene expression data are deposited in the Gene Expression Omnibus (GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/). The differences between the control and the experimental groups were evaluated with the Student's t-test. P-values less than 0.05 were considered to be significant. The Web-based application GOstat (http://gostat.wehi.edu.au/) was used to identify statistically overrepresented Gene Ontology (GO) terms.

Results and discussions

Effect levels

Endpoint	Effect level	Based on	Sex	Basis for effect level / Remarks
NOAEL	> 0.12 mg/m³ air (analytical)	test mat.	male	Although slight inflammatory response was observed in the lungs, no histopathlogical abnormalities were observed in the liver, kidney, spleen, cerebrum, cerebellum, testis, or nasal cavity tissues.Therefore, it was considered that NOAEL (systemic) of fullerene C60 is greater than 0.12 mg/m3.

Results of examinations

Clinical signs and mortality
no data

Body weight and weight gain
no data

Food consumption
not examined

Food efficiency
not examined

Water consumption
not examined

Ophthalmoscopic examination
not examined

Haematology
not examined

Clinical chemistry
not examined

Urinalysis
not examined

Neurobehaviour
not examined

Organ weights
no effects

Gross pathology
no effects

Histopathology: non-neoplastic
yes

Histopathology: neoplastic
no effects

Details on results
ORGAN WEIGHTS:
- There were no statistical differences in the weights of the body, lung, liver, or brain between C60 fullerene or NiO exposure groups and clean air exposure group (negative control).

GROSS PATHOLOGY:
- No obvious morphological changes were observed in the clean air or C60 fullerene exposure groups at 3 days and 1 month post-exposure. Nodule-like lesions were observed in animals that were exposed to NiO nanoparticles at both 3 days (n=2/5) and 1 month (n=1/5) post-exposure.
-
HISTOPATHOLOGY:
- Lungs: Although slight macrophage accumulation and a small number of inflammatory cells were observed in the alveoli at 3 days in the C60-exposed groups, these findings were not seen and the lung sections were similar to the negative control at 3 months. Some alveolar macrophages with brown pigment granules were observed in the alveoli of the C60 inhalation groups. There was no granuloma, emphysematous change, or fibrosis during the post-exposure period. In the inhalation study of nano-NiO as a positive control substance, macrophage accumulation in the alveoli with infiltration of inflammatory cells at 3 days after the termination of exposure. Hyperplasia of the terminal bronchiole and alveolar epithelial cells was also seen. These findings decreased 3 months after inhalation.- Other organs: No histopathlogical abnormalities were observed in the liver, kidney, spleen, cerebrum, cerebellum, testis, or nasal cavity tissues in each group of clean air, C60 fullerene or NiO exposure (n=5).

OTHER FINDINGS:
- Cell count and ALP in BALF: The total cell count of C60 fullerene inhalation group was almost the same as that of the control group. Also, almost no neutrophil count increase was observed in the control and C60 groups. The ALP release in BALF was significantly in the NiO exposure group, while there was no difference between C60 fullerene and control groups.
- CINC concentration in the lung: There was no difference in the CINC-1 concentration between the C60 inhalation group and the control group. On the other hand, CINC-1 concentration in lung tissue was increased at 3 days and 1 month in the NiO exposure group as compared to the control group. As in the case of CINC-1 concentration, CINC-2αβ concentration in lung tissue was not significantly increased in the C60 exposure group. No significant difference in CINC-3 concentration was observed among the 3 groups throughout the observation period.
- Gene expression of CINC mRNA in the lung: There was no significant changes of gene expression of CINC-1 between the C60 and the control groups. CINC-2αβ gene expression was unchanged in C60 exposure groups throughout the observation period, while that in the NiO exposure group increased significantly at 3 days and 1 month. No significant gene expression of CINC-3 was observed between the

C60 and the control groups. On the other hand, gene expression of CINC-3 mRNA markedly increased at 3 days in the NiO exposure group.
- C60 fullerene and nickel in lung tissue: LC-UV or ICP-MS analyses indicated that C60 fullerene or nickel remained in lung tissues at 3 days post-exposure, and decreased at 1 month exposure (Table 1).
- DNA microarray analysis: Gene expression profiles revealed that few genes involved in the inflammatory response, oxidative stress, apoptosis, and metalloendopeptidase activity were up-regulated at both 3 days and 1 month post-exposure of C60 fullerene. only some genes associated with the immune system process, including major histocompatibility complex (MHC)-mediated immunity were up-regulated. These results were significantly different from those of NiO ultra-fine particles which included high expression of genes associated with chemokines, oxidative stress, and matrix metalloprotease 12 (Mmp12), suggesting that NiO ultra-fine particles lead to acute inflammation for the inhalation exposure period.

Any other information on results incl. tables

Table 1 C60 fullerene and nickel burden in lung tissues after inhalation exposure

Post-exposure	C60 fullerene		Nickel	
	3 days	1 month	3 days	1 month
Control	u.l.	u.l.	0.13±0.04	0.08±0.02
C60 fullerene	3.40±0.37	1.91±0.34	n.d.	n.d.
NiO	n.d.	n.d.	4.99±0.19	2.86±0.27

The particle weight per left lung tissue was expressed as mean ± S.E.M (μg)
C60 detection limit:<0.012μg; Ni detection limit: <0.021μg;
u.l. : undetectable levels; n.d. : not determined.

Overall remarks, attachments

Attached full study report

Illustration (picture/graph)

