Committee on MUTAGENICITY

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COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COT)

Guidance on Genotoxicity Testing Strategies for Manufactured Nanomaterials

Background

1. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) has a remit to provide UK Government Departments and Agencies with advice on the most suitable approaches to testing chemical substances for genotoxicity. The COM views regarding the most appropriate strategy for genotoxicity testing are outlined in full in the COM (2011)[RB1] "*Guidance On A Strategy For Genotoxicity Testing Of Chemical Substances*".

~~1.~~2. In brief, the COM recommend a staged approach to genotoxicity testing. **Stage 0**, in the absence of test data from adequately designed and conducted genotoxicity tests, consists of preliminary considerations of the test chemical substance, including, physico-chemical properties, Structure Activity Relationships (SAR), and information from screening tests. **Stage 1** consists of *in vitro* genotoxicity tests that provide information on three types of genetic damage (namely, gene mutation, chromosomal damage and aneuploidy) and gives appropriate sensitivity to detect chemical genotoxins. **Stage 2** consists of *in vivo* genotoxicity tests which are chosen on a case-by-case basis to address any genotoxic endpoints identified in Stage1; investigate genotoxicity in tumour target tissue(s) and/or site of contact tissues; investigate potential for germ cell genotoxicity; and investigate potential genotoxicity for chemicals where high/moderate and prolonged exposure is anticipated, even if negative in Stage 1.

3. As part of an update of the full COM guidance, a number of additional topics have been included for consideration. One such area addresses genotoxic testing strategies for manufactured nanomaterials. A brief summary of this area is provided in the full guidance document, while this document outlines in more detail the initiatives that have been carried out in the area. It is recognised by the Committee that this is an area that is rapidly developing. As such, COM will keep a watching brief and update this guidance document with new information as it becomes available.

OECD initiatives

~~2.~~4. OECD nanomaterial research is conducted through the Working Party on Manufactured Nanomaterials (WPMN) and the Working Group of National Coordinators of the Test Guidelines Programme (WNT). OECD research has focused on the development of regulatory test guidelines, guidance on nanosafety, to support the regulatory safety needs of member countries (Steinhäuser *et al.*, 2017).

Working Party on Manufactured Nanomaterials

~~3.~~5. The OECD WPMN was set up in 2006, with the aim '*to ensure that the approach for hazard, exposure and risk assessment is of a high, science-based and internationally harmonised standard*' (OECD, 2018a).

~~4.~~6. A WPMN Workshop on the Genotoxicity of Manufactured Nanomaterials was held in 2013 to reflect the completion of the WPMN Testing Programme dossiers, the publication of the final report by EU NANOGENTOX Joint Action, an EU funded collaboration project coordinated by ANSES, and the current process within the OECD TG Programme to revise seven genotoxicity test guidelines (TGs)[1] (OECD, 2014).

~~5.~~7. The purpose of the Workshop was to review the genotoxicity data from the OECD Testing Programme and EU NANOGENOTOX Joint Action, to explore if there is a need to adapt existing OECD Test Guidelines on genotoxicity for testing the genotoxicity of nanomaterials, and/or investigate the need to develop new Test Guidelines or guidance material. The workshop also aimed to identify any knowledge gaps and regulatory needs in the area of genotoxicity testing of nanomaterials (OECD, 2014; OECD, 2018a).

WPMN Testing Programme

~~6.~~8. The WPMN Testing Programme was led by the Joint Research Centre (JRC) and the International Council for Animal Protection in OECD Programmes (ICAPO). The aim of the programme was to generate datasets on a number of endpoints, including genotoxicity endpoints, for a list of nanomaterials. By evaluating which of the existing OECD TGs were used for testing nanomaterials, what other non-guideline methods were applied, and the potential limitations of each assay used, information was obtain to contribute to nanomaterial-relevant updates of the TGs (OECD, 2018a; Rasmussen *et al.*, 2016).

~~7.~~9. The nanomaterials tested included gold and silver nanoparticles (Au NP/Ag), zinc oxide (ZnO), titanium dioxide (TiO_2), single walled carbon nanotubules (SWCNTs), multiwalled carbon nanotubules (MWCNTs), cerium oxide (CeO_2), silicon dioxide (SiO_2), fullerenes C60.

[1] Four *in vivo* TGs (TGs 474, 475, 478, and 483), two *in vitro* TGs (TGs 473 and 487), the mammalian cell gene mutation assay, and the introduction to the test guidelines on genotoxicity.

~~8.~~10. A number of *in vitro* assays (TG and non-TG) were assessed, namely TGs 471(Bacterial Reverse Mutation Test), 473 (*In vitro* Mammalian Chromosomal Aberration Test), 476 (*In vitro* Mammalian Cell Gene Mutation Tests), 487 (*In vitro* Mammalian Micronucleus Test), the Comet assay and double strand breaks assay (H2AX phosphorylation). *In vivo* assays that were assessed included, TG 474 (*In vivo* Mammalian Erythrocyte Micronucleus test) and TG 489 (*In vivo* Mammalian Alkaline Comet assay).

Bacterial Reverse Mutation Test (OECD TG 471)

~~9.~~11. The bacterial reverse mutation test (Ames test) uses amino-acid requiring strains of *Salmonella typhimurium* and *Escherichia coli*, to detect point mutations, involving substitution, addition or deletion of DNA base pairs. The test is relatively easy to perform, is rapid, and inexpensive and is often used as an initial screen of genotoxicity, in particular, to detect point mutation-inducing activity. Extensive data have shown that many chemicals which are Ames-positive are also positive in other genotoxicity tests such as micronuclei tests. There are, however, some mutagenic chemicals that are not detected by the Ames test, mainly due to the specific nature of the endpoint detected, differences in metabolic activation, or differences in bioavailability between bacterial and mammalian cells (OECD, 1997).

~~10.~~12. The mutagenic potential of Au, Ag, ZnO, SiO_2 and TiO_2 nanomaterials, SWCNTs, MWCNTs and fullerenes was assessed in the WPMN Testing Programme using the Ames test, in most cases, with and without metabolic activation (S9 mixture) (OECD, 2018a).

Specific requirements for nanomaterials

~~11.~~13. TG 471 does not contain any nanomaterial-specific considerations. One of the studies noted the lack of uptake of the nanomaterials tested into bacteria, and thus the possibility of false negative results (OECD, 2018a).

Results and discussion

~~12.~~14. Of the eight types of nanomaterials tested, none showed mutagenic activity in the bacterial reverse mutation test under the conditions of TG 471 (OECD, 2018a).

~~13.~~15. The Ames test was previously discussed at the OECD Expert Meeting on Genotoxicity of Manufactured Nanomaterials [ENV/JM/MONO(2014)34] and it was concluded that '*The use of the Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of nanomaterials. The test guidelines programme should consider modifying the applicability domain within this test guideline accordingly'* (OECD, 2014). A report by the Scientific Committee on Consumer Safety (SCCS) on the Guidance on Safety Assessments of Nanomaterials in Cosmetics also concluded that '*although reports can be found on positive bacterial reverse mutation test, there are doubts if the Ames test is an accurate representative test for genotoxicity*'(SCCS, 2012). OECD noted that this is

because bacterial cells lack the ability to uptake nanomaterials through endocytosis, and some nanomaterials have bactericidal activity. Therefore the Ames test is not regarded as being suitable for the genotoxicity testing of nanomaterials (OECD, 2018a).

In vitro Mammalian Chromosomal Aberration Test (OECD TG 473)

~~14.~~16. The *in vitro* chromosomal aberration test aims to identify substances that cause structural chromosomal aberrations in cultured mammalian cells. Following exposure to the test chemical, the cells are treated with Colcemid® or colchicine to induce metaphase arrest and analysed for chromosomal aberrations. There are two types of structural aberrations seen, namely chromosome or chromatid aberrations. Although polyploidy2 could also arise in chromosome aberration assays *in vitro,* alone it does not indicate aneugenic potential and could simply indicate cell cycle perturbation or cytotoxicity (OECD, 2016d).

~~15.~~17. The mutagenic potential of Au, Ag, ZnO and SiO_2 nanomaterials, SWCNTs, MWCNTs and fullerenes was assessed using the *in vitro* mammalian chromosomal aberration test, in most cases with and without metabolic activation (S9 mixture). Most of the nanomaterials were tested with the Chinese hamster ovary (CHO) cell line, however, Chinese hamster lung V79 cells, Chinese hamster lung (CHL)/IU, human lymphocytes, and (human embryonic lung cells (WI-38) were also used. A 4 h exposure period was predominantly used in conjunction with metabolic activation and between 4 h and 24 h in the absence of metabolic activation; other exposure times were also used in some instances (OECD, 2018a).

