Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

03-Dec-2014

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

GENOTOXICITY OF MANUFACTURED NANOMATERIALS : REPORT OF THE OECD EXPERT MEETING

Series on the Safety of Manufactured Nanomaterials
No. 43

JT03367819

ENV/JM/MONO(2014)34
Unclassified
English - Or. English

OECD Environment, Health and Safety Publications

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GENOTOXICITY OF MANUFACTURED NANOMATERIALS : REPORT OF THE OECD EXPERT MEETING

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris, 2014

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FOREWORD

The OECD Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology (the Joint Meeting) held a Special Session on the Potential Implications of Manufactured Nanomaterials for Human Health and Environmental Safety (June 2005). This was the first opportunity for OECD member countries, together with observers and invited experts, to begin to identify human health and environmental safety related aspects of manufactured nanomaterials. The scope of this session was intended to address the chemicals sector.

As a follow-up, the Joint Meeting decided to hold a Workshop on the Safety of Manufactured Nanomaterials in December 2005, in Washington, D.C. The main objective was to determine the "state of the art" for the safety assessment of manufactured nanomaterials with a particular focus on identifying future needs for risk assessment within a regulatory context.

Based on the conclusions and recommendations of the Workshop [ENV/JM/MONO(2006)19] it was recognised as essential to ensure the efficient assessment of manufactured nanomaterials so as to avoid adverse effects from the use of these materials in the short, medium and longer term. With this in mind, the OECD Council established the OECD Working Party on Manufactured Nanomaterials (WPMN) as a subsidiary body of the OECD Chemicals Committee in September 2006. This programme concentrates on human health and environmental safety implications of manufactured nanomaterials (limited mainly to the chemicals sector), and aims to ensure that the approach to hazard, exposure and risk assessment is of a high, science-based, and internationally harmonised standard. This programme promotes international co-operation on the human health and environmental safety of manufactured nanomaterials, and involves the safety testing and risk assessment of manufactured nanomaterials.

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.

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PART I : WORKSHOP REPORT ON THE GENOTOXICITY OF MANUFACTURED NANOMATERIALS

Background and Objectives

The OECD WPMN Workshop on the Genotoxicity of Manufactured Nanomaterials was hosted by Health Canada and was held in Ottawa, Canada on 18-19 November 2013. The workshop was one of the horizontal expert workshops agreed to be held as part of the program of work of the WPMN Testing Programme. A total of 41 participants from 20 delegations attended. The meeting was chaired by Tim Singer (Health Canada).

The timing of the Workshop was carefully chosen to reflect a number of factors: a) the completion of the WPMN Testing Programme dossiers, which for a number of the representative materials included genotoxicity testing results; b) the conclusion of the EU NANOGENOTOX Joint Action and publication of its final report; and c) the current process within the OECD Test Guidelines Programme to revise seven genotoxicity test guidelines.

The purpose of the Workshop was to review the genotoxicity results from the OECD Testing Programme[1] and the EU NANOGENOTOX Joint Action with the intention of discussing whether there is a need to include specific adaptations relevant to testing the genotoxicity of nanomaterials within the existing OECD Test Guidelines on genotoxicity, and/or a need to develop new Test Guidelines or guidance material. The workshop also had as an objective the identification of any knowledge gaps and regulatory needs in the area of genotoxicity testing of nanomaterials.

In order to maximise the potential for recommendations to be taken up within the Test Guidelines Programme, the meeting was held back-to-back with a meeting *of the OECD Expert Group on the revision of the genotoxicity test guidelines*. A number of participants at the Workshop were members of the genotoxicity expert group and attended both meetings.

The meeting was structured to begin with a presentation setting the stage and several plenary presentations, followed by the discussion of pre-identified questions in two breakout groups – *in vitro* assays, and *in vivo* assays. The workshop agenda and break-out group questions are found in Appendix A.