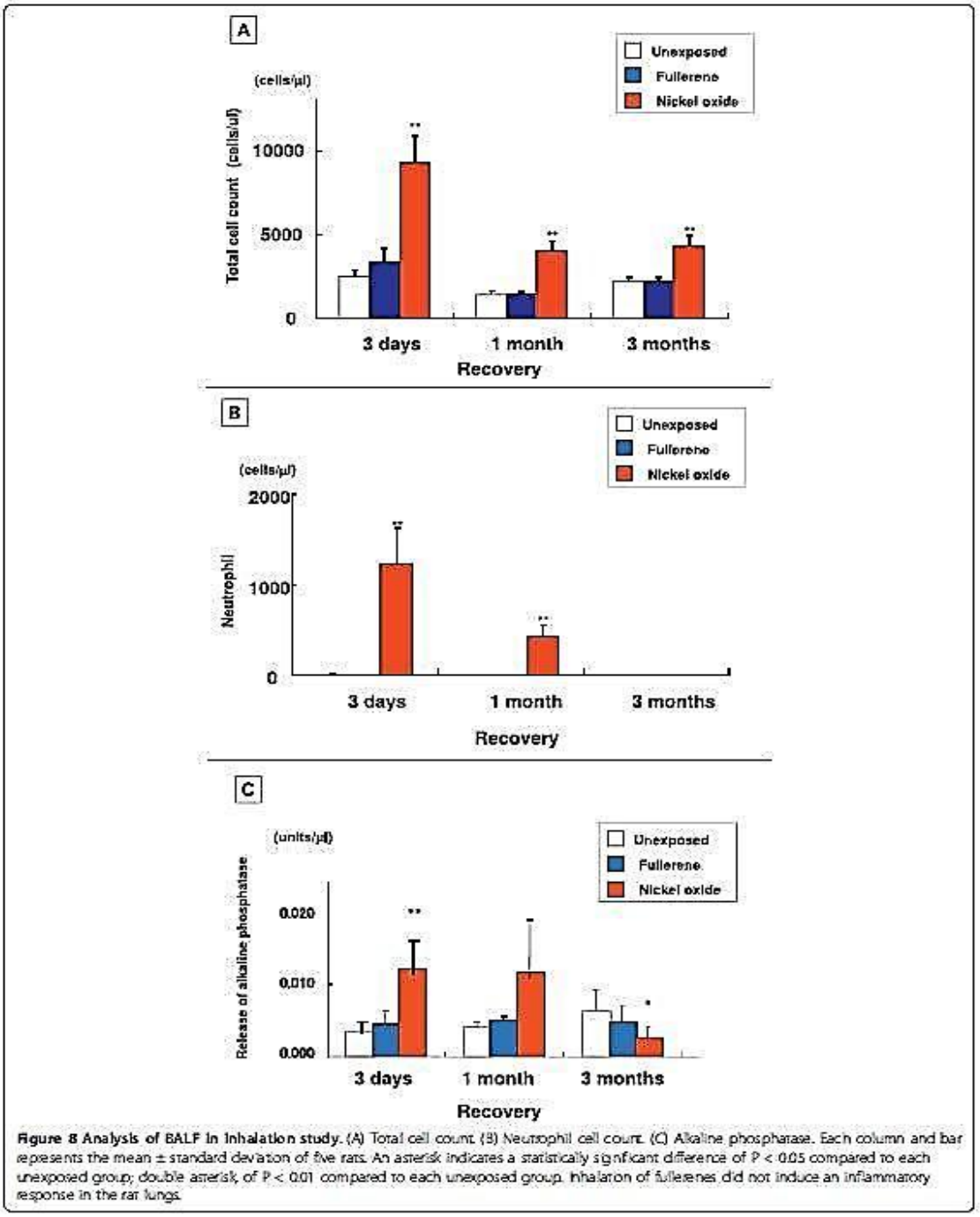

Figure 8 Analysis of BALF in inhalation study. (A) Total cell count (B) Neutrophil cell count (C) Alkaline phosphatase. Each column and bar represents the mean ± standard deviation of five rats. An asterisk indicates a statistically significant difference of $P < 0.05$ compared to each unexposed group; double asterisk, of $P < 0.01$ compared to each unexposed group. Inhalation of fullerenes did not induce an inflammatory response in the rat lungs.

Applicant's summary and conclusion

Conclusions

Based on data for histopathological examination, BALF examination, chemokine analysis in lung tissue and DNA microarray analysis, It was suggested that C60 fullerene might not have a severe pulmonary toxicity after 4 weeks inhalation exposure in rats. Although slight inflammatory response was observed in the lungs, no histopathlogical abnormalities were observed in the liver, kidney, spleen, cerebrum,

cerebellum, testis, or nasal cavity tissues in C60 inhalation group. Therefore, it was concluded that NOAEL (systemic) of fullerene C60 under this test condition is greater than 0.12 mg/m3.

7.5.3 Repeated dose toxicity: dermal

7.5.4 Repeated dose toxicity: other routes

7.6 Genetic toxicity

7.6.1 Genetic toxicity in vitro

Endpoint study record: Genetic toxicity in vitro.001

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Matsumoto, K. et al.	2008	Final Report of Bacterial Reverse Mutation Test of Fullerene (with irradiation) (Japanese)		The Institute of Environmental Toxicology	IET 07-0128	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2008-02-18
study report	Matsumoto, K. et al.	2008	Final Report of Bacterial Reverse Mutation Test of Fullerene (without irradiation) (Japanese)		The Institution of Environmental Toxicology	IET 07-129	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2008-01-28
publication	Sinohara, N. et al.	2009	In vitro and in vivo genetoxicity tests on fullerene C60 nanaoparticles	Toxicol. Lett., vol. 191, 289-296 (2009)					

Data access

data published

Materials and methods

Type of genotoxicity
gene mutation

Type of study
bacterial reverse mutation assay (e.g. Ames test)

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 471 (Bacterial Reverse Mutation Assay)	
according to	JAPAN: Guidelines for Screening Mutagenicity Testing Of Chemicals	

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
yes

Test material identity

Identifier	Identity
common name	Fullerene C60 nanoparticles

Test material form
nanomaterial

Details on test material
- Name of test material (as cited in study report): C60 nanoparticles
- Analytical purity: > 99.5%
- Storage condition of test material: stored in cold and dark place.
- Other: Supplier: Frontier Carbon Co., Ltd., Japan.

Method

Species/strain

Species/strain	S. typhimurium TA 1535, TA 1537, TA 98 and TA 100
Details on mammalian cell lines (if applicable)	- Type and identity of media: 2.5% nutrient broth (Oxoid nutrient broth No. 2; Oxoid Ltd., UK)
Metabolic activation	with and without

Metabolic activation system	Rat liver S9 fraction prepared from male SD rats pretreated with phenobarbital and 5,6-benzoflavone.
Species/strain	E. coli WP2 uvr A pKM 101
Details on mammalian cell lines (if applicable)	- Type and identity of media: 2.5% nutrient broth (Oxoid nutrient broth No. 2; Oxoid Ltd., UK)
Metabolic activation	with and without
Metabolic activation system	Rat liver S9 fraction prepared from male SD rats pretreated with phenobarbital and 5,6-benzoflavone.

Test concentrations

50, 100, 200, 400 and 1000 μg/plates

Vehicle

- Vehicle used: CMC-Na (sodium carboxymethyl cellulose)
- Justification for choice of vehicle: not cytotoxic.

Sample preparationThe principal fullerenes are stirred and pulverized in Ultra Apex Mill (50μm zirconia beads) together with CMCNa (carboxymethylcellulose sodium) solution for 30 minutes after having raveled in a mortar with a small quantity of CMCNa solutions. The supernatant of the dispersion from centrifugation is concentrated by Ultrafiltration Membrane and filtrated by a sterile filter of 45μm.

Controls

Negative controls	yes
Solvent / vehicle controls	yes
True negative controls	no
Positive controls	yes

Details on test system and conditions

METHOD OF APPLICATION:
- Modified plate incorporation method under irradiation;
- Preincubation method under dark conditions

DURATION
- Preincubation period: 20 min.
- Exposure duration: 48 hrs.

NUMBER OF REPLICATIONS: 2

OTHER:
- For the test without S9 mix, 0.1-0.5 mL of the C60 suspension, a negative control solution, or a positive control solution was added to a mixture of 0.5 mL of 100 mM sodium phosphate buffer (pH 7.4) and 0.1 mL of a given bacterial suspension.

- For the test with S9 mix, 0.5 mL of S9 mix was used instead of the sodium phosphate buffer.1. The test without irradiation (pre-incubation method)
- For the test under dark conditions, each mixture was cultured for 20 min at 37°C with shaking. Following pre-incubation, 2 mL of molten top agar at 45 °C was added to the mixture and incubated for 48 hrs at 37°C. Then the number of the revertant colonies was counted. Every procedure was conducted under a yellow lamp to prevent light interference. CMC-Na solution was used as a negative control and the following solutions were used as positive controls.Strain without metabolic activation (μg/plate) with metabolic activation (μg/plate)TA100 AF-2 (0.01) 2-AA (1)TA1535 NaN3 (0.5) 2-AA (2)WP2 uvrA/pKM101 AF-2 (0.005) 2-AA (2)TA98 AF-2 (0.1) 2-AA (0.5)TA1537 9-AA (80) 2-AA (2)AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; NaN3: sodium azaide; 9-AA: 9-aminoacridine hydrochloride; 2-AA: 2-aminoanthracene2. The test with irradiation (plate incorporation method)For the test under irradiation, each mixture was irradiated with visible light using two fluorescent lamps (FHF32EDX-P-NU, EDX,Ra92; 32W, color temperature=8000K, 400-720 nm) at 5000lx for 1hr. Following irradiation, top agar was added and incubated for 48 hrs at 37°C. Then the number of the revertant colonies was counted. CMC-Na solution was used as a negative control and the following solutions were used as positive controls.Strain without metabolic activation (μg/plate) with metabolic activation (μg/plate)TA100 MB (1) MB (8)TA1535 MB (0.5) MB (4)WP2 uvrA/pKM101 MB (0.5) MB (2)TA98 MB (2) MB (8)TA1537 PF (1) PF (2)MB: methylene blue; PF: proflavin hemisulfate

Evaluation criteria

The results were judged positive if a two fold or more increase above the concurrent negative control in the number of revertant colonies was observed in the treatment test groups with a dose-response relationship.

Results and discussions

Test results

Species/strain	S. typhimurium TA 1535, TA 1537, TA 98, TA 100 and E. coli WP2
Metabolic activation	with and without
Test system	other: under dark conditions
Genotoxicity	negative
Cytotoxicity	no, but tested up to limit concentrations (At 400 and 1000 μg/plate, the color of agar plate was too dark (because of high concentration of C60 suspension) to check for cytotoxicity and precipitation.)
Vehicle controls valid	yes
Negative controls valid	yes
Positive controls valid	yes
Species/strain	S. typhimurium TA 1535, TA 1537, TA 98, TA 100 and E. coli WP2
Metabolic activation	with and without
Test system	other: under irradiation
Genotoxicity	negative
Cytotoxicity	no, but tested up to limit concentrations (At 400 and 1000 μg/plate, the color of agar plate

was too dark (because of high concentration of C60 suspension) to check for cytotoxicity and precipitation.)