Specific requirements for nanomaterials

~~16.~~18. TG 473 states that specific adaptations may be needed for the testing of nanomaterials, but such adaptations are not described (OECD, 2018a).

Results and discussion

~~17.~~19. Of the seven types of nanomaterials tested in the WPMN Testing Programme, none showed mutagenic activity in the *in vitro* mammalian chromosomal aberration test under the conditions of TG 473 (OECD, 2018a).

~~18.~~20. No information regarding potential interference of the nanomaterials with the assay was reported.

In vitro Mammalian Cell Gene Mutation Tests using Hprt and xprt genes (OECD TG 476)

~~19.~~21. The *in vitro* mammalian cell gene mutation test aims to detect gene mutations induced by the test substance in cultured mammalian cells. The cell lines measure

[2] Numerical chromosomal aberrations in cells or organisms involving entire set(s) of chromosomes, as opposed to an individual chromosome or chromosomes (aneuploidy).

forward mutations in reporter genes, such as the endogenous hypoxanthine-guanine phosphoribosyl transferase (HPRT; Hprt in rodent cells, HPRT in human cells), and the xanthine-guanine phosphoribosyl transferase (XPRT), although the latter is currently less widely used for regulatory purposes. Mutant cells deficient in HPRT or XPRT enzyme activity are resistant to the cytostatic effects of the purine analogue 6-thioguanine. The Hprt or gpt-proficient cells, in the HPRT or XPRT test respectively, are sensitive to 6-thioguanine, causing inhibition of cellular metabolism and halting further cell division. Therefore, mutant cells are able to proliferate in the presence of 6-thioguanine, whereas normal cells, which contain the Hprt or gpt enzyme, are not able to proliferate. Both the HPRT and XPRT mutation tests detect different mutations. As well as the detection of base pair substitutions, frameshifts, small deletions and insertions in the HPRT test, the autosomal location of the gpt transgene may allow mutations resulting from large deletions and possibly mitotic recombination to be detected (OECD, 2016e).

~~20.~~22. The mutagenic potential of ZnO, SiO_2 and TiO_2 nanomaterials and MWCNTs was assessed by using the *in vitro* mammalian cell gene mutation tests, in most cases, both with and without metabolic activation (S9 mixture). All nanomaterials were tested using the L5178Y TK +/- cells and CHO cells were used in two studies to test SiO2 (OECD 2018).

Specific requirements for nanomaterials

~~21.~~23. TG 476 does not contain any nanomaterial-specific considerations.

Results and discussion

~~22.~~24. Of the four types of nanomaterials tested, none showed mutagenic activity in the *in vitro* mammalian cell gene mutation test, under the conditions used.

~~23.~~25. No information regarding potential interference with the assay was reported for MWCNTs, SiO_2 and TiO_2. For ZnO, an increased turbidity was reported at higher concentrations, which could potentially influence the outcome of the test (OECD 2018).

~~24.~~26. The WPMN Testing Programme report concluded that '*the in vitro mammalian cell gene mutation tests (TG 476) is considered as an alternative for the bacterial reverse mutation test (TG 471), as no report has yet identified specific limitations when testing nanomaterials with TG 476'* (OECD, 2014; OECD, 2018a).

In vitro Mammalian Micronucleus Test (OECD TG 487)

~~25.~~27. The *in vitro* micronucleus test aims to detect micronuclei in the cytoplasm of interphase cells following exposure to the test substance, which represents DNA damage that has been transmitted to daughter cells. Both aneugens and clastogens can be detected in cells that have undergone cell division during or after exposure to the test chemical. Micronuclei may originate from acentric chromosome fragments,

or from whole chromosomes that are unable to migrate to the poles during cell division. Hence the *in vitro* micronucleus test is a comprehensive test for investigating chromosome damaging potential of a test substance. To analyse the induction of micronuclei, it is essential that mitosis has occurred in both treated and untreated cultures. The TG allows for the test to be carried out with or without cytokinesis block, providing there is evidence that mitosis has occurred in the cells being analysed (OECD, 2016f).

~~26.~~28. Micronuclei may also arise from lagging chromosomes[3], hence this test also allows the detection of substances that induce aneuploidy that are otherwise difficult to study using conventional chromosomal aberration tests, e.g. OECD TG 473. However, TG 487 specifies that techniques such as Fluorescence *In Situ* Hybridization (FISH) should be used to differentiate between substances inducing changes in chromosome number and/or ploidy from those inducing clastogenicity (OECD, 2018a). FISH can also provide additional information on the mechanisms of chromosome damage and micronuclei formation (OECD, 2016f).

~~27.~~29. The mutagenic potential of SiO_2 and TiO_2 nanomaterials was assessed in the WPMN Testing Programme using the *in vitro* mammalian micronucleus test, without metabolic activation. Both nanomaterials were tested using human bronchial epithelial cells (16-HBE), adenocarcinomic human alveolar basal epithelial cells (A549), human bronchial epithelium cells (BEAS-2B), and human primary lymphocytes, and TiO_2 nanomaterials were tested using normal human keratinocyte cells (NHK). The exposure time used across all cell lines was between 30 h and 52 h, and cytokinesis was blocked, in most assays, using cytochalasin B (OECD, 2018a).

Specific requirements for nanomaterials

~~28.~~30. TG 487 states that specific adaptations may be needed for the testing of nanomaterials, but such adaptations are not described (OECD, 2018a).

Results and discussion

~~29.~~31. Neither SiO_2 nor TiO_2 nanomaterials induced micronuclei formation in 16-HBE cells under the conditions of the assay. Some positive results were observed in the other cell lines tested, as detailed below.

~~30.~~32. All TiO_2 nanomaterials induced a dose-dependent increase in the frequency of binucleated cells with micronuclei in NHK cells. With BEAS-2B cells, whilst four out of six studies reported negative results with some TiO_2 nanomaterials, one study reported an increase in micronucleus frequency in binucleated cells, and one reported an equivocal result as an increase in micronuclei was only observed at some concentrations. Moreover, in two studies using human blood lymphocytes,

[3] Lagging whole chromosomes are those that are not included in the daughter nuclei produced by mitosis due to incorrectly attaching to the spindle during the segregation of chromosomes in anaphase.

TiO_2 nanomaterials induced damage at the lower concentrations tested, but not at higher concentrations. TiO_2 did not induce aneugenic or clastogenic damage in A549 or Caco-2 cells.

~~31.~~33. Using the cytokinesis block version of the assay, SiO_2 nanomaterials (NM-200, NM201 and NM-202) caused a dose-dependent increase in the frequency of binucleated cells with micronuclei, when using Caco-2 cells, in two out of three studies. NM-201 and NM-202 were also positive in A549 cells. NM-203 nanomaterials gave conflicting findings in A549, Caco-2 and BEAS-2B cells, as some studies reported negative results whilst others reported a dose-dependent increase in the frequency of binucleated cells with micronuclei. SiO_2 did not induce damage in human lymphocytes (OECD, 2018a).

~~32.~~34. No information regarding potential interference of the nanomaterials with the assay was reported.

~~33.~~35. The OECD Expert meeting on Genotoxicity of Manufactured Nanomaterials (OECD, 2014) concluded that '*the extent of cellular uptake is a critical factor to consider when interpreting test results. In some circumstances, a lack of uptake in a mammalian cell may indicate a low intrinsic hazard from a direct genotoxicity perspective*' and recommended that '*the test guidelines program should consider modification of the in vitro micronucleus assay to recommend, where cyto B is used, its addition using a post-treatment or delayed co-treatment protocol, in order to ensure a period of exposure of the cell culture system to the nanomaterial in the absence of cyto B*'.

~~34.~~36. The Scientific Committee on Consumer Safety (SCCS, 2012) noted that '*cytochalasin B, which is often used in to inhibit cytokinesis, may inhibit endocytosis and hence has been suggested to lead to false negative outcomes with particles. For several types of nanoparticles (e.g. titanium dioxide, multi-walled carbon nanotubes), the microscopic evaluation of cytokinesis-block proliferation index and micronucleus identification was found to be inappropriate at high testing concentrations due to the overload of agglomerates*'.

In vitro Comet Assay (single-cell gel electrophoresis)

~~35.~~37. The Comet assay aims to detect single and double-stranded DNA damage and repair in eukaryotic cells following treatment with the test substance, by measuring the migration of DNA from individual nuclei following alkaline treatment. The assay involves encapsulating cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline conditions, followed by electrophoresis of the suspended lysed cells, to migrate the DNA fragments through the electrophoresis gel. It is thought that the comet pattern observed is due to the loops containing a break losing their supercoiling properties, becoming free to extend toward the anode (OECD, 2018a).