Presentations

NanoGenotoxicity Testing for Safety Assessment – Dr. Shareen Doak (Swansea University, UK)

Dr. Shareen Doak presented by WebEx and the following text summarises her presentation. She noted that evidence in the literature indicating that some manufactured nanomaterials may be genotoxic was beginning to accumulate, but that there are many inconsistencies that hinder drawing conclusions. Although a battery of well-defined assays with OECD Test Guidelines are routinely used to support regulatory approval of pharmaceutical and chemical compounds, the testing strategy for nanomaterials has

1. OECD Sponsorship Programme for the Testing of Manufactured Nanomaterials

yet to be defined. Dr. Doak identified three questions that remain to be resolved: 1) is the current regulatory testing regime suitable for nanomaterials; 2) what combination of tests is optimal; and 3) do nanomaterials require their own testing protocols or the development of new methods?

The decreased size and greatly increased surface area of nanomaterials provide the potential for much greater activity, for a given mass, compared to larger particles of the same chemical composition. While being a desirable property for commercial applications, such increased reactivity may potentially increase the probability of interactions and interference with genotoxicity assay components. *In vitro* assay components that may potential interact with nanomaterials include serum content in culture media, as well as cytochalasin B (cyto B) used in the cytokinesis-block micronucleus assay.

Dr. Doak presented four recommendations related to the *in vitro* micronucleus assay (OECD TG 487).

- Firstly, regarding the use of cyto B, it was recommended to apply it separately, either by first applying the nanomaterial to the cells, then removal of media and replacement with media containing cyto B, or by delayed co-treatment where the nanomaterial is applied to the cells for 3-24 hours, followed by addition of cyto B to the cells plus medium for the remaining treatment time. Which one of these two procedures is preferable has not yet been conclusively determined.

- Secondly, regarding exposure time, a 24-hour exposure typically results in a higher micronucleus frequency and a lower effective dose than shorter or longer treatment durations. This suggests that 24 hours is sufficient to determine a genotoxic response.

- Thirdly, regarding the use of serum, reduced serum concentrations can often enhance nanomaterial uptake, but low serum content is not necessarily representative of the *in vivo* situation where there are high protein levels present. This may increase sensitivity, but at the expense of a higher proportion of false positives.

- Finally, a large variation in the response of various cell lines has been observed and it is not possible at present to make recommendations on the optimal cell type to use; however, it is important to use genetically stable, p53 competent cell lines as one way of avoiding misleading results.

The Ames test (OECD TG 471) is an essential component of the genotoxicity testing battery, but it may not be suitable for detecting genotoxicity induced by nanomaterials. This is because the bacterial cells used lack the ability to perform endocytosis and because nanomaterial diffusion across the bacterial cell wall may be limited, both of which limit nanomaterial uptake; as well, some nanomaterials have antibacterial properties. Dr. Doak noted that the majority of Ames tests report negative results for a range of nanomaterial types, despite these having induced positive genotoxic responses in other *in vitro* test systems. She presented a summary of results for eight nanomaterials with negative Ames results that have been found genotoxic in a variety of mammalian cell assays[2]. As an alternative *in vitro* gene mutation assay, Dr. Doak recommended the *in vitro* mammalian cell gene mutation assay (OECD TG 476), where no reports have yet identified specific limitations when testing nanomaterials.

When considering the existing literature in assessing best practices for testing nanomaterials for genotoxicity, a number of limitations have reduced the utility of the results. These include: the use of cell lines with high background levels of DNA damage and high genetic instability, the absence of positive or negative control data that hinders assessment of data quality, the absence of data on cellular or target tissue

2. See Doak et al., Mutat Research. 2012 Jun 14;745(1-2):104-11

uptake, no data provided to support dose selection, limited physical-chemical characterisation, insufficient cell numbers assessed, or no description of dispersion protocols used.

In developing an interim *in vitro* genotoxicity testing approach, Dr. Doak made the following recommendations:

- Provide detailed descriptions of the physical-chemical properties of the nanomaterial being tested, the dispersion protocols used, the exact treatment conditions employed and the positive and negative control data.
- Use cells with genetically stable backgrounds and that are p53 competent.
- Conduct extended dose-response investigations, relating doses to physiologically relevant levels where possible and avoid excessively high doses.
- Justify dose selection with toxicity or cytotoxicity data.
- Confirm that cells or the target tissue were actually exposed to the nanomaterial.
- Employ the micronucleus test adaptations previously described and avoid the Ames test in favour of the *in vitro* mammalian cell gene mutation assay.