Vehicle controls valid yes

Negative controls valid yes

Positive controls valid yes

Additional information on results

ResultsRegardless of metabolic activation and irradiation, the growth inhibition was not found in all strains and dose amounts. Although the precipitation of the test material was not seen with 50, 100, 200 μg/plate, that with 400, 1000 μg/plate was difficult to judge because the sample solution was black. However, clear precipitation was seen at the 1000 μg/plate dose in the test with irradiation. Regardless of metabolic activation and irradiation, more than double of the reverse mutation colonies was not found comparing with the negative control group in any strains and dose levels tested.

Any other information on results incl. tables

Table 1 Results of bacterial reverse mutation test of Fullerene under irradiation

S9 mix	Dose (μg/plate)	Number of revertant colonies / plate [Mean]									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2*uvrA* /pKM101		TA98		TA1537	
-	0 (sterile water)	138	(136)	9	(8)	117	(127)	29	(25)	6	(8)
		133		7		137		20		10	
	50	123	(128)	11	(12)	125	(123)	22	(23)	8	(8)
		133		13		120		24		8	
	100	129	(128)	2	(9)	116	(114)	15	(16)	10	(10)
		127		15		111		17		9	
	200	124	(139)	9	(6)	132	(123)	14	(16)	4	(5)
		153		3		113		17		5	
	400	123	(126)	9	(9)	113	(122)	20	(21)	5	(6)
		128		9		130		22		7	
	1000‡	157	(155)	8	(9)	134	(127)	18	(20)	6	(6)
		153		9		119		22		5	
+	0 (sterile water)	123	(129)	9	(7)	128	(122)	19	(21)	13	(13)
		134		5		116		23		12	
	50	126	(136)	14	(11)	160	(147)	18	(16)	9	(12)
		145		7		134		14		14	
	100	128	(131)	10	(7)	144	(144)	18	(16)	9	(13)
		133		3		143		14		16	
	200	147	(139)	10	(8)	131	(131)	18	(15)	5	(9)
		131		6		131		11		12	
	400	168	(155)	13	(12)	132	(141)	17	(16)	9	(10)
		141		10		150		15		10	
	1000	164	(171)	10	(10)	155	(154)	19	(22)	6	(7)
		178		10		153		25		8	
Positive control (S9mix -)	Substance	MB		MB		MB		MB		PF	
	Dose (μg/plate)	1		0.5		0.5		2		1	
	w light	314	(305)	24	(24)	245	(221)	82	(97)	427	(420
		295		23		196		112		413	

	w/o light	154	(133)	10	(8)	97	(98)	18	(18)	11	(15)
		111		5		98		18		19	
Positive control (S9mix +)	Substance	MB		MB		MB		MB		PF	
	Dose (μg/plate)	8		4		2		8		2	
	w light	427	(458)	52	(50)	202	(230)	137	(163)	394	(386)
		489		47		258		189		377	
	w/o light	149	(149)	13	(12)	95	(108)	19	(18)	22	(21)
		149		10		120		16		19	

MB: methylene blue FP: Proflavine Hemisulfate
‡: Precipitation (at the start and end of treatment)

Table 2 Results of bacterial reverse mutation test of Fullerene under dark conditions

S9 mix	Dose (μg/plate)	Number of revertant colonies / plate [Mean]									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2*uvrA* /pKM101		TA98		TA1537	
-	0 (1%CMC.Na)	153	(154)	12	(10)	88	(93)	12	(15)	8	(7)
		154		7		98		17		6	
	50	175	(162)	13	(9)	106	(108)	19	(17)	8	(8)
		149		5		110		14		8	
	100	161	(160)	8	(9)	102	(93)	13	(15)	6	(6)
		159		9		84		16		6	
	200	157	(164)	14	(11)	98	(99)	16	(17)	1	(2)
		170		8		100		17		3	
	400	180	(178)	13	(12)	103	(105)	23	(21)	5	(4)
		175		11		106		18		3	
	1000	188	(181)	7	(7)	152	(141)	18	(16)	3	(4)
		173		6		129		14		4	
+	0	157	(165)	11	(9)	158	(156)	22	(25)	22	(17)
		172		7		153		27		11	
	50	136	(145)	13	(11)	142	(158)	24	(24)	28	(23)
		154		8		173		23		17	
	100	145	(156)	11	(8)	159	(146)	23	(20)	25	(20)
		166		4		133		16		15	
	200	188	(185)	15	(12)	136	(161)	20	(22)	12	(19)
		181		9		185		24		25	
	400	178	(173)	7	(6)	193	(178)	22	(21)	24	(19)
		168		5		163		19		13	
	1000‡	156	(163)	8	(8)	211	(217)	18	(21)	19	(15)
		170		8		223		24		10	
Positive control(S9mix -)	Substance	AF2		NaN3		AF2		AF2		9AA	
	Dose (μg/plate)	0.01		0.5		0.005		0.1		80	
	colonies / plate	665	(703)	519	(484)	1562	(1539)	482	(501)	850	(857)
		741		449		1515		520		863	
Positive control (S9mix(S9mix +)	Substance	2AA		2AA		2AA		2AA		2AA	
	Dose (μg/plate)	1		2		2		0.5		2	
	colonies / plate	931	(877)	186	(208)	663	(691)	329	(326)	119	(126)
		823		230		718		322		133	

Applicant's summary and conclusion

Interpretation of results
negative

Conclusions
It was concluded that in vitro bacterial mutagenicity of fullerene C60 nanoparticles is negative regardless of metabolic activation and irradiation.

Endpoint study record: Genetic toxicity in vitro.002

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Matsumoto, K. et al.	2008	Final Report of in vitro Mammalian Chromosome Aberration Test (with irradiation) (Japanese)		The Institute of Environmental Toxicology	IET 07-0130	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2008-02-18
study report	Matsumoto, K. et al.	2008	Final Report of in vitro Mammalian Chromosome Aberration Test (without irradiation) (Japanese)		The Institute of Environmental Toxicology	IET 07-0131	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2008-02-18
publication	Shinohara, N. et al.	2009	In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles	Toxicol. Lett., vol.191, 289-296 (2009)					

Data access
data published

Materials and methods

Type of genotoxicity
chromosome aberration

Type of study
in vitro mammalian chromosome aberration test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 473 (In vitro Mammalian Chromosome Aberration Test)	
according to	JAPAN: Guidelines for Screening Mutagenicity Testing Of Chemicals	

GLP compliance
no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
yes

Test material identity

Identifier	Identity
common name	Fullerene C60 nanoparticles

Test material form
nanomaterial

Details on test material
- Name of test material (as cited in study report): C60 nanoparticles
- Analytical purity: > 99.5%
- Storage condition of test material: stored in cold and dark place.
- Other: Supplier: Frontier Carbon Co., Ltd., Japan.

Method

Species/strain

Species/strain	other: Chinese Hamster Lung (CHL/IU) cells
Details on mammalian cell lines (if applicable)	- Properly maintained: yes. The cell were obtained from National Institute of Hygiene Science of Japan and used at 12 passages.- Periodically checked for Mycoplasma contamination: yes. The cells used was checked as Mycoplasma-free.
Metabolic activation	with and without

Metabolic activation system	Rat liver S9 fraction prepared from male SD rats pretreated with phenobarbital and 5,6-benzoflavone.

Test concentrations

In a preliminary test, precipitation of C60 occurred at a concentration of 85 μg/mL without irradiation or 200 μg/mL with irradiation after treatment. Therefore, 100 and 200 μg/mL were selected as the highest dose in the test without irradiation and test with irradiation, respectively.Test concentration used in the chromosomal aberration test:(i) Short-term treatment assay:Under dark conditions, the concentrations used were 12.5, 25, 50 and 100 μg/mL without S9 mix, and 25, 50 and 100 μg/mL with S9 mix, respectively.Under irradiation, the concentration used were 50, 100 and 200 μg/mL without s9 mix, and 25, 50, 100 and 200 μg/mL with S9 mix, respectively. 0.1% CMC-Na was used as a negative control, and Mitomycin C (MMC), benzo[a]pyrene (B[a]P) or acridine orange (AO) were used as positive controls. (ii) Continuous treatment assay:Under dark conditions, the test concentrations used were 12.5, 25, 50 and 100 μg/mL without S9. Under irradiation, 25, 50, 100 and 200 μg/mL without S9. 0.1% CMC-Na was used as a negative control, and Mitomycin C (MMC), benzo[a]pyrene (B[a]P) or acridine orange (AO) were used as positive controls.