~~36.~~38. The assay may be modified by using lesion-specific endonucleases, such as formamido-pyrimidine-DNA glycosylase (FPG) and endonuclease III (ENDOIII, also known as Nth), that can detect oxidative damage in DNA bases. This is known as the alkaline Comet assay.

~~37.~~39. The mutagenic potential of TiO_2 and SiO_2 nanomaterials and SWCNTs was assessed in the WPMN Testing Programme using the Comet assay with various cell lines. SiO_2 nanomaterials were tested in A549, BEAS-2B, Caco-2 cells and 16-HBE cells and primary rat alveolar macrophages; TiO_2 nanomaterials in EpiDerm^{TM}, 16-HBE, A549, Caco-2, V79 and BEAS-2B cells and normal human epidermal keratinocytes; and SWCNTs in V79, Mouse FEI-MML epithelial cells, BEAS-2B and RAW 264.7 cells, normal human mesothelial cells, malignant human mesothelial cells, human peripheral blood lymphocytes and primary mouse embryo fibroblasts (OECD, 2018a).

Specific requirements for nanomaterials

~~38.~~40. No comments were made regarding the applicability of the Comet assays for nanomaterials or whether any adaptations were e needed (OECD, 2018a).

Results and discussion

~~39.~~41. For most of the nanomaterials tested, studies reported largely negative or equivocal results. If positive results were found, in many cases a dose response relationship could not be established, or effects were only seen at one exposure time. Hence, authors note that it was difficult to conclude on the genotoxicity of the nanomaterials tested. Only SWCNTs (Sigma-Aldrich CNT) induced a dose-dependent increase in DNA damage in BEAS-2B cells after 48 and 72 hours, noted by the authors to be the longest exposure period of all studies carried out.

~~40.~~42. In addition, due to the large variability in the experimental conditions used, such as cell line, the concentration range tested, exposure time and dispersion protocols, the authors concluded that comparison between results is difficult.

~~41.~~43. Authors noted that '*in contrast to other in vitro genotoxicity studies, the DNA damage picked up by the Comet assay may be repaired at later cell cycles. Therefore, the results from the Comet Assay can only give an indication of potential genotoxicity of environmental chemicals'* (OECD, 2018a).

Double-Strand Breaks assay (Histone H2AX phosphorylation)

~~42.~~44. DNA double-strand breaks (DSBs) are one of the most critical types of DNA damage, formed as a result of genotoxic insult. Following DSB formation, phosphorylation of the histone H2AX, forming γ-H2AX, is one of the earliest molecular responses. Many copies of γ-H2AX are generated at DSBs and can be detected *in vitro* using immuno-histochemical methods employing specific monoclonal and/or polyclonal antibodies against the H2AX C-terminal phosphorylated peptide.

~~43.~~45. Total γ-H2AX levels can be measured directly, or in lysates from cells or tissues. If cells or tissues are used, γ-H2AX levels are measured directly in cell nuclei by microscopy or fluorescence-activated cell sorting (FACS). Many γ-H2AX molecules are formed at DNA break sites, which create bright foci, allowing the detection of individual DSBs. If measurement is made in lysates , immunoblotting or enzyme-linked immunosorbent assays (ELISAs) can be used to establish the overall γ-H2AX levels in the lysates (OECD, 2018a).

~~44.~~46. In the WPMN Testing Programme, microscopic detection and counting of γ-H2AX foci was employed

~~45.~~47. The mutagenic potential of SWCNTs only was assessed, through use of the DSB assay with normal human dermal fibroblasts or normal and malignant mesothelial human cells (OECD, 2018a).

Specific requirements for nanomaterials

~~46.~~48. No comments were made by the authors regarding the applicability or adaptation of the DSB assay for use with nanomaterials (OECD, 2018a).

Results and discussion

~~47.~~49. Limited data were presented as only two studies were reported. A 2.7-fold increase in the number of γ-H2AX foci was reported in fibroblast cells exposed to SWCNTs, compared to negative controls, although interpretation is difficult as no positive controls were included. In mesothelial cells, a nominal and moderate increase in phosphorylation of H2AX was reported in normal and malignant mesothelial cells, respectively.

~~48.~~50. Due to the variability in the experimental conditions used, including cell lines, concentrations of nanomaterial tested, source of SWCNTs, surface modifications, dose-metrics (µg/cm2 or µL/mL) and lack of positive controls, the study authors concluded that comparison of data is difficult. It was also noted that although interactions of the nanomaterials with the assay were not reported, it could be assumed that nanomaterials with autofluorescence properties may interfere with the quantification of foci (OECD, 2018a).

In vivo Mammalian Erythrocyte Micronucleus test (OECD TG 474)

~~49.~~51. The mammalian *in vivo* micronucleus test aims to detect cytogenetic damage induced by a test chemical, resulting in the formation of micronuclei, containing either lagging chromosome fragments or whole chromosomes, in erythrocytes from the bone marrow or peripheral blood cells of exposed animals.

~~50.~~52. Following acute exposure to the test substance, bone marrow or blood are harvested at time points when treatment-related induction of micronucleated immature erythrocytes can be detected. Preparations are analysed for the presence

of micronuclei, either by visualisation using a microscope, image analysis, flow cytometry, or laser scanning cytometry (OECD, 2016g).

~~51.~~53. The mutagenic potential of fullerenes C60, SWCNTs, MWCNTs, Au and SiO_2 nanomaterials was assessed in the WPMN Testing Programme using the *in vivo* mammalian erythrocytes micronucleus test. The dossiers for TiO_2 and ZnO are not available hence it is unknown if they were tested using this assay. However, in a review of work performed by the OECD WPMN, Rasmussen listed TiO_2 and ZnO as being tested using OECD 474, although no data were presented (Rasmussen *et al.*, 2016).

Specific requirements for nanomaterials

~~52.~~54. No comments were made by the authors regarding the applicability of the *in vivo* mammalian erythrocyte micronucleus test for nanomaterials or if any adaptations are needed

Results and discussion

~~53.~~55. None of the nanomaterials tested was associated with an increased incidence of micronuclei formation, suggesting they are not genotoxic under the test conditions (OECD, 2016a; OECD, 2016b; OECD, 2016c; OECD, 2017a; OECD, 2017b).

In vivo Mammalian Alkaline Comet assay (OECD TG 489)

~~54.~~56. The Comet assay aims to detect single and double-stranded DNA damage and repair in eukaryotic cells following treatment with the test substance, by measuring the migration of DNA from individual nuclei following alkaline treatment (see para 36).

~~55.~~57. The *in vivo* alkaline Comet assay is especially relevant to assessing genotoxic potential as the response observed is dependent upon *in vivo* absorption, distribution, metabolism and excretion, and on DNA repair processes, which may vary among species, tissues and the types of DNA damage (OECD, 2016h).

~~56.~~58. The mutagenic potential of SiO_2 nanomaterials, fullerenes C60, SWCNTs and MWCNTs was assessed in the WPMN Testing Programme using the *in vivo* mammalian alkaline Comet assay.

Results and discussion

~~57.~~59. No mutagenic effects were reported for SiO_2, fullerenes C60 and SWCNTs. However, rats exposed to the highest concentration of MWCNT by inhalation showed a statistically significant increase in lung DNA damage (OECD, 2016a; OECD, 2016b; OECD, 2016c; OECD, 2017a; OECD, 2017b).

General observations

~~58.~~60. A number of observations were made by the report authors on conclusion of the WPMN Testing Programme:

- The Testing Programme identified a variety of *in vitro* methods that had been previously used to assess the genotoxicity of nanomaterials, however many were not carried out to OECD TGs;

- None of the nanomaterials selected were tested using the full set of *in vitro* assays;

- The dossiers published by OECD as part of the Series on the Safety of Manufactured Nanomaterials contained various inconsistencies and omissions related to the use of existing OECD TGs, doses used and dose metrics, physico-chemical parameters assessed, consideration of nanomaterials interference with assay parameters, and protocols used (e.g. sample preparation, cell types used, dose-ranges applied, time of exposure, use of positive/negative controls, use of metabolic activation systems), and there were also various errors in reporting;

- Characterisation of nanomaterials under *in vitro* conditions (e.g. in culture media) and cellular uptake were seldom reported.