Additional research is necessary to clarify the unresolved questions, including

- Determining what *in vitro* assays are most informative, least likely to produce misleading positive results and best correlate with their *in vivo* counterparts.
- Determine which cell lines should be used for *in vitro* assays.
- Better characterise the extent and impact of nanomaterial degradation within *in vitro* systems.
- Develop better approaches to consider the consequences of chronic exposures
- Develop assays better able to identify and characterise DNA damage induced by secondary mechanisms, such as oxidative stress caused by chronic inflammation.
- Develop a more detailed understanding of structure-activity and property-activity relationships to better predict genotoxicity based on physical-chemical features.

ITS-NANO: Research Prioritisation to Deliver an Intelligent Testing Strategy for Engineered Nanomaterials – Prof. Vicki Stone (Heriot-Watt University, UK)

Prof. Stone presented by WebEx the outcomes of the EU FP7 project ITS-NANO and the following text summarises her presentation. This project aimed to develop a research prioritisation tool with the objective of contributing towards the development of an intelligent testing strategy (ITS) for nanomaterials, recognising that it is impossible to consider assessing the risks of every nanomaterial for every exposure scenario on a case-by-case basis. The overall premise of the project is that modelling in combination with testing can predict the risks of nanomaterials. Achieving this objective would be based

on conducting various research initiatives on a short-term, long-term, and distant future basis. In the short-term (<5 years), research would focus on developing an understanding of the connection between physical-chemical properties, exposure and hazard, which enables grouping or ranking based on these properties. In the longer-term (10-15 years), the development of modelling approaches would lead to a continual reduction in requirements for *in vivo* and then *in vitro* testing. In the distant future (>15 years), risk assessment of nanomaterials will be based on modelling and extrapolation, with focused physicochemical, exposure and hazard testing where necessary.

Prof. Stone described the three priority research components of the ITS, those being physicochemical identification, exposure identification and hazard identification, and described research priorities within each component area along with the timeframe for implementing these. By addressing these priorities, one moves towards an ITS by being able to make rational grouping and ranking decisions, leading to the development of modelling and computational approaches that will ultimately reduce reliance on testing.[3]

OECD Test Guidelines on Genotoxicity – Ms. Nathalie Delrue (OECD)

Ms. Delrue presented an overview of the organisation and functioning of the OECD Test Guidelines Programme and the 1981 Council Act on the Mutual Acceptance of Data. The mutual acceptance of data principle, whereby the results from tests conducted according to the OECD Test Guidelines (TG) and under GLP are accepted in all adhering countries, helps to avoid unnecessary duplication of testing, saving resources for industry and society as a whole, reduces the use and suffering of laboratory animals needed for *in vivo* tests and helps to minimise non-tariff trade barriers. Since the adoption of the first TG in 1981, there have been about 150 new or updated TGs adopted, of which about 45 have been adopted in the last five years. TGs are freely available as are related documents (including Detailed Review Papers, validation reports and guidance documents) in the Series on Testing and Assessment.

The Working Group of National Coordinators of the Test Guidelines Programme (hereafter WNT) is responsible for oversight of the OECD Test Guidelines 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 supported by *the OECD Expert Group on the revision of the genotoxicity test guidelines* (Hereafter The Expert Group). These projects include the deletion of several unused and unneeded TGs, the revision of four *in vivo* TGs (TGs 474, 475, 478, and 483), revision of two *in vitro* TGs (TGs 473 and 487), the revision of the mammalian cell gene mutation assay, and the revision of the introduction to the test guidelines on genotoxicity. The Expert Group was to meet immediately following the Workshop and, amongst other issues, was to consider the Workshop outcomes in the context of the revisions to the Test Guidelines that are underway.