Vehicle

- Vehicle used: CMC-Na (sodium carboxymethyl cellulose)
- Justification for choice of vehicle: not cytotoxic.

Controls

Negative controls	yes
Solvent / vehicle controls	yes
True negative controls	no
Positive controls	yes
Positive control substance	mitomycin C benzo(a)pyrene other: acridine orange
Remarks	Acridine orange was used in the test under dark conditions.

Details on test system and conditions

METHOD OF APPLICATION:
- The CHL/IU cells were cultured on 60-mm tissue culture plates at 37 °C in a humidified atmosphere of 5% CO2 in air. The growth medium was MEM (minimum essential medium) supplemented with 10% inactivated newborn calf serum. In the short-term treatment assay, the culture medium was changed to 2.7 mL of fresh medium with or without S9 mix, and then, 0.3 mL of each concentration of C60 suspension, a negative control solution, or a positive control solution was added to the plate. After a 6-h treatment, the cells were rinsed with phosphate-buffered saline (PBS) and recultured in fresh medium for a further 18h. In the continuous treatment assay, the culture medium was changed to 4.5 mL of fresh medium without S9 mix, and then, 0.5 mL of each concentration of C60 suspension added to the plate. The cells were continuously treated for 24h. In the test under irradiation, irradiation was carried out after 1h from the start of the treatment and the cells were exposed for 1h.

DURATION
- Exposure duration: (Short-term treatment assay): 6 hours with and without S9; (Continuous treatment assay): 24 hours without S9
- Expression time (cells in growth medium): 24 hours after the start of exposure.

SPINDLE INHIBITOR (cytogenetic assays):
- Colcemid, added to the cultures at the final concentration of 0.2 μg/mL 2 hr prior to harvesting to collect metaphase cells.
STAIN (for cytogenetic assays):
- The chromosome preparations stained with 2% Giemsa solution.

NUMBER OF REPLICATIONS: 2

NUMBER OF CELLS EVALUATED: 200 well-spread metaphase cells

DETERMINATION OF CYTOTOXICITY:
- Method: relative total growth

OTHER:

Results and discussions

Test results

Species/strain	other: Chinese Hamster Lung (CHL/IU) cells
Metabolic activation	with and without
Test system	other: Short-term treatment, under dark conditions
Genotoxicity	negative
Cytotoxicity	no
Vehicle controls valid	yes
Negative controls valid	yes
Positive controls valid	yes
Species/strain	other: Chinese Hamster Lung (CHL/IU) cells
Metabolic activation	with and without
Test system	other: Short-term treatment, under irradiation
Genotoxicity	negative
Cytotoxicity	no, but tested up to limit concentrations (Cytotoxicity observed only at 200 μg/mL with S9 mix.)
Vehicle controls valid	yes
Negative controls valid	yes
Positive controls valid	yes
Species/strain	other: Chinese Hamster Lung (CHL/IU) cells
Metabolic activation	without
Test system	other: Continuous treatment, under dark conditions
Genotoxicity	negative

Cytotoxicity no
Vehicle controls valid yes
Negative controls valid yes
Positive controls valid yes
Species/strain other: Chinese Hamster Lung (CHL/IU) cells
Metabolic activation without
Test system other: Continuous treatment, under irradiation
Genotoxicity negative
Cytotoxicity no
Vehicle controls valid yes
Negative controls valid yes
Positive controls valid yes

Additional information on results

Results:Fine precipitations were observed at 100 μg/mL under the dark conditions and at 200 μg/mL with S9 mix under irradiation. No cytotoxicity was observed in the cultures treated with C60 except at 200 μg/mL with S9 mix under irradiation. The incidence of cells with structural chromosomal aberrations was less than 5% and that of polyploid cells was 0-1.0%, showing no statistically significant increase at any dose compared with the vehicle control. No increase in the incidence of either type of aberration was observed at any dose regardless of metabolic activation and irradiation.

Any other information on results incl. tables

In vitro chromosomal aberration test of Fullerenes with cultured mammalian cells under irradiation
- Direct method (S9 mix -)-

Treatment time[a)](h)	Dose (mg/mL)	Growth rate (%)	No. of cells	Number of cells with structural aberration[b)]						Total (%)		Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap	-gap	
6-18[a]	0(sterile water)	100	200	3	0	1	0	0	0	2.0	0.5	1.0
	50	94	200	1	0	1	0	0	0	1.0	0.5	1.0
	100	92	200	3	1	1	0	0	0	2.0	1.0	0.0
	200	90	200	1	0	2	0	0	0	1.0	1.0	0.0
	Pstv Cntrl (-)[b]	99	200	1	0	1	0	0	0	1.0	0.5	1.5
	Pstv Cntrl (+)[c]	86	200	11	24	65	0	0	0	39.0	35.0	2.0
24-0[a]	0(sterile water)	100	200	2	0	1	0	0	0	1.5	0.5	0.5
	50	94	200	1	0	3	0	0	0	2.0	1.5	0.0
	100	99	200	0	0	1	0	0	0	0.5	0.5	0.0
	200	93	200	0	1	3	0	0	0	2.0	2.0	0.0
	Pstv Cntrl (-)[b]	84	200	0	2	0	0	0	0	1.0	1.0	0.0
	Pstv Cntrl (+)[c]	74	200	5	17	57	4	1	0	34.5	32.5	4.0

- Direct method (S9 mix +)-

Treatment time[a)](h)	Dose (mg/mL)	Growth rate (%)	No. of cells	Number of cells with structural aberration[b)]						Total (%)		Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap	-gap	
6-18[a]	0(sterile water)	100	200	0	0	0	0	0	0	0.0	0.0	0.0
	25	96	200	2	1	2	0	0	0	2.5	1.5	0.5
	50	94	200	2	1	1	1	1	0	3.0	2.0	1.0
	100	84	200	1	0	2	0	0	0	1.5	1.0	0.0
	200	20	200	0	0	2	0	0	0	1.0	1.0	0.5
	Pstv Cntrl (-)[b]	92	200	1	0	1	0	0	0	1.0	0.5	1.0
	Pstv Cntrl (+)[c]	30	200	4	53	108	5	0	64	88.5	88.5	0.5

gap; Chromatid and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange, oth; Multiple aberrant cell, fragmentation, etc.

a) Treatment time - Recovery time

b)Acridine orange without lighting

c)Acridine orange with lighting

In vitro chromosomal aberration test of Fullerenes with cultured mammalian cells under dark conditions

- Direct method (S9 mix -)-

Treatment time[a)](h)	Dose (mg/mL)	Growth rate (%)	No. of cells	Number of cells with structural aberration[b)]						Total (%)		Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap	-gap	
6-18[a]	0(sterile water)	100	200	1	0	0	0	0	0	0.5	0.0	1.0
	12.5	97	200	1	1	0	0	0	0	1.0	0.5	0.0
	25	95	200	1	0	1	0	0	0	1.0	0.5	0.5
	50	92	200	0	1	2	1	0	0	2.0	2.0	0.5
	100	88	200	1	0	0	1	0	0	1.0	0.5	0.0
	Pstv Cntrl[b]	91	200	6	19	19	2	1	0	21.5	19.5	0.0
24-0[a]	0(sterile water)	100	200	0	1	0	0	0	0	0.5	0.5	0.0
	12.5	101	200	1	2	1	1	2	0	2.5	2.5	0.0
	25	98	200	1	2	0	0	0	0	1.5	1.0	0.0
	50	87	200	0	0	0	0	0	0	0.0	0.0	0.0
	100	80	200	2	0	0	0	0	0	1.0	0.0	0.0
	Pstv Cntrl (+)[b]	98	200	8	30	68	4	1	0	45.0	42.0	0.0

- Direct method (S9 mix +)-

Treatment time[a)](h)	Dose (mg/mL)	Growth rate (%)	No. of cells	Number of cells with structural aberration[b)]						Total (%)		Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap	-gap	
6-18[a]	0(sterile water)	100	200	0	0	1	0	0	0	0.5	0.5	0.5
	25	95	200	0	0	0	0	0	0	0.0	0.0	0.0
	50	96	200	2	0	0	0	0	0	1.0	0.0	0.0
	100	89	200	0	0	0	0	0	0	0.0	0.0	0.0
	200	88	200	3	1	0	1	0	0	2.5	1.0	0.0
	Pstv Cntrl (+)[c]	68	200	5	12	79	2	3	0	44.0	43.5	0.0

gap; Chromatid and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange, oth; Multiple aberrant cell, fragmentation, etc.

a) Treatment time - Recovery time

b)MMC

c)B(a)P

Applicant's summary and conclusion

Interpretation of results

negative

Conclusions
It was concluded that in vitro chromosomal aberration test of fullerene C60 nanoparticles is negative regardless of metabolic activation and irradiation.