Summary and next steps

~~59.~~61. TG 471 (Bacterial reverse mutation test) may be amended but it was acknowledged that it is not applicable for most types of nanomaterials as there is no uptake into the bacteria. This correlates with the report from the OECD Expert Meeting on 'Genotoxicity of Manufactured Nanomaterials (OECD, 2014), in which it was concluded that '*Ames test (TG 471) is not a recommended test method for the investigation of the genotoxicity of nanomaterials. The TG programme should consider modifying the applicability domain within this guideline accordingly'*.

~~60.~~62. Specific recommendations regarding addition of cytochalasin B and the verification of intracellular uptake of nanomaterials should be considered in the modification of TG 487 (*In vitro* micronucleus assay). Again, this is in line with the outcome of the OECD expert meeting on Genotoxicity of Manufactured nanomaterials (OECD, 2014). The JRC is currently leading the development of an OECD Guidance Document that will support the implementation of the existing *in vitro* Mammalian Cell Based Genotoxicity OECD TGs.

~~61.~~63. Prioritisation of assays for harmonisation should be carried out. Assays that should be considered include the micronucleus test (TG 487) and the *in vitro* mammalian cell gene mutation test (TG 476). TG 476 is considered as an alternative to the bacterial reverse mutation test (TG 471), as specific limitations when testing nanomaterials have not yet been identified (OECD, 2014).

Working Group of National Coordinators of the Test Guidelines Programme

~~62.~~64. The WNT is responsible for overseeing the OECD Test Guideline Programme. In 2011, five projects related to the review of the TGs on genotoxicity were added to the WNT workplan, led by Canada, France, the Netherlands and the United States. The revision of the genotoxicity test guidelines was supported by the OECD Expert Group on the revision of the genotoxicity test guidelines (hereafter called the Expert Group) (OECD, 2014). Not all revisions were related to the testing of nanomaterials.

~~63.~~65. The five projects included the deletion of several TGs, the revision of four *in vivo* TGs (TGs 474, 475, 478, and 483), two *in vitro* TGs (TGs 473 and 487), and the mammalian cell gene mutation assay, as well as the revision of the introduction to the test guidelines on genotoxicity (OECD, 2014). TGs 474, 473 and 487 were also included in the WPMN Testing programme hence are relevant to the testing of nanomaterials.

~~64.~~66. The Expert Group met immediately following the WPMN Workshop on the Genotoxicity of Manufactured Nanomaterials and considered any outcomes of the Workshop in the context of the adaptations to the Test Guidelines and/or a need to develop new Test Guidelines or guidance material (see para 66-70) -(OECD, 2014).

Post WPMN workshop developments

~~65.~~67. Following the WPMN workshop, the Expert Group considered the comments arising from the workshop with the view of revising the genotoxicity TGs. The group agreed to develop a list of characterisation, and other nano-related, parameters that could be listed within the genotoxicity TGs to be included in study reports. As some characterisation methods do not yet exist or are not standardised, there are implications on Mutual Acceptance of Data if these parameters are to be included within the TGs. Therefore a guidance document specifying the recommended characterisation parameters was initiated by the OECD Test Guidelines Programme Secretariat as an interim measure (OECD, 2014).

~~66.~~68. The Expert Group also considered the need for including further nano-related guidance in the introduction document to the genotoxicity TGs, or within separate guidance documents being prepared for the Series on Testing and Assessment.

~~67.~~69. The draft TGs 473 and 487 initially excluded insoluble materials from the applicability domain. As both assays have been shown to be useful in testing the genotoxicity of nanomaterials, the relevant sections of these guidelines were re-worded so they do not exclude nanomaterials.

~~68.~~70. TGs 473, 487, 474 and 475 were approved by the WNT in April 2014 but do not contain any nano-specific adaptations.

~~69.~~71. The Ames test (TG 471) is not under revision. However, the Test Guidelines Programme is considering how to address the applicability domain issues for this assay, which may include opening the guideline for revision in the future (OECD, 2014).

OECD TGP workplan

~~70.~~72. In September 2018, OECD published the test guidelines programme (TGP) workplan (OECD, 2018b). It outlined a number of nanomaterial-related activities, including project 4.95: Guidance Document on the Adaptation of *In Vitro* Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials, being led by the European Commission. A workshop was held at the Joint Research Centre in Ispra, Italy early in 2019 (OECD 2019)[4].

~~71.~~73. The workplan outlined that the appropriate parameters needed for an optimised micronucleus test had been previously discussed, and that '*the project aims to develop a Guidance Document that will support the existing genotoxicity Test Guidelines by indicating where protocol modifications and special considerations should be applied when testing manufactured nanomaterials'*.

~~72.~~74. The project should enable the Expert Group to start an experimental inter-laboratory comparison to optimise the micronucleus test protocol, and later, to propose any modifications to TGs and to develop a Guidance Document (OECD, 2018b).

EU NanoSafety Programme

~~73.~~75. In its 7th Framework Programme the EU identified the safety of nanomaterials as a key area of research and subsequently initiated the NanoSafetyCluster (NSC), including the Nanogenotox and NANoREG projects.

NANOGENTOX Joint Action

~~74.~~76. The NANOGENOTOX Joint Action (JA) was an EU funded collaborative project that was completed in 2013. It was coordinated by ANSES and involved 16 associated partners and 15 collaborating partners from across Europe.

~~75.~~77. The objective of the JA was to develop standardised methods for characterisation and determination of *in vitro* and *in vivo* genotoxicity of nanomaterials. Several genotoxicity test methods were applied, modified for use with nanomaterials and a round robin test was carried out. Furthermore, the correlation of *in vivo* and *in vitro* methods was investigated and the toxicokinetic behaviour of nanomaterials was also studied. Ultimately, the aim was to '*work towards establishing a robust methodology to assess the potential genotoxicity of*

[4] http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2019)11&doclanguage=en

nanomaterials and to generate data on the genotoxic effects of certain commonly used nanomaterials' (ANSES, 2013a).

~~76.~~78. The project focused on 15 representative nanomaterials, namely five types of TiO_2 nanomaterials, four types of synthetic amorphous silica (SAS) and six types of MWCNTs, as well as ZnO as a control. Seven work packages (WP) were included, covering *in vitro* testing (WP5) and *in vivo* testing (WP6) (ANSES, 2013a).

Work package 5

~~77.~~79. The objective of WP5 was to generate *in vitro* genotoxicity data using both standard and modified assays (in some but not all cases OECD TGS were used), with a round robin test on selected nanomaterials using the most promising assays. Three genotoxicity endpoints were selected for the initial phase, namely DNA damage (using alkaline and FpG-modified Comet assay), micronuclei formation (using cytokinesis-block micronucleus assay and micronucleus assay without cyto B) and mutation formation (using the mouse lymphoma assay). Various cell lines were used including pulmonary (bronchial epithelial BEAS 2B and 16 HBE; alveolar A549), intestinal (Caco-2, primarily undifferentiated cells) and epidermal (NHEK keratinocytes), in addition to human primary lymphocytes for the micronucleus assay and L5178Y TK +/- cells for the mouse lymphoma assay. Protocols for each cell line and endpoint were agreed and harmonised in advance (ANSES, 2013b).

Comet assay

Methodology

~~78.~~80. The Comet assay was carried out using 3 h or 24 h exposures. For each nanomaterial, 4-6 doses were included to obtain a minimum of 3 analysable doses. No metabolic activation system was used (ANSES, 2013b).

Results and discussion

The Comet assay was positive for all TiO_2 nanomaterials in Caco-2 cells after 24 hr, except for NM-104. NM-102 was positive at both 3 and 24 hr in all cell lines apart from 16HBE, and NM-105 was positive in all cell lines except BEAS 2B and 16HBE. The FpG-modified Comet assay was positive for NM-104 and NM-105 in BEAS 2B and Caco-2 cells and for NM-104 was in A549 cells, but all other TiO_2 were negative in 16HBE cells (

~~79.~~81. Table 1).

All SAS were positive in the Comet assay following 3 hr of treatment in BEAS 2B cells. NM-200 was positive in all other cell lines. NM-201, NM-202 and NM-203 were positive in A459 cells, and NM-203 in Caco-2 cells, both after 3 and 24 hr treatment (

~~80.~~82. Table 1).

The Comet assay was negative for all MWCNTs (

~~81.~~83. Table 1).

In the FpG-modified Comet assay, all SAS nanomaterials were positive in Caco-2 cells after 3 or 24 hr; NM-202 and NM-203 were positive in BEAS 2B cells after 3 hr, and A549 cells after both 3 and 24 hr. All types of SAS and MWCNTs were negative in 16 HBE cells (

~~82.~~84. Table 2) (ANSES, 2013b).