NANOGENOTOX Joint Action – Dr. Hannu Norppa (Finnish Institute of Occupational Health, Finland) and Dr. Nathalie Thieriet (ANSES, France)

The NANOGENOTOX Joint Action was an EU funded collaborative project coordinated by ANSES (France) that ran from March 2010 to February 2013 and involved 16 associated partners and 15 collaborating partners (7 national ministries and 8 institutes) from across Europe. The objective of the Joint Action (JA) was to work towards establishing a robust methodology to assess the potential genotoxicity of nanomaterials and to generate data on the genotoxic effects of certain commonly used nanomaterials. The

3. A report on this project has been published as Stone et al., Part Fibre Toxicol. 2014 Feb 13;11:9.

project stemmed from a recognition that there was a clear need for data given that many published studies have reported results on a variety of poorly characterised materials, there has not been a systematic effort to correlate *in vitro* and *in vivo* studies, the majority of cell types employed have been pulmonary cells, and there were no standard operating procedures for testing.

The JA focused investigation on 16 representative nanomaterials – 5 types of nano titanium dioxide (TiO_2), 4 types of synthetic amorphous silica (SAS), 6 types of multiwall carbon nanotubes (MWCNT) and one type of zinc oxide (ZnO). Seven work packages made up the JA, amongst which included physicochemical characterisation, *in vitro* testing, *in vivo* testing, and toxicokinetics and tissue distribution.

Work Package 5 (WP5) aimed to generate *in vitro* genotoxicity data on the representative nanomaterials using standard tests and modified assays using specific cell models, and to perform, based on the *in vitro* genotoxicity and physicochemical characterisation data obtained, an *in vitro* round robin test on selected nanomaterials using the most promising *in vitro* assays. The genotoxicity tests employed assessed DNA damage (alkaline comet assay, FpG-modified in a few labs), micronuclei (cytokinesis-block micronucleus assay and micronucleus assay without cyto B), and mutations (mouse lymphoma assay). The cell systems chosen included human pulmonary cells, human intestinal cells, human dermal cells and lymphatic cells from humans and mice. Protocols for each cell line and endpoint were agreed upon in advance.

On the basis of the WP5 results, a number of general conclusions were made. These included:

- Cell lines that take up nanomaterials can be used for genotoxicity testing.
- The genotoxic activity that some nanomaterials induced *in vitro* could be the result of indirect mechanisms that are not presently well understood.
- Some of the genotoxic effects observed were slight and not always reproducible.
- The pulmonary-derived BEAS 2B cells performed slightly better than the intestinal-derived Caco-2 cells at detecting genotoxicity.
- While full thickness 3D skin models are not practical for genotoxicity testing, they are suitable for studying skin penetration.
- The preparation of the dispersion is key, as it is expected to affect agglomerate size, which in turn affects sedimentation, cell exposure, cytotoxicity, dose selection and genotoxicity.

Work Package 6 (WP6) aimed to determine the *in vivo* genotoxicity of the representative nanomaterials and to make comparisons between *in vitro* and *in vivo* results. Three complementary tests were employed: the comet assay in rats, the micronucleus assay in rats and the lacZ transgenic gene mutation assay in mice. Twelve nanomaterials were studied (4 SAS, 4 TiO_2 and 4 MWCNT) and were administered by oral gavage and intratracheal instillation, with four materials also administered intravenously. Inflammation and oxidative stress were examined by bronchoalveolar cell count, histology, the comet assay using FpG to detect oxidative DNA damage, and measurement of some indicators of lipid peroxidation in plasma. Doses administered were selected based on the dispersion protocol and the results from toxicokinetic investigations.

On the basis of the WP6 results, a number of general conclusions were made. These included:

- Most data indicated no *in vivo* genotoxicity, except in a few organs. These results need to be confirmed.

- Within the same nanomaterial family, genotoxicity appeared to vary by nanomaterial.

- Negative results were generally observed when using the bone marrow micronucleus assay (TG 474).

- Non-OECD guideline methods require acceptability criteria because of inter-laboratory variability.

- Even cases causing clear pulmonary neutrophilia are not necessarily associated with genotoxicity.