7.6.2 Genetic toxicity in vivo

Endpoint study record: Genetic toxicity in vivo.001

Administrative Data
Purpose flag key study

Study result type experimental result **Study period** 2008

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies Guideline study.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Matsumoto, K. et al.	2008	Final Report of Micronucleus Test of Fullerene In Mice (Japanese)		The Institute of Environmental Toxicology	IET 07-0092	National Institute of Advanced Industrial Science and Technology (AIST), Japan		2008-02-18
publication	Shinohara, N. et al.	2009	In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles	Toxicol. Lett., vol. 191, 289-296 (2009)					

Data access
data published

Materials and methods

Type of genotoxicity
chromosome aberration

Type of study
micronucleus assay

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 474 (Mammalian Erythrocyte Micronucleus Test)	

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
common name	Fullerene C60 nanoparticles

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): C60 nanoparticles
- Analytical purity: > 99.5%
- Storage condition of test material: stored in cold and dark place.
- Other: Supplier: Frontier Carbon Co., Ltd., Japan.

Test animals

Species

mouse

Strain

ICR

Sex

male

Details on test animals and environmental conditions

TEST ANIMALS
- Source: Charles River, Japan.
- Age at study initiation: 8 weeks-old (definitive study)
- Weight at study initiation: 31.7-41.4 g (definitive study)
- Assigned to test groups randomly: yes, under following basis: body weight
- Fasting period before study: fasted for 3 hours each before and after administration.

- Housing: The mice were housed in groups of 5 animals per cage in aluminum cages (215W x 330D x 180H mm) with wire-mesh floor.
- Diet (e.g. ad libitum): MF (Oriental Yeast Co.), ad libitum
- Water (e.g. ad libitum): tap water, ad libitum
- Acclimation period: 14 days (definitive study)

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 21.5-22.5 °C
- Humidity (%): 46.7-62.8 %
- Air changes (per hr): at least ten air changes per hour
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Administration / exposure

Route of administration

oral: gavage

Vehicle(s)

- Vehicle used: 1% Tween 80
- Justification for choice of vehicle: To obtain a homogenous suspension since the test material was insoluble in water.
- Concentration of test material in vehicle: 1.12, 2.27 and 4.41 mg/mL
- Amount of vehicle (if gavage or dermal): 20 mL/kg bw
- Lot/batch no. (if required): R23305

Details on exposure

REPARATION OF DOSING SOLUTIONS:
The available highest concentration of C60 in 0.1% Tween 80 (vehicle) was 4.41 mg/mL. This suspension was used for the dosing solution for the high dose group. And the suspension was diluted with vehicle to give the dosing solutions for the middle and low dose groups.

Duration of treatment / exposure

Following 3 hours fasting, the mice were twice administered by gavage at 24-h intervals.

Frequency of treatment

Once a day, two consecutive days

Post exposure period

At 48 hrs after the first administration, the mice were sacrificed.

Doses / concentrations

0, 22, 45, 88 mg/kg bw/day

Basis actual ingested

No. of animals per sex per dose

5 animals per dose

Control animals

yes, concurrent vehicle

Positive control(s)

Mitomycin C- Justification for choice of positive control(s): Commonly used in this test system.
- Route of administration: oral by gavage
- Doses / concentrations: 10 mg/kg bw
- The mice in the positive control group were sacrificed 24h after the administration.

Examinations

Tissues and cell types examined

Bone marrow cells from femur.

Details of tissue and slide preparation

CRITERIA FOR DOSE SELECTION:
The available highest concentration of C60 in 0.1% Tween 80 was approx. 4.4 mg/mL. The highest dose was set as the maximum applicable dose.

TREATMENT AND SAMPLING TIMES (in addition to information in specific fields):
At 48 h after the first administration, five mice from each dose group were sacrificed by cervical dislocation. The mice in the positive control group were sacrificed 24 h after the administration. Fetal calf serum was instilled from one end of the femur to immediately collect bone marrow into a glass tube. Bone marrow cells were separated by centrifugation and excess serum was removed.

DETAILS OF SLIDE PREPARATION:
A drop of suspended bone marrow cells was placed on a slide glass and evenly smeared with a cover glass. Two bone marrow smears per animal were air-dried, fixed in methanol for 5 min, and stained with 3% Giemsa solution for 30 min at room temperature.

METHOD OF ANALYSIS:
For an analysis of induction of micronucleated polychromatic erythrocytes (MNPCEs), 2000 polychromatic erythrocytes (PCEs) for each mouse were examined under 1000-fold magnification using a light microscope. As an index of bone marrow inhibition, the proportion of PCEs among total (polychromatic and normochromatic) erythrocytes was determined for each mouse by counting a total of 1000 erythrocytes.

OTHER:

Evaluation criteria

The result was judged positive if a statistically significant increase in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) was observed in at least one dose level of treatment groups, accompanied by statistically significant dose-response relationship.

Statistics

Statistical analysis of frequency of MNPCEs was according to the method with numerical list by

Kastenbaum-Bowman (treatment groups) or chi-square test (positive control group). As for the proportion of PCEs, Wilcoxon rank-sum test was used.

Any other information on materials and methods incl. tables

Table 1 Results of micronucleus test of fullerene in male mice - Main test -

Dose (mg/kg)	Number of doses	Number of animals	MNPCE[a] / PCE[b](%) Mean±S.D. (Min.-Max.)		PCE / (PCE + NCE[c]) (%)Mean±S.D. (Min.-Max.)	
0[d]	2	5	0.28 ± 0.07 (0.20 - 0.35)	---	55.1 ± 3.7 (50.2 - 60.4)	---
22	2	5	0.18 ± 0.06 (0.10 - 0.25)	NS[f]	55.7 ± 8.7 (43.6 - 66.2)	NS
45	2	5	0.26 ± 0.13 (0.10 - 0.40)	NS	57.9 ± 5.9 (50.3 - 64.1)	NS
88	2	5	0.21 ± 0.11 (0.10 - 0.35)	NS	58.6 ± 5.2 (54.8 - 67.7)	NS
10 (MMC[e])	1	5	5.36 ± 1.50 (3.25 - 6.60)	***[g]	53.2 ± 14.2 (28.0 - 61.4)	NS

a) MNPCE: Micronucleated polychromatic erythrocytesb) PCE: Polychromatic erythrocytes
c) NCE: Normochromatic erythrocytesd) Negative control, 1% Tween 80e) Positive control, Mitomycin C
f) NS: Not significantly different from the concurrent vehicle control (P > 0.05)
g) *: Significantly different from the concurrent vehicle control (P≤0.001)**

Results and discussions

Test results

Sex male
Genotoxicity negative
Toxicity no effects
Vehicle controls valid yes
Negative controls valid yes
Positive controls valid yes

Additional information on results

RESULTS OF RANGE-FINDING STUDY (Toxicity study)
- Dose range: 0, 20, 40 and 78 mg/kg bw/day, two consecutive days
- Clinical signs of toxicity in test animals: No deaths occurred. No signs of toxicity observed until 24 hrs after the second administration.
- Other: Three mice of both sexes were tested in a range-finding study. Because of no sex difference in the toxicity, only males were used in the definitive study.
RESULTS OF DEFINITIVE STUDY
- Induction of micronuclei (for Micronucleus assay): The incidence of MNPCEs in the C60-treatment groups (0.18-0.26%) was not different from that in the negative control group (0.28%), but the incidence in the positive control group (5.36%) was statistically higher than that in the negative control group.
- Ratio of PCE/(PCE+NCE) (for Micronucleus assay): The incidence of PCEs in the C60-treatmnet groups (55.7-58.6%) was not different from that in the negative control group (55.1%).