Table 1 Genotoxicity of nanomaterials in the *in vitro* Comet assay

	Caco-2 (3/24 hr)	**BEAS 2B (3 hr)**	**16 HBE (3/24 hr)**	**A549 (3/24 hr)**	**NHEK (3/24 hr)**
TiO_2					
NM-102	-/+	+/+	+/-	+/-	+/+
NM-103	-/+	-/-	-/-	-/-	+/+
NM-104	-/-	-/-	-/-	-/-	+/+
NM-105	-/+	-/-	-/+	+/-	+/+
SAS					
NM-200	+/+	+	+/-	(+)/-	NT
NM-201	-/(+)	(+)	-/-	+/(+)	NT
NM-202	(+)/(+)	+	-/-	+/(+)	NT
NM-203	+/+	+	-/-	-/+	NT
MWCNT					
NM-400	-/-	-/-	-/-	-/-	-/-
NM-401	-/-	-/-	-/-	-/-	-/-
NM-402	-/-	-/-	-/-	-/-	-/-
NM-403	-/-	-/-	-/-	-/-	-/-

NT – not tested; + positive: a statistically significant increase with ≥2 doses or a statistically significant increase at high dose and a dose-dependent increase; (+) equivocal: a statistically significant increase with 1 dose, not dose-dependent increase; - negative

Table 2 Genotoxicity of nanomaterials in the *in vitro* FpG-modified Comet assay

	Caco-2 (3/24 hr)	BEAS 2B (3 hr)	16 HBE (3/24 hr)	A549 (3/24 hr)	NHEK (3/24 hr)
TiO_2					
NM-102	NT	NT	NT	NT	NT
NM-103	NT	NT	NT	NT	NT
NM-104	NT	NT	NT	NT	NT
NM-105	NT	NT	NT	NT	NT
SAS					
NM-200	(+)/+	(+)	-/-	-/-	NT
NM-201	-/+	-	-/-	-/(+)	NT
NM-202	+/-	+	-/-	+/-	NT
NM-203	+/(+)	+	-/-	-/+	NT

NT – not tested; + positive: a statistically significant increase with ≥2 doses or a statistically significant increase at high dose and a dose-dependent increase; (+) equivocal: a statistically significant increase with 1 dose, not dose-dependent increase; - negative

Micronucleus assay

Methodology

~~83.~~85. For the micronucleus assay, a longer exposure period was used, covering 1.5-2 cell cycles. Cytochalasin B was generally added 6 hr after the initiation of exposure, but after 24 hr in Caco-2 cells[5]. Doses of the nanomaterials tested were chosen on the basis of cytotoxicity measurements, mostly using cell count relative to controls, relative increase in cell counts, or relative population doubling. The highest dose selected was either at the cytotoxicity limit of 55 % (+/-5 %) or as otherwise justified. In the case of nanomaterials with low cytotoxicity, the maximum dose was derived from the WP4 dispersion protocol (256 µg/ml) or was based on technical limitations (e.g. inhibition of analysis because cells were covered with nanomaterials) (ANSES, 2013b).

[5] The reason for using a different protocol with Caco-2 cells was not provided.

Results and discussion

All TiO_2 nanomaterials were positive in NHEK cells, some were positive in human lymphocytes, but all were negative in all other cells (

~~84.~~86. Table 3).

SAS induced micronuclei in Caco-2 cells although the effect was not repeatable and NM-201 and NM-202 induced micronuclei in A459 cells. All other SASs were negative in all other cells (

~~85.~~87. Table 3).

All MWCNTs were mostly positive in BEAS 2B, A459 (NM-401 and NM-403 were negative) and Caco-2 cells (NRCWE0-006 was negative) but all were negative in 16 HBE cells (

~~86.~~88. Table 3) (ANSES, 2013b).

Table 3 Genotoxicity of nanomaterials in the *in vitro* micronucleus assay

	Caco-2 (48 hr)[a]	BEAS 2B (48 hr)[b]	16 HBE (48 hr)[c]	A549 (48 hr)[b,c]	NHEK (48 hr)[d]	Human lymphocytes (48 hr)[e]
NM-102	-	-	-	-	+	(+)
NM-103	-	-	-	-	+	+
NM-104	-	-	-	-	+	+
NM-105	-	-	-	-	+	-
SAS						
NM-200	+/-	-	-	-/-	NT	-
NM-201	+/-	-	-	+/+	NT	-
NM-202	+/-	-	-	+/+	NT	-
NM-203	+/-	(+)	-	-/(+)	NT	-
MWCNT						
NM-400	(+)	(+)	-	(+)	NT	-
NM-401	+	+	-	-	NT	-
NM-402	+	+	-	+	NT	(+)
NM-403	(+)	+	-	-	NT	+

[a]Treatment for 52 h, Cyt-B added at 24 h; [b]Treatment for 48 h, Cyt-B added at 6 h; [c]Treatment for 41 h, no Cyt-B used; [d]Treatment for 54 h, Cyt-B added at 6 h; [e]Treatment for 30 h, Cyt-B added at 6 h.

NT – not tested; + positive: a statistically significant increase with ≥2 doses or a statistically significant increase at high dose and a dose-dependent increase; (+) equivocal: a statistically significant increase with 1 dose, not dose-dependent increase; - negative; / Separates results of two experiments with different exposure times

Mouse lymphoma assay

Methodology

~~87.~~89. No information about the methodology for the mouse lymphoma assay was provided (ANSES, 2013b).

Results and discussion

~~88.~~90. All types of TiO_2, SAS and MWCNTs tested were negative (ANSES, 2013b).

Round robin study

~~89.~~91. Following the first phase, a round robin study was carried out to assess the reproducibility of the cytokinesis block micronucleus assay and the alkaline Comet assay to assess the genotoxicity of TiO_2, SAS and MWCNT, plus ZnO to assess its use as a positive control. The study was carried out to agreed protocols by twelve independent laboratories, half using BEAS-2B cells and the other using Caco-2 cells.

Results and discussion

~~90.~~92. For TiO_2, the Comet assay was positive in BEAS 2B cells in five out of the six laboratories, correlating to the results shown in the first phase, and was negative in three laboratories and positive in two when using Caco-2 cells (Table 4). The first phase in Caco-2 cells was negative.

~~91.~~93. The micronucleus assay using BEAS 2B cells was negative in four of the six laboratories, again in agreement with the first phase. The other laboratories report one positive and one equivocal result. With the Caco-2 cells, the presence of particle agglomerates on the microscopic slides hampered the assay in three laboratories. For the others, the assay was negative in two laboratories and positive in one (Table 4). The first phase of the study reported negative data (ANSES, 2013a; ANSES, 2013b).

Table 4 Genotoxicity of TiO_2 NM-102 in the *in vitro* Comet and micronucleus assay in the round robin study

	Comet assay		Micronucleus assay	
	Caco-2	**BEAS 2B**	**Caco-2**	**BEAS 2B**
ANSES	-	+	-	+
NRCWE	+	-	NT	-
BfR	-	+	+	(+)
IPL	-	+	-	-
RIVM	NT	+	NT	-
INRS	+	+	NT	-

+ positive; - negative; (+) equivocal; NT; not tested

~~92.~~94. In BEAS 2B cells, SAS were negative in the Comet assay in three laboratories but positive in three. Using Caco-2 cells, SAS was negative in three and positive in two laboratories (5). In the first phase, the Comet assay was negative in BEAS 2B cells and positive in Caco-2 cells.

~~93.~~95. For the micronucleus assay in BEAS 2B cells, SAS was positive in three laboratories and negative in three laboratories, compared to equivocal results in the first phase. In the Caco-2 cells, three laboratories reported positive and three reported negative data (5). Conflicting results had also been obtained in the first phase.

Table 5 Genotoxicity of SAS NM-103 in the *in vitro* Comet and micronucleus assay in the round robin study

	Comet assay		Micronucleus assay	
	Caco-2	**BEAS 2B**	**Caco-2**	**BEAS 2B**
ANSES	-	-	+	+
NRCWE	+	+	+	-
BfR	-	+	-	+
IPL	+	+	-	-
RIVM	NT	-	-	+
INRS	-	-	+	-

+ positive; - negative; (+) equivocal; NT; not tested

For MWCNT, the Comet assay in BEAS 2B cells was negative in three and positive in three laboratories, whereas it was negative in the first phase. In Caco-2 cells, four laboratories reported negative data and one reported positive results (

~~94.~~96. Table 6). The first phase reported negative data.

~~95.~~97. The micronucleus assay in BEAS 2B cells was negative in five of the six laboratories, in contrast to the positive results in the first phase. In Caco-2 cells results were positive in three laboratories, negative in two and equivocal in one. The first phase also reported equivocal data (ANSES, 2013a; ANSES, 2013b).