In conclusion, Dr. Norppa highlighted a number of points, which included the need for resolution of various technical issues related to testing, characterising the limitations of the assays used in terms of their predictivity and false positive and negative rates, the importance of intracellular dose and its relation to the exposure and dispersion techniques, the need for further comparative data for evaluating the performance of *in vitro* assays, the need to better understand whether there are potential nanospecific mechanisms of genotoxicity, and the association of inflammation and oxidative stress with genotoxicity and the question of whether *in vitro* assays are capable of identifying secondary genotoxicity caused by such mechanisms.

Finally, Dr. Norppa highlighted a number of suggestions for further research that arose in response to the results of the JA. These included:

- The development of techniques to measure cellular uptake in order to identify a dose metric for comparison between experiments and test systems.

- The development of measures additional to cytotoxicity to better define the dose or concentration range to be tested. In addition, the development of criteria to determine the upper bounds on the dose range and whether this should be based on upper limits on uptake or on cytotoxicity, and as an extension, the development of a means to differentiate direct cytotoxicity from indirect cytotoxicity caused by impacts of the nanomaterial on culture conditions.

- Low-dose effects.

- The influence of dispersant on the outcome of the test.

- Whether poorly soluble nanomaterials could produce a Trojan horse effect, as well as the influence of culture media on test results with poorly soluble nanomaterials.

- Better understanding nanomaterial-induced genotoxic mechanisms.

Genotoxicity Test Methods and Assessment Challenges: A Regulatory Perspective – Mrs. Myriam Hill (Health Canada) and Dr. Frank Le Curieux (European Chemicals Agency)

Mrs. Hill presented on the regulatory challenges associated with assessing the genotoxicity of nanomaterials. She noted the impracticality of conducting traditional mammalian toxicity testing for all variations of a given nanomaterial and the need to reassess some well-established tests for their

applicability for characterising the hazard of nanomaterials. Most jurisdictions require genotoxicity testing at some point before full commercialisation or registration of products or chemicals, including nanomaterials. In Canada, genotoxicity testing requirements for new substances are tiered and follow Health Canada's mutagenicity guidelines, which established, "…an ordered approach using a limited number of well-defined tests that complement each other in terms of endpoints, and that permits a systematic assessment of genotoxicity." Canada does not have nano-specific regulations and nanomaterials are evaluated according to the same criteria as for new chemicals or polymers. Data requirements are tiered based on tonnage, with an *in vitro* test for gene mutations required at 1000 kg/yr; an additional *in vitro* test for chromosomal aberrations and *an in vivo* genotoxicity test are required at 10000 kg/yr. Canada has received very few new substance notifications of nanomaterials

Waivers (particularly for *in vivo* studies) may present a particular challenge to regulators, since in many jurisdictions they are normally justified scientifically based on negative *in vitro* results. If the *in vitro* studies are not conducted in a manner that produces valid results, it is difficult to judge whether there are valid grounds to grant a testing waiver.

Dr. Le Curieux presented a brief overview of REACH. Under REACH, nanomaterials are evaluated according to the same criteria as substances and there are no nano-specific provisions. ECHA has received only a few dossiers for clearly identified nanomaterials. The genotoxicity requirements under REACH are also tonnage-based and include tests from the standard genotoxicity test battery that are applied in a tiered manner. Dr. Le Curieux noted that the European Commission has launched a public consultation on the options for possible amendment of REACH Annexes for the registration of nanomaterials.

Following from 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 and what adaptations would be appropriate.

Mrs. Hill noted that, in the context of genotoxicity testing of nanomaterials, discrepancies in results have been attributed to various factors, including interference with various assay components, incomplete or inappropriate characterisation and variations in the methods for nanomaterial synthesis. There are also unresolved questions that hinder regulatory assessment of genotoxicity test results including whether the exposure times are suitable, determining the appropriate dose metrics, the typical lack of characterisation data presented in most test reports, and the reliability of the Ames test. Regulators are confronted with uncertainties regarding which of the test methods are most suitable in providing robust results that can be relied on to identify genotoxic hazards, and furthermore regulators do not yet have a firm scientific basis in recommending that registrants conduct one *in vitro* genotoxicity test over another. Additional uncertainties result from the current lack of understanding of appropriate sample preparation procedures, particularly for *in vitro* tests. As it will be impossible to consider testing all forms of nanomaterials for genotoxicity, the question of how to use greater mechanistic understanding as a basis for creating groups or categories of nanomaterials for assessment becomes increasingly important.