Applicant's summary and conclusion

Interpretation of results
negative

Conclusions
It was conclude that micronucleus induction potential of the fullerene C60 in male ICR mice is negative under this experimental conditions.

Endpoint study record: Genetic toxicity in vivo.002

Administrative Data

Purpose flag	key study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Acceptable, well-documented publication which meets basic scientific principles

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Ema, M. et al.	2012	Genotoxicity evaluation of fullerene C60 nanoparticles in a comet assay using lung cells of intratracheally instilled rats	Regul. Toxicol. Pharmacol., 62, 419-424 (2012)	Biosafety Research Center, Foods, Drugs and Pesticides (BSRC)				

Data access
data published

Materials and methods

Type of genotoxicity
DNA damage and/or repair

Type of study
single cell gel/comet assay in rodents for detection of DNA damage

Test guideline

Qualifier	Guideline	Deviations
no guideline followed		

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
common name	fullerene C60 nanoparticles (Nanom purple SU)

Test material form

nanomaterial

Details on test material

- Name of test material (as cited in study report): C60 nanoparticles
- Analytical purity: > 99.5%
- Other: Supplier: Frontier Carbon Co. Ltd.

Test animals

Species

rat

Strain

other: Crl: CD(SD)

Sex

male

Details on test animals and environmental conditions

TEST ANIMALS
- Source: Charles River, Japan, Inc.
- Age at study initiation: 8 weeks-old
- Diet (e.g. ad libitum): CRF-1 (Oriental Yeast Co., Ltd.), ad libitum
- Water (e.g. ad libitum): tap water, ad libitum
- Acclimation period: 7 days

ENVIRONMENTAL CONDITIONS
- Temperature (°C): 20-26 °C
- Humidity (%): 35-75 %
- Air changes (per hr): 12 air changes per hour
- Photoperiod (hrs dark / hrs light): 12 hrs dark / 12 hrs light

Administration / exposure

Route of administration
intratracheal

Vehicle(s)
- Vehicle(s)/solvent(s) used: Tween 80

Details on exposure
PREPARATION OF DOSING SOLUTIONS:
- Fullerene C60 nanoparticles were dispersed in distilled water containing 0.1% Tween 80 and milled in an agar mortar for 30 min. The milled fullerene material was suspended with 50-µm zirconium particles using a high-performance dispersion machine. The C60 nanoparticle suspension was separated by centrifugation at 8000g for 60 min. The concentration was determined by high-performance liquid chromatography.

Duration of treatment / exposure
Single instillation groups and repeated instillation groups

Frequency of treatment
Once a week for 5 weeks in repeated instillation groups.

Doses / concentrations
0, 0.5, 2.5 mg/kg (single instillation)

Basis actual ingested

0, 0.1, 0.5 mg/kg (repeated instillation)

Basis actual ingested

No. of animals per sex per dose
5 animals per dose

Control animals
yes, concurrent vehicle

Positive control(s)
ethylmethanesulphonate (EMS)
- Justification for choice of positive control(s): Used commonly in this assay system.
- Route of administration: oral by gavage
- Doses / concentrations: 500 mg/kg / 50 mg/mL

Examinations

Tissues and cell types examined

lung tissue

Details of tissue and slide preparation

CRITERIA FOR DOSE SELECTION:

Dosage levels were determined based on the results of a preliminary study in which male rats were given a single intratracheal instillation of C60 nanoparticles at 0.5 or 2.5 mg/kg. Rales were heard in one of the three rats at 0.5 and 2.5 mg/kg and one rat died immediately after the instillation at 2.5 mg/kg.

TREATMENT AND SAMPLING TIMES:

In the single instillation groups, rats were sacrificed 3 or 24 h after the instillation. In the repeated instillation groups, they were sacrificed 3 h after the last instillation. At higher doses, eight rats were given C60 nanoparticles to secure five rats per group for comet assay. At lower doses, five rats per group for each time point were instilled. As a negative control, five rats were given Tween 80 at 1 mg/mL/kg by a single oral or repeated intratracheal instillation similar to the C60 nanoparticles. As a positive control, five rats were orally given a single dose of EMS at 500 mg/kg at 3 h before sacrifice. In five rats of each group, the left lobes of the lungs were used for histopathological examination and the lobes were used for the comet assay.

DETAILS OF SLIDE PREPARATION:

The left lobes of the lungs were fixed in 10% neutral buffered formalin for histopathological examination. Tissues were routinely processed, embedded in paraffin, sectioned at 4-6 μm, and stained with hematoxylin and eosin (HE).

METHOD OF ANALYSIS: COMET ASSAY

The comet assay was conducted in accordance with the standard protocol “International Validation of the In Vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the Japanese Center for the Validation of Alternative Methods (JaCVAM). Briefly, the right lobes of the lungs were washed out with homogenizing buffer (Hanks' balanced salt solutions containing 25 mmol/L EDTA-2 Na and 10% DMSO) and homogenized in 5 mL of the homogenizing buffer using a Downs homogenizer. Cell suspensions were chilled on ice for about 5 min and centrifuged at 800 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. Ten micro liters of the single cell suspension was mixed with 90 μL of 0.5% low-melting agarose gel, and 90μL of the mixture was placed on a slide pre-coated with 1.0% agarose ge1. Another 90μL of low melting agarose was added. Two slides were prepared from each rat. The slides were transferred to lysing solution (2,5 mol/L NaCl, 100 mmol/L EDTA-2Na, 10mmol/L, pH 10 Tris buffer, 10% DMSO and 1% TritonX-100) for at least one night at 4°C in the dark. The slides were next covered with chilled electrophoresis buffer (pH>13) for 20min to allow DNA to unwind. Electrophofesis was conducted at a constant voltage of 0.7 V/cm (25V) (initial current=300 mA) for20 min. The slides were transferred into neutralization buffer and held for about 10min. Subsequently, they were dehydrated with ethanol, and air-dried. The slides were stained with SYBR Gold nucleic acid gel stain which was diluted 5000-fold with TE buffer solution. Images of DNA migration were examined using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The final magnification was 200×. The images were analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments Ltd., Suffolk, UK). The comet parameter to measure DNA damage in the cells was the percentage of DNA in the tail (% Tail DNA), because % Tail DNA could be considered meaningful and easy to conceptualize. Images of 100 (50×2) cells per rat were analyzed. The mean of the % Tail DNA value (mean value of 100 cells) of each group was calculated.

Statistics

Data for C60 nanoparticle-treated groups and negative and positive control groups were analyzed using Dunnett's multiple comparison test. Data for positive control was compared to that for the negative control with Aspin-Weltch's t-test.