Table 6 Genotoxicity of MWCNT MN-403 in the *in vitro* Comet and micronucleus assay in the round robin study

	Comet assay		Micronucleus assay	
	Caco-2	**BEAS 2B**	**Caco-2**	**BEAS 2B**
ANSES	-	+	+	+
NRCWE	+	-	+	-
BfR	-	+	+	-
IPL	-	-	-	-
RIVM	NT	-	-	-
INRS	-	+	(+)	-

+ positive; - negative; (+) equivocal; NT; not tested

Conclusions from WP5

~~96.~~98. A number of conclusions were drawn following WP5:

- Cell lines that take up nanomaterials can be used for genotoxicity testing;
- The genotoxic activity seen *in vitro* could have resulted from indirect mechanisms that are not presently understood;
- Slight genotoxic effects were often observed that were not always reproducible;
- The pulmonary-derived BEAS 2B cells performed slightly better than the intestinal-derived Caco-2 cells in the Comet assay, based on ??? (ANSES, 2013b; OECD, 2014).

Work package 6

~~97.~~99. The objective of WP6 was to assess *in vivo* genotoxicity and to make comparisons between *in vitro* and *in vivo* findings in rodent models. Three complementary tests were selected, namely the Comet and micronucleus assay in rats, and the lacZ transgenic gene mutation assay in mice. Twelve nanomaterials were studied (four SAS, four TiO_2 and four MWCNTs). All were administered by oral gavage and intratracheal instillation, and four were also administered intravenously (OECD, 2014). All studies were carried out using three doses, selected according to the toxicokinetics data from WP7. The highest dose was that which did not cause mortality or obvious adverse effect; 20 mg/kg/day SAS, 12.8 mg/kg/day TiO_2 and

51.2 mg/kg/day CNTs for gavage and 12 mg/kg/day SAS, 4.6 mg/kg/day TiO_2 and 0.48 mg/kg/day CNTs for instillation. The lower doses were obtained through factor 2 dilutions.

~~98.~~100. To detect genotoxic effects, animals were administered nanomaterials on 3 consecutive days, and tissue sample collection occurred three hours following the final administration (ANSES, 2013c).

Comet assay in rats

Methodology

~~99.~~101. The organs collected for analysis was dependent on the route of exposure. Following oral exposure, liver, kidney, blood, bone marrow, intestine and colon were collected for Comet assay analysis, whilst for the instillation route, lung tissue and bronchoalveolar lavage (BAL) were collected in place of intestine and colon tissues.

~~100.~~102. Oxidative DNA damage was investigated using a Modified Comet assay with FpG enzyme recognising some specific oxidative lesions (ANSES, 2013c).

Results and discussion

~~101.~~103. Following exposure by instillation, only one of the four TiO_2 nanomaterials (NM-105) was associated with the induction of DNA damage in BAL cells, as measured by the Comet assay; two TiO_2 nanomaterials (NM-102 and -103) gave equivocal responses in liver tissue samples. None of the remaining TiO_2 nanomaterials showed genotoxic effects in lung, spleen or kidney tissues. Similarly, none of the SAS nanomaterials were positive for genotoxicity in any organ following exposure via instillation. For MWCNT, some positive effects were seen in kidney (NM-400) and some equivocal effects were reported (NM-401 in lung and spleen; NM-403 in BAL, lung and kidney (Table 7).

No oxidative DNA damage induced by SAS was detected using the modified FpG Comet assay. For MWCNT NM-401 and NM-402, induction of DNA damage was noted in kidney tissue. Equivocal results were reported in spleen tissue for NM-401(

~~102.~~104. Table 8) (ANSES, 2013a; ANSES, 2013c).

Table 7 Genotoxicity of nanomaterials in the *in vivo* Comet assay following instillation

	BAL fluid	Lung	Blood	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	-	-	NT	NT	-	(+)	-
NM-103	-	-	NT	NT	-	(+)	-
NM-104	-	-	NT	NT	-		-
NM-105	++	-	NT	NT	-		-
SAS							
NM-200	-	-	-	-	-	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
MWCNT							
NM-400	NT	**	NT	NT	ID	-	++
NM-401		(+)	NT	NT	(+)	-	-
NM-402		-	NT	NT	-	-	-
NM-403	(+)	(+)	NT	NT	-	-	(+)

NT not tested; * technical issue; ID invalidated data

Table 8 Genotoxicity of nanomaterials in the *in vivo* FpG- Comet assay following instillation

	BAL fluid	Lung	Blood	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	NT	NT	NT	NT	NT	NT	NT
NM-103	NT	NT	NT	NT	NT	NT	NT
NM-104	NT	NT	NT	NT	NT	NT	NT
NM-105	NT	NT	NT	NT	NT	NT	NT
NM-200	-	-	-	-	(+)*	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
NM-400	NT	**	NT	NT	ID	-	-
NM-401	-	-	NT	NT	(+)	-	+
NM-402	-	-	NT	NT	-	-	+
NM-403	ID	ID	NT	NT	-	-	ID

NT not tested; * technical issue; ID invalidated data

~~103.~~105. Following gavage, positive genotoxic effects were seen with TiO_2 in spleen (NM-102, NM-104 and NM-105), intestine (NM-103), colon (NM-102 and -105) and bone marrow (NM-104), measured by the Comet assay. None were associated with positive genotoxic effects in the liver or blood. Similarly, none of the SAS nanomaterials were positive for genotoxicity in any organ tissues following oral gavage. For the MWCNT nanomaterials, some equivocal effects were reported for NM-401 in liver and kidney tissue samples. All other findings were negative for the remaining organ tissue samples collected (Table 9).

Results from the FpG modified assay did not show any specific oxidative damage for any nanomaterial in any tissue sample (

~~104.~~106. Table 10) (ANSES, 2013a; ANSES, 2013c)

Table 9 Genotoxicity of nanomaterials in the *in vivo* Comet assay following gavage

	Intestine	Colon	Blood	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	-	++	-	-	+++	-	-
NM-103	++	-	-	-	(+)	-	-
NM-104	-	-	-	++	+++	-	-
NM-105	-	++	-	-	+	-	-
SAS							
NM-200	-	-	-	-	-	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
MWCNT							
NM-400	NT	-	NT	NT	-	-	-
NM-401	NT	-	NT	NT	-	(+)	(+)
NM-402	NT	ID	NT	NT	-	-	-
NM-403	NT	-	NT	NT	-	-	-

NT not tested; * technical issue; ID invalidated data

Table 10 Genotoxicity of nanomaterials in the *in vivo* FpG-Comet assay following gavage

	Intestine	Colon	Blood	Bone marrow	Spleen	Liver	Kidney
TiO_2							
NM-102	-	-	NT	NT	NT	NT	NT
NM-103	-	-	NT	NT	NT	NT	NT
NM-104	-	-	NT	NT	NT	NT	NT
NM-105	-	-	NT	NT	NT	NT	NT
SAS							
NM-200	-	-	-	-	-	-	-
NM-201	-	-	-	-	-	-	-
NM-202	-	-	-	-	-	-	-
NM-203	-	-	-	-	-	-	-
MWCNT							
NM-400	NT	-	NT	NT	-	-	(+)
NM-401	NT	ID	NT	NT	-	(+)	ID
NM-402	NT	ID	NT	NT	ID	-	ID
NM-403	NT	-	NT	NT	-	-	-

NT not tested; * technical issue; ID invalidated data

Micronucleus assay in rats

Methodology

~~105.~~107. According to OECD TG 474, bone marrow and colon samples were collected from the same animals used for the Comet assay. Additional studies were carried out using intravenous administration to 'increase the potency to reach systemic organs' (ANSES, 2013c).

Results and discussion

~~106.~~108. Following instillation, micronuclei were not induced by TiO_2, SAS or CNTs in bone marrow. Additional studies using intravenous administration were also negative.

~~107.~~109. Following gavage, none of the SAS induced micronuclei formation in bone marrow, however, two (NM-202 and -203) of the four SAS induced an increase of micronuclei in colon samples at the lowest dose.

~~108.~~110. TiO_2 and SAS were also negative following intravenous administration. With SAS, a slight increase in micronuclei formation was observed at the highest dose tested (20 mg/kg), but as this dose induced mortality in three out of six animals the findings of genotoxicity were not considered relevant (ANSES, 2013c).

lacZ transgenic gene mutation assay in mice.