Finally, Mrs. Hill presented a list of regulatory needs for improving genotoxicity assessments including that the physical-chemical properties of the nanomaterial under assessment are thoroughly characterised and presented in the dossier, that genotoxicity has been assessed via a scientifically reliable test, and that there are reliable, efficient and reproducible biological model systems developed to allow the assessment of secondary genotoxicity in order to anticipate potential carcinogenicity.

Genotoxicity of Nanomaterials: Refining Strategies and Tests for Hazard Identification – Results from an Environmental Mutagen Society workshop – Dr. Stefan Pfuhler (The Procter and Gamble Company)

Dr. Pfuhler presented the outcomes of a workshop that was held at the Environmental Mutagen Society (now the Environmental Mutagenesis and Genomics Society) meeting in Fort Worth, Texas, USA on October 23, 2010. The workshop had 80 participants from the regulatory, academic and industrial sectors from a variety of countries. The workshop was structured with initial presentations covering the scientific and regulatory views, three breakout groups to allow for in-depth discussion and then a final plenary session to capture consensus views. The meeting was chaired by Drs. Stefan Pfuhler (Procter & Gamble) and Rosalie Elespuru (US FDA).

The purpose of the workshop was to consider the utility of standard *in vitro* and *in vivo* assays for identifying nanomaterial induced genotoxicity, discuss whether standard protocols are suitable to detect the anticipated modes of action, discuss the needs related to the integration of new technologies, identify the gaps left by the standard assays and the research needs that must be filled to move forward.

The conclusions of the workshop have been published as Pfuhler et al., Environ Mol Mutagen. 2013 May;54(4):229-39. Following on from the workshop, a Nanomaterials Working Group has been formed under the Health and Environmental Sciences Institute (HESI) Genetic Toxicology Technical Committee to address some of the issues identified. This group of more than 20 members has started a comprehensive literature review with the target of completing the review in 2014.

Discussions

Most experts felt that there remained a large number of unknowns that made it difficult to make concrete and specific recommendations in the areas of concern that were outlined during the presentations. As well, the WPMN Testing Programme dossiers and the NANOGENOTOX JA were just completed earlier in 2013 and it was felt there was a need to conduct more detailed analyses of these results in order to draw additional conclusions. It was determined early during the Workshop that it was not going to be possible to address all the challenge questions set out for the two breakout groups due to the lack of data. As a result, the workshop participants focused on discussing broader issues on which it was felt a consensus could be achieved.

There were seven consensus statements agreed during the meeting:

1. 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.

2. Measures of cytotoxicity based on cell proliferation that are described in the test guidelines are appropriate for determining the top concentration to be applied for *in vitro* tests of nanomaterials. It is appropriate in some cases to consider wider concentration spacing than the standard $\sqrt{10}$ in order to ensure the concentration-response relationship is well characterized, and at concentrations not associated with cytotoxicity.

3. Characterisation of the materials should be undertaken in the cell culture medium used both at the beginning of treatment and, where methodologies exist, after treatment. The intent when applying nanomaterials to a cell culture medium is to create conditions that are comparable, to the extent possible, with the biological and physiological conditions within the *in vivo* system.

4. 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.

5. 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.

6. Prior to conducting an *in vivo* genotoxicity study, there is a need to conduct some toxicokinetic investigations to determine if the nanomaterial reaches the target tissue, where the target issue is not the site of contact. In the absence of data to the contrary, the test is not applicable for detecting primary genotoxicity if the nanomaterial does not reach the target tissue.

7. There are insufficient data to recommend one route of administration over another. The basis for selecting the route of administration for testing should be to consider the route most applicable to human exposure(s).