Results and discussions

Test results

Sex	male
Genotoxicity	negative (Single instillation groups, 3 hr after instillation)
Negative controls valid	yes
Positive controls valid	yes
Sex	male
Genotoxicity	negative (Single instillation groups, 24 hr after instillation)
Negative controls valid	yes
Sex	male
Genotoxicity	negative (Repeated instillation groups, 3 hr after instillation)
Negative controls valid	yes
Positive controls valid	yes

Additional information on results

Single instillation (autopsy at 3h after instillation) No changes were observed in clinical signs and body weights of rats given C60 nanoparticles at 0.5 and 2.5 mg/kg. At autopsy, brown-patches on the lungs were found in three rats at 0.5mg/kg and in all rats at 2.5mg/kg, whereas no brown-patches were noted in the rats given Tween 80 or EMS. In the histopathological examination of the lungs, the degree of the changes were slight. The histopathological examinations revealed the focal accumulation of macrophages in one rat of the Tween 80-treated control group, the focal accumulation of macrophages in one rat at 0.5mg/kg, and hemorrhage in one rat at 2.5mg/kg. The results of comet assays using the lung cells of rats given C60 nanoparticles are shown in Table 1. The average value of percent Tail DNA in the lung cells was 2.42 in the Tween 80-treated control group, and 2.09 and 3.07 in the group instilled with C60 nanoparticles at 0.5 and 2.5mg/kg, respectively. There was no significant difference in %Tail DNA between the Tween 80-treated control and C60 nanoparticle-treated groups. The average % Tail DNA value was 16.98 in the EMS-treated positive control group, significantly higher than that in the Tween 80-treated control group.Single instillation (autopsy at 24h after instillation) There was no change in clinical signs and body weights of rats given C60 nanoparticles at 0.5 and 2.5 mg/kg. At autopsy, a single brown-patch was noted in the lungs of one rat given Tween 80. A single brow D-patch on the lungs was observed in two rats at 0.5mg/kg, and multiple brown-patches on the lungs were found in all rats at 2.5mg/kg. The degree of all these changes was slight. The focal accumu1ation of macrophages and hemorrhage in the alveoli in one rat each were noted in the Tween 80-treated control group. The focal accumulation of macrophages in the alveoli in two rats was observed at 0.5mg/kg. Multifocal hemorrhages in the alveoli in four rats, deposition of the test substances in macrophages of the alveoli and cellular in filtration of neutrophils and macrophages in the alveoli in five rats, thickening of the alveolar wall in two rats, and acute pneumonia with focal deposition of the hematoidin crystals in one rat were found at 2.5mg/kg. The results of comet assays using the lung cells of rats given C60 nanoparticles are summarized in Table. The average %Tail DNA value was 3.13 in the Tween 80-treated control group, and 2.53 and 3.07 in the group given C60 nanoparticles at 0.5 and 2.5mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C60 nanoparticle-treated groups. Repeated instillation (autopsy

at 3 h after instillation) No difference was found in clinical signs and body weights between the Tween 80-treated control group and groups given C60 nanoparticles at 0.1 and 0.5 mg/kg. At autopsy, no brown-patches were found in the lungs of rats given C60 nanoparticles at 0.1 mg/kg or Tween 80. Multiple brown-patches on the lungs were observed in all rats given C60 at 0.5mg/kg. The degree of all these changes was slight. No histopathologica1 changes in the lungs were noted in rats of the Tween 80-teated control group. Acute focal pneumonia in two rats and focal hemorrhages in the alveoli in one rat were observed at 0.1 mg/kg. The focal accumulation of macrophages and hemorrhage in the alveoli in one rat each and focal or multi focal deposition of the test substances in the macrophages in the alveoli and cellular infiltration of the macrophages in the alveoli in five rats were found at 2.5 mg/kg. The results of comet assays of the lung cells are also summarized in Table. The average value for % Tail DNA was 4.65 in the Tween 80-treated control group, and 6.80 and 5.08 in the group instilled with C60 nanoparticles at 0.5 and 2.5 mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C60 nanoparticle-treated groups. The average value was 16.66 in the EMS-treated positive control group, significantly higher than in the C60-treated groups.

Any other information on results incl. tables

Table 1 Effects of C60 nanoparticles on % tail DNA in lung cells following a single or repeated intratracheal instillation

Treatments	Groups (compounds)	No. of rats	No. of cells analyzed/rat	% Tail DNA
A single intratracheal instillation (autopsy at 3h after instillation)	Negative control (Tween 80)	5	100	2.42 ± 0.76
	C60 (0.5 mg/kg)	5	100	2.09 ± 0.62
	C60 (2.5 mg/kg)	5	100	3.07 ± 0.93
	Positive control (EMS)	5	100	16.98 ± 7.08*
A single intratracheal instillation (autopsy at 24h after instillation)	Negative control (Tween 80)	5	100	3.13 ± 0.76
	C60 (0.5 mg/kg)	5	100	2.53 ± 0.62
	C60 (2.5 mg/kg)	5	100	3.07 ± 0.93
Repeated intratracheal instillation (autopsy at 3h after instillation)	Negative control (Tween 80)	5	100	4.65 ± 1.56
	C60 (0.1 mg/kg)	5	100	6.80 ± 1.76
	C60 (0.5 mg/kg)	5	100	5.08 ± 0.83
	Positive control (EMS)	5	100	16.66 ± 1.94*

Values are given as mean± S.D.
***: Significantly different from the negative control group (p< 0.05)**

Overall remarks, attachments

Remarks on results including tables and figures

In conclusion, the present findings showed that C60 nanoparticles did not induce DNA damage in the lung cells of rats intratracheally instilled with C60 nanoparticles even at doses that elicited inflammatory responses.

Illustration (picture/graph)

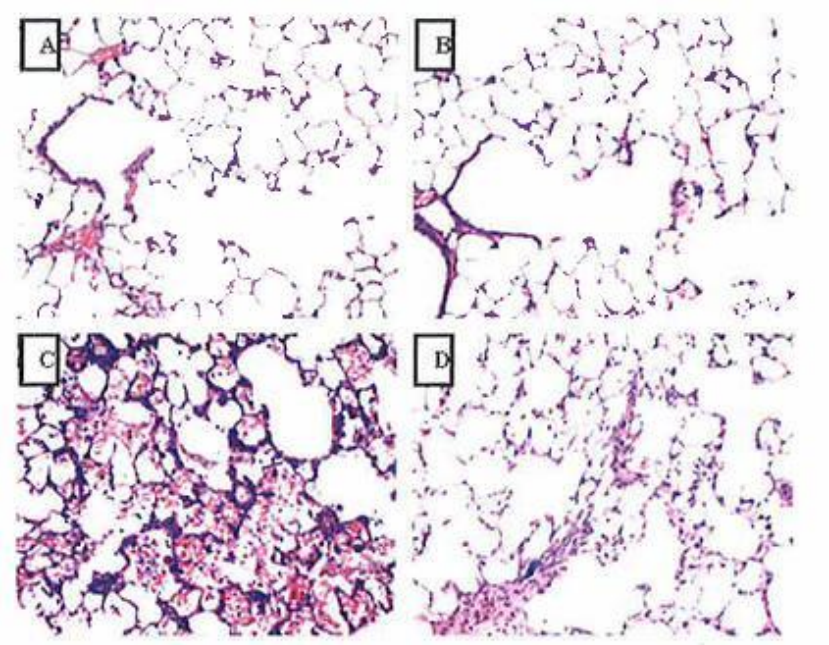

Fig. HE staining of lung histopathology sections (magnification, 20x),
(A) negative control: no significant lesion, (B) a single intratracheal instillation at 2.5 mg/kg (autopsied 3h after instillation): no significant lesion, (C) a single intratracheal instillation at 2.5 mg/kg (autopsied 24h after instillation): hemorrhage, infiltration of alveolar macrophages and neutrophils in the alveolus, thickening of alveolar wall, and deposition of the test substances in alveolar macrophages, (D) repeated intratracheal instillation for 5 weeks at o.5 mg/kg/week (autopsied 3h after the last instillation): hemorrhage, infiltration of alveolar macrophages and neutrophils in the alveolus, thickening of alveolar wall, and deposition of the test substances in alveolar macrophages.

Applicant's summary and conclusion

Interpretation of results

negative

Conclusions

It was concluded that C60 nanoparticles had no potential for DNA damage in comet assays using the lung cells of rats given C60 even at doses causing inflammation.

7.7 Carcinogenicity

7.8 Toxicity to reproduction

7.9 Specific investigations

7.10 Exposure related observations in humans

7.11 Toxic effects on livestock and pets

7.12 Additional toxicological information

Endpoint study record: Intratrach Instill Additional toxicological information. 001

Administrative Data

Purpose flag key study

Study result type experimental result **Study period** 2008

Reliability 2 (reliable with restrictions)

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	NEDO project(P06041):Research and development of a nanoparticle characterisitic evaluation technology	2010		Nanomaterials risk assessment document-Fullerene(C60)-	University of Occupational and Environmental Health, Japan				
publication	Morimoto, Y. ,et al.	2010	effect of well-characterized fullerenes in inhalation and intratracheal instillation studies	Particle andFibre Toxicology 2010, 7:4	University of Occupational and EnvironmentAl Health, Japan				
publication	Ogami et al.	2011	Pathological features of rat lung following inhalation and intratracheal instillation of C60 fullerene	Inhal. tox., 2011; 23 (7): 407-416					

Materials and methods

GLP compliance

no

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom purple

Confidential details on test material

The pristine fullerenes are disintegrated in a mortar with adding Tween80 solution. Then they are stirred and dispersed by beads mill (surface treated zirconia beads by plasma, 50μm of average diameter) after adding Tween80 solution. The dispersion was carried out under an anaerobic atmosphere and light shield to avoid oxidation. The supernatant (concentration 0.5mg/mL) is retrieved after centrifuging. The particle size, the state of agglomeration, the stability of dispersion, the crystal structure and the oxidation state are measured by dynamic light scattering, TEM and liquid chromatography. The mass based average diameter

in dispersion is 33 nm.