Methodology

~~109.~~111. A gene mutation assay on LacZ mice was performed with TiO_2 according to the transgenic rodent mutation assay (OECD TG 488). Animals were treated intravenously with NM-102 (10 and 15 mg/kg bw/day) for 2 consecutive days. After 28 days, the DNA of liver and spleen tissue was extracted and mutant frequencies were determined.

~~110.~~112. A Comet assay on the liver and spleen tissue, and a micronucleus assay on bone marrow samples were also performed. To verify that the exposure of the mice in the organs was effective, samples from liver tissue were also collected for Transmission Electron Microscopy and histopathology analyse (ANSES, 2013c).

Results and discussion

~~111.~~113. Following TiO_2 exposure via gavage and instillation, the Comet and micronucleus assays in liver, spleen and bone marrow samples respectively, were negative in lacZ transgenic mice. The lacZ mutation assay in liver and spleen tissue was also negative for mutagenicity, as was the Comet assay in the liver and spleen tissues and micronucleus assay in the bone marrow samples, following iv administration (ANSES, 2013c).

Conclusions from WP6

~~112.~~114. A number of conclusions were drawn following WP6:

- There were limited indications of *in vivo* genotoxicity in a few organs, which need to be confirmed in follow-up studies;

- The Comet assay was largely negative with all nanomaterials. Where positive responses were seen, these showed no dose-response and hence it is difficult to conclude on the genotoxicity of these.

- Genotoxicity of different nanomaterials within the same nanomaterial family varied;

- The bone marrow micronucleus assay (TG 474) predominantly gave negative results. However, as the nanomaterials have low bioavailability it is possible that these may not have reached the bone marrow. Therefore, the micronuclei test may not be suitable for testing nanomaterials;

- Test methods need to include historical data or acceptability/reproducibility criteria because of inter-laboratory variability (ANSES, 2013c; OECD, 2014).

~~113.~~115. Following the OECD Council Recommendation on the safety testing and assessment of manufactured nanomaterials, which recommended that, "*members apply the OECD Test Guidelines, adapted as appropriate to take into account the specific properties of manufactured nanomaterials*…" it was noted that regulators are looking for further guidance on how the Test Guidelines should be adapted for the testing of nanomaterials, and what adaptations would be appropriate (OECD, 2014).

NanoReg project

~~114.~~116. The NANoREG project was one of the largest projects in the NSC. It was coordinated by Ministry of Environment and Infrastructure, in the Netherlands and was completed in 2017.

~~115.~~117. In NANoREG over 85 institutional partners from EU member states, associated states, the Republic of Korea and Brazil collaborated in developing reliable, reproducible and relevant methods for testing and assessing the effects of nanomaterials on human health and environment in a regulatory context.

~~116.~~118. The project ended in 2017 and all results have been made available via the NANoREG Results Repository (https://www.rivm.nl/en/about-rivm/mission-and-strategy/international-affairs/international-projects/nanoreg) , which serves as a central point of access to all the results of the project by providing links to all relevant documents, datasets and other information of interest (Steinhäuser *et al.*, 2017).

~~117.~~119. The project consisted of a number of WPs, of which WP4 (Biokinetics and toxicity testing *in vivo*) included some assessment of genotoxicity of nanomaterials. For each WP a number of deliverables are available, of which deliverable 4.09 (*in vivo* results on genotoxicity and biological markers), D4.11 (Identification of hazards and NOAELs for amorphous silica after subchronic oral exposure), D4.13 (Mode of toxic action of high aspect ratio nanomaterials), and

D4.16 (Immunotoxic and genotoxic effects (Repeated dose after short-term inhalation of fibrous nanomaterials) all included some genotoxicity assessment.

~~118.~~120. Within the results repository where reports for each WP are available, deliverable 4.09 was not included and was also not included in the final report (NANoREG, 2016). The reason for this is unknown.

~~119.~~121. The deliverable report and fact sheet for D4.13 are not yet available in full but were summarised in the final report. Ten commercial MWCNT, in three groups of different dimensions, were tested in mice following intratracheal installation Pulmonary inflammation and genotoxicity were determined on day 1, 28 or 92. Specific surface area (BET) and therefore diameter, significantly predicted genotoxicity in BAL fluid cells and lung tissue such that lower BET surface area or correspondingly larger diameter was associated with increased genotoxicity. A single dose of ten MWCNTs were also administered to mice and effects were evaluated after a year. when genotoxicity in liver and spleen was reported (NANoREG, 2016).

~~120.~~122. A short report for deliverable 4.11 is available (NANoREG, Unknown-b). Genotoxicity endpoints were assessed following a 90 day oral toxicity assay in rats, exposed to SiO_2 (no further information about the genotoxicity tests used were provided). No genotoxicity was reported. The assay was also mentioned in the final report (NANoREG, 2016).

~~121.~~123. A short report for deliverable D4.16 is available (NANoREG, Unknown-a). The Comet assay was carried out on lung cells and BAL fluid and the micronucleus assay on bone marrow polychromatic erythrocytes after single pharyngeal aspiration to female mice. Exposure to nine out of ten of the nanofibrillated cellulose materials tested, and all the MWCNTs, resulted in DNA damaged as measured by the Comet assay. No nanomaterial was associated with systemic genotoxicity as determined by the micronucleus assay (NANoREG, Unknown-a). These assays were also briefly mentioned in the final report, that simply stated ‘there was no evidence of genotoxic effects in livers and spleens’ (NANoREG, 2016).

~~122.~~124. NANoREG mainly focussed on the impact of manufactured nanomaterials on human health and applied *in vivo* (chapter 5.6) and *in vitro* (chapter 5.7) methods to assess the potential risks, including genotoxicity.

~~123.~~125. Chapter 5.6 largely discusses hazard identification, dosimetry, route of exposure and exposure duration in *in vivo* studies. Regarding mutagenicity of nanomaterials, authors noted that ‘*the key issue was the appropriateness of administered doses since most of them are done as acute in vitro studies’*, an issue that was not addressed in the OECD workshop held in 2014. They also stated that *in vitro* mutagenicity tests should be validated by *in vivo* tests but *in vivo* acute or short-term mutagenicity studies generally also use unrealistically high doses (Oberdorster, 2017; Steinhäuser *et al.*, 2017).

~~124.~~126. Chapter 5.7 discusses various assays that were assessed to investigate genotoxicity of nanomaterials (Drasler *et al.*, 2017; Rothen-Rutishauser and Drasler, 2017; Steinhäuser *et al.*, 2017).

Bacterial Reverse Mutation Test (OECD TG 471)

~~125.~~127. The Ames test (OECD TG 471) was not recommended for the genotoxicity testing of nanomaterials due to the possible lack of nanomaterial uptake in bacterial cells. Although no studies under review used bacterial cells, other studies showed uptake of the nanomaterials TiO_2, ZnO, CeO2, SWCNTs and MWCNTs into bacterial cells, although mutagenicity results were negative (Drasler *et al.*, 2017; Rothen-Rutishauser and Drasler, 2017).

~~126.~~128. Authors agreed with the outcome of the OECD workshop (OECD, 2014) regarding the inappropriateness of the bacterial based tests. This is due to the uptake of nanomaterials in bacterial cells being limited due to lack of the endocytoticability of bacteria and diffusion of nanomaterials across the bacterial cell wall may be limited. Moreover, some nanomaterials may have antibacterial properties which may lead to the misinterpretation of data, and in some cases, the bacteria may be smaller than the nanomaterial (Drasler *et al.*, 2017; Rothen-Rutishauser and Drasler, 2017).

In vitro Mammalian Cell Gene Mutation Tests using Hprt and xprt genes (OECD TG 476)

~~127.~~129. Authors cited findings from the OECD workshop (OECD, 2014), stating that there have been no reports identified regarding any specific limitations of the assays when used to test nanomaterials. Advantages of using the *in vitro* mammalian cells gene mutation assay included the lack of interference with nanomaterials; closer resemblance of the mammalian cell model to human physiology; simple selection of mutants with 6-thioguanine; the capacity to characterise a diverse range of mutations; and inactivation of only one allele is required for the mutation to be expressed.

~~128.~~130. One study that was reviewed reported a lower number of hprt mutants in nanomaterial-treated cells, compared with viable cell populations. This was thought to be due to higher concentrations of nanomaterials inducing larger genetic malformations thereby reducing cell viability leading to a lower mutant frequency. However, to date there has been no evidence of interference by nanomaterials in the assay and therefore the assay was recommended, although authors suggested further testing should be carried out (Rothen-Rutishauser and Drasler, 2017{Drasler, 2017 #26)}.

In vitro Mammalian Micronucleus Test (OECD TG 487)

~~129.~~131. The recommendation from the OECD workshop (OECD, 2014) regarding the OECD TG 487 was discussed in terms of the use of cytochalasin-B,

which is used to prevent cytokinesis in the test, to allow scoring of micronuclei. As cytochalasin-B affects the uptake of nanomaterials, post-treatment or delayed co-treatment was suggested (Drasler *et al.*, 2017; Rothen-Rutishauser and Drasler, 2017).

In vitro Comet Assay (single-cell gel electrophoresis)

~~130.~~132. Regarding the Comet assay, authors noted the method is highly sensitive but is capable of detecting damage only over a narrow range of breaks per cells, as it is limited by the cytotoxicity of the nanomaterial. False positives have been reported due to nanomaterials being in the comet head, which raised concerns of potential interference. Therefore, co-incubation of the nanomaterials with untreated and treated cells in the gel is recommended. Overestimation of the DNA damage due to additional damage occurring during the assay was also noted, especially with high doses of nanomaterials (CeO2, TiO2, SiO2, and polystyrene NPs) (Drasler *et al.*, 2017; Rothen-Rutishauser and Drasler, 2017).

Recommendations

~~131.~~133. In line with the OECD workshop report (OECD, 2014), the following recommendations were made:

- Assays: Bacterial-based tests, i.e. the Ames test, are not appropriate, because non-soluble nanomaterials cannot penetrate the bacterial wall. Therefore, the comet assay and micronucleus assay performed with mammalian cells are recommended;

- Use of mammalian tests: Bacterial cells have limited uptake ability for nanomaterials, hence mammalian cells are recommended;

- Non-cytotoxic concentrations: Appropriate cytotoxicity tests should be part of the genotoxicity testing strategy;

- Time: In some assays, longer treatment (at least 24 h) is recommended to ensure nanomaterial uptake by cells and access to DNA as a standard 4 h treatment may not be sufficient to induce genotoxic effects. However, in the Comet assay, 2-3 h exposure is sufficient to induce genotoxicity;

- Controls: Controls should always be included in assays for quality control purposes as a demonstration of correct performance of the assay, and to ensure reproducibility. Negative controls should demonstrate the background level of DNA damage; positive controls should show significant effects; and solvent/supernatant controls should be included to avoid false positive results due to coating/solvent/stabilising effect or ion dissolution. As far as possible, nano-specific positive controls should be used, but no generally accepted candidate has been found and no consensus has been reached;

- OECD activities: The OECD has started to develop a 'Guidance Document on the Adaptation of In Vitro Mammalian Cell Based Genotoxicity TGs for Testing of Manufactured Nanomaterials' which is focused on the adaption of the micronucleus test TG 487. This activity should be further pursued and extended to the *in vitro* gene mutation test TG 476 (Drasler *et al.*, 2017; Rothen-Rutishauser and Drasler, 2017).

Prosafe project

~~132.~~134. The ProSafe project (02/2015-04/2017), run by the National Institute for Public Health and the Environment (RIVM), is an EU Horizon 2020 coordination and support action (CSA), which '*coordinated and strengthened existing and new initiatives in the field of nanosafety (risk assessment, management and governance)*'. In ProSafe, twelve organisations from nine EU member and associated states collaborated to support the review of regulatory relevant results from NANoREG, the OECD WPMN and other projects of the NanoSafety Cluster funded with the EU FP7, in the form of a White Paper which was the main outcome of the study. The findings were translated into fifteen recommendations for policy makers and regulators (ProSafe, 2017; Prosafe/OECD, 2017)

135. In the White Paper, one of the recommendations by the authors was to improve data quality and data management by using harmonised and validated test methods to generate data that are reliable and comparable. However, specific assays for different endpoints, including genotoxicity, were not detailed (ProSafe, 2017).

RiskGONE project

136. ANSES are involved in the H2020 project RiskGONE[6] on governance of nanomaterials, especially on in vitro genotoxicity and high throughput methods.

NanosolveIT project

~~133.~~137. Birmingham University are coordinating NanoSolveIT[7], a project which is developing a validated, multi-scale nanoinformatics IATA, for assessment of potential adverse effects of NM on human health and the environment. A part of the project, case studies utilising OECD IATA will be carried out, including a case study on grouping and read-across for nanomaterials on genotoxicity of nano-TiO2

Summary[RB2]

~~134.~~138. A number of projects and initiatives have been conducted over recent years to evaluate and harmonise methodologies to assess the genotoxicity of nanomaterials. These include the OECD WPMN, NANOGENTOX, NANoREG,

[6] https://riskgone.wp.nilu.no/
[7] https://www.nanosolveit.eu/

ProSafe, RiskGONE and NanosolveIT that have evaluated test methodologies for genotoxicity testing and their applicability for nanomaterials.

~~135.~~139. Overall, the bacterial reverse mutation test (Ames test, OECD TG 471) is not recommended due to the limited uptake of nanomaterials in bacteria, as non-soluble nanomaterials cannot penetrate the bacterial wall. TG 476 is considered a suitable alternative for testing, as specific limitations when testing nanomaterials have not yet been identified.

~~136.~~140. The WPMN, WNT, NANoREG and the report from the OECD expert meeting on Genotoxicity of Manufactured Nanomaterials concluded that the *in vitro* micronucleus assay (OECD TG 487) using mammalian cells is recommended for use with nanomaterials, however modification regarding the addition of cytochalasin B should be included. The Expert Group also recommended that the TG should be reworded so as not to exclude nanomaterials, and OECD also stated that specific adaptations may be needed, although such adaptations were not described. Using TG 487, mutagenic activity was reported in the WPMN Testing Programme for some nanomaterials tested in certain cell lines.

~~137.~~141. The WNT also approved TGs 473 (In vitro Mammalian Chromosomal Aberration Test), 474 (*In vivo* Mammalian Erythrocyte Micronucleus test) and 475 (Mammalian Bone Marrow Chromosomal Aberration Test) for testing nanomaterials, although acknowledging that some of these may need adapting. Such adaptations were not specified.

~~138.~~142. Using TG 473 (*In vitro* Mammalian Chromosomal Aberration Test), none of the nanomaterials tested showed mutagenic activity in any of the cell lines in the WPMN Testing Programme. As with TG 487, the Expert Group also recommended that the guideline be reworded so as not to exclude nanomaterials.

~~139.~~143. Using TG 474 (*In vivo* Mammalian Erythrocyte Micronucleus test), none of the nanomaterials were positive for genotoxicity in the WPMN Testing Programme in any of the cell lines tested. Similarly, the assay predominantly gave negative results in the NANoREG project and NONOGENTOX Joint Action. The latter noted that nanomaterials have low bioavailability hence may not reach bone marrow, limiting the use of the *in vivo* micronuclei test for testing of nanomaterials.

~~140.~~144. The NANoREG project recommended the use of the Comet assay with nanomaterials. In the WPMN testing Programme, most nanomaterials gave negative or equivocal results in the *in vitro* Comet assay, or if positive results were seen, no dose-response relationship could be established. Authors noted that it was therefore difficult to conclude on the genotoxicity of the nanomaterials tested. Similarly, few mutagenic effects were seen in the *in vivo* Comet assay. MWCNTs did cause an increase in lung DNA damage following inhalation, but no OECD TG was followed in assessing this.

~~141.~~145. The NANoREG project recommended that a combination of tests is needed, including two to three representative cell lines, five concentrations of nanomaterials and negative and positive controls. It was also recommended comparison of different methods is needed. From a regulatory perspective, different genotoxicity and mutagenic endpoints are required, to ensure that any important event has not been missed.

Questions for the Committee

- Do members have any additional data for inclusion.
- Do members consider that the paper can be published as COM guidance?

NCET at WRc/IEH-C under contract supporting the PHE COM Secretariat
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Glossary[RB3]

BAL	Bronchoalveolar lavage
CeO_2	Cerium oxide
DSBs	DNA double-strand breaks
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FISH	Fluorescence *In Situ* Hybridization
FPG	Formamido-pyrimidine-DNA glycosylase
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
ICAPO	International Council for Animal Protection in OECD Programmes
JA	Joint Action
JRC	Joint Research Centre
MWCNTs	Multiwalled carbon nanotubules
RIVM	National Institute for Public Health and the Environment
SAS	Synthetic amorphous silica
SiO_2	Silicon dioxide
SSCS	Scientific Committee on Consumer Safety
SWCNTs	Single walled carbon nanotubules
TG	Test guidelines
TGP	Test guidelines programme
TiO_2	Titanium dioxide
WP	Work packages
WPMN	Working Party on Manufactured Nanomaterials
XPRT	Xanthine-guanine phosphoribosyl transferase
ZnO	Zinc oxide

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