Method

Any other information on materials and methods incl. tables

The dispersions of fullerenes in distilled water (containing Tween80) with concentrations of0 (control),0.1mg (0.33mg/kg), 0.2mg (0.66mg/kg) and 1mg (3.3mg/kg) are intratracheally instilled in a single doses to Wistar male rats. On3 days, 1 week, 1, 3, 6, 12 and 24 months after instillation, lung weights, biomarkers in blood, BAL, and lung representing lung inflammation and fibrosis, histopathological observation of lungs tissues (quantification of inflammation and fibrosis) and other organs (brain, nasal cavity, testis, liver, kidney and spleen)are examined.

Results and discussions

Any other information on results incl. tables

Lung wet weight increase was observed only at 1 week after instillation. As finding of BAL examination, total cell number has increased until 1 week; and the number of neutrophil cell has increased until 3 months after instillation. For 3.3 mg/kg dose group, the increase was extremely small compared with negative control group, which could be considered toxicologically negligible small. Stehenia is observed until 1 week as HO-1 gene expression in lung tissue. Inflammation degree has increased until 3 days for 0.33 and 0.66 mg/kg dose group and until 1 week for 3.3 mg/kg dose group then returned to the same level with negative control group. As inflammation in blood, no increase is observed for number of leukocyte and neutrophil. No abnormal finding was observed in other organs for the cerebrum, the cerebellum, the nasal cavity, the spermary, the liver, the kidney and the spleen.

Table 1 Summary of histopathological findings in the lungs of rats intratracheally instilled with C60 or NiO

Group	Control (0.1%Tween 80)						C60 0.1 mg						C60 0.2 mg						C60 1 mg						NiO 0.2 mg					
Time point after instillation	3d	1w	1m	3m	6m	12m	3d	1w	1m	3m	6m	12m	3d	1w	1m	3m	6m	12m	3d	1w	1m	3m	6m	12m	3d	1w	1m	3m	6m	12m
Alveolar macrophages accumulation		±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	+	±	+	+	+	+	+	+	+	+	+	++	+	+
Inflammatory cell infiltration		±				±		±	±	±	±		±	±	±		±	±	±		±			±	±	+	±	+	±	±
Epithelial cell hyperplasia																			±						+	±		+		±

Collagen deposition at alveolar wall																		±	±	±			±	±			±		
Micrognuloma																		±						±	±	+	+	±	±
Foamy macrophage																								±	±	+	++	+	+

d, day; w, week; m, month
Grade of changes : , none;±,slight;+,moderate; ++, marked.

Overall remarks, attachments

Attached full study report

Endpoint study record: (In vitro cytotoxicity) Additional toxicological information.002

Administrative Data

Purpose flag key study

Study result type experimental result

Reliability 2 (reliable with restrictions)

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	Nedo project (AIST)								
	M. Horie et al	2010	In vitro investigation of cellular responses induced by fullerene C60. Free Radical Biology and Medicine	J Biochem (2010) 148 (3): 289-298.					

Materials and methods

Test guideline

Qualifier	Guideline	Deviations
no guideline available		

Principles of method if other than guideline

In vitro tests using three kinds of cell lines, human lung adenocarcinoma cell line (A549); human keratinocyte cell line (HaCaT); and human acute monocytic leukemia cell line (THP-1)

Test materials

Test material identity

Identifier	Identity
CAS number	99685-96-8 Nanom Purple

Method

Any other information on materials and methods incl. tables

*In vitro*tests using three kinds of cell lines, human lung adenocarcinoma cell line (A549); human keratinocyte cell line (HaCaT);and human acute monocytic leukemia cell line (THP-1), have been executed to examine cell viability, oxidative stress and apoptosis as endpoints. Along with the endpoints, absorption of protein and stability of dispersion have been measured as a material characterization.

C60 powder was dispersed in FBS solution at concentration of 10 mg/mL and stirred for 2 h. Next, 60 mL of this dispersion was added to 250 mL of DMEM-FBS, and the mixture was stirred for 30 min with 50 μm-zirconia beads by using a bead mill (79.1 m/s) under nitrogen purge. Then, 300 mL of DMEM was added to the mixture in the bead mill and stirred for 1 h. Three hundred milliliters of this mixture was used as high-concentration C60 dispersion, designated as No.2. Next, 60 mL of freshly prepared C60-FBS dispersion and 250 mL of DMEM were added to the C60-DMEM-FBS dispersion remaining in the bead mill, and the solution was stirred for 60 min; 500 mL of this C60-DMEM-FBS dispersion was collected as a high-concentration C60 dispersion, designated as No.1. Both these dispersions, namely, No.1 and No.2, were centrifuged at 1000 × g for 10 min. The supernatant was collected and centrifuged at 2000 × g for 10 min, 4000 × g for 10 min, and 8000 × g for 45 min. The supernatant was collected and used as the C60 dispersion. To prevent the depletion of the medium due to adsorption of the medium components such as proteins onto C60, 5% FBS (v/v) was added to the C60 dispersion and the dispersions were passed through a filter of pore size 0.45 μm („). A low-concentration C60 dispersion was obtained by preparing 5-fold and 10-fold dilutions of the high-concentration C60 dispersion No.2 with DMEM-FBS. The particle size of C60 in dispersion was 100-130 nm by DLS measurement and stable for more than 4 days.

HaCaT and A549 cells were exposed to the above dispersions for 6 and 24 hrs and viabilities of cells were measured by MTT assay. LDHrelease, level of oxidative stress (ROS level in cells, lipid oxidation level) and various apoptosis-associated markers (Caspase-3) were also measured. In addition, analysis of DNA damage and TEM observation were conducted.

Results and discussions

Any other information on results incl. tables

Cellular effects of homogeneous and stable dispersion of C60 nanoparticle in culture medium without surfactant and organic solvent were tested. There was no LDH release from cells exposed to C60 for 24 hours In addition, apoptosis and necrosis cells were not increased compared by control cell in flow cytometry measurement. At the same time cell proliferation rate were decreased. Although those results suggest that administration of C60 to cell causes decrease of the proliferation rate but its influence on cell death is very small. ROS level and lipid oxide increases with time in the cells exposed to C60. Exposure to high concentrations of C60 led to an increase HO-1 expression on HaCaT cell. These results suggest that

the administration of C60 induce the oxidative stress on the cell. Furthermore, the DNA damage of cell significantly increased depending on the concentration of C60. Because the C60 particles did not translocate into the nucleus, the DNA damage may be caused by indirect influence induced by oxidative stress rather than direct reaction between C60 and nucleus. As the oxidative stress does not affect the viability of cells and the expression of antioxidants is observed, the effect of C60 could be repaired by various DNA repair enzymes within a few hours though it causes the oxidative stress to the cells. However, the long-term influence of DNA damage cannot be denied. In this experiment, the oxidation stress was observed at higher concentrations in this test than in the past studies.
In conclusion,C60 did not influence viabilities and LDH activities of HaCaT and A549 cells. Apoptosis was not increased but cell proliferation rate slightly decreased. The oxidative stress marker and DNA damage were observed. There was no evidence that C60 will cause adverse effect, but possibility of the long-term effect due to the DNA damage cannot be denied.

8. ANALYTICAL METHODS

9. RESIDUES IN FOOD AND FEEDINGSTUFFS

10. EFFECTIVENESS AGAINST TARGET ORGANISMS

11. GUIDANCE ON SAFE USE

12. LITERATURE SEARCH

13. ASSESSMENT REPORTS

14. INFORMATION REQUIREMENTS