In addition, workshop participants acknowledged that there remained a number of knowledge gaps that will need to be addressed in order to resolve many of the outstanding issues. Examples of these knowledge gaps include:

- What are appropriate nano-specific positive and negative controls? The NANOGENOTOX JA assessed nano-TiO_2 as a potential universal positive control; however, it was found not to be suitable in all cases.

- Are there (or could there be developed) *in vitro* test methods suitable for detecting secondary genotoxicity?

- Can we identify appropriate metrics, complementary to existing toxicity/cytotoxicity measures, to allow better definition of the dose/concentration range to be tested?

- What are the most suitable cell lines to use?

- What is the influence of dispersants (e.g., BSA) on test outcomes?

- What is the effect of exogenous metabolic activation (S9)?

- Can we identify the biological mechanisms underlying the genotoxicity of nanomaterials?

Post-Workshop Developments

The workshop consensus statements were considered on 20 November by the Expert Group (EG) for the revision of the genotoxicity TGs. As a result, the EG, with the help of some of the Workshop participants, agreed to begin the development of a list of characterisation and other nano-related parameters that could be listed within the genotoxicity TGs as required information to be included within the study report produced by the testing lab. The list would be based around the eight characterisation parameters described in ISO/TR 13329:2012. Because some characterisation methods do not yet exist or are not yet standardised, there are implications on Mutual Acceptance of Data if these parameters were now to be included within the TGs; therefore, a guidance document (not subject to MAD) specifying the recommended characterisation parameters was initiated by the OECD Test Guidelines Programme Secretariat as an interim measure and it is currently under revision.

The EG considered the needs for including further nano-related guidance within the introduction document to the genotoxicity TGs or within separate guidance/interpretation documents that are already being prepared for the Series on Testing and Assessment. These documents are intended to offer guidance on the general use of the genotoxicity guidelines and interpretation of the data, but could be expanded to also offer nano-specific guidance. This area presents a good collaborative opportunity between the WPMN Steering Group on Testing and Assessment and the WNT genotoxicity EG.

The draft TG 473 and 487 excluded insoluble materials from the applicability domain of the guidelines. These two TGs have demonstrated utility in testing the genotoxicity of nanomaterials and, in order to prevent any confusion, the relevant sections of these guidelines were re-worded carefully so they do not exclude nanomaterials. Test Guidelines 473, 487, 474 and 475 were approved by the WNT in April 2014. These approved TGs do not contain any nano-specific adaptations.

At the moment, the Ames test (TG 471) is not under revision. However, the Test Guidelines Programme is considering how best to address the applicability domain issues for this guideline (amongst other more general issues), which may include opening the guideline for revision in the future.

Additional Recommendations

As the lack of data prevented workshop participants from being able to make concrete and specific recommendations on any adaptations to the genotoxicity TGs that may at present be necessary, it is recommended that analysis of the Testing Programme dossiers continue, along with data from the NANOGENOTOX JA and the general scientific literature. Analyses should continue with the focused objective of being able to address the unresolved questions, to the extent possible, in order to identify any adaptations that may be needed.

Direct collaboration between the WPMN and WNT will be critical to adapting the genotoxicity TGs where appropriate; collaboration would be greatly facilitated through the engagement of the Secretariat for the Test Guidelines Programme and the Nanosafety Programme. A particular immediate collaborative opportunity between the Steering Group on Testing and Assessment and the Genotoxicity EG may arise from the development of guidance documents. The current timeframe for the planned genotoxicity guidance documents would see these completed and sent for WNT approval in April 2015, meaning there is a short window of opportunity if the two groups are to take advantage of the existing process to insert nano-specific guidance into these more general documents. Alternatively, and particularly if more time is needed, the groups could decide together to propose a new project related to the creation of separate guidance material and submit a workplan project proposal (SPSF) to the WNT accordingly.

Finally, it was noted that other groups, such as the HESI Genetic Toxicology Technical Committee, are also engaged in considering nano-genotoxicity testing issues. It is therefore recommended to consider these efforts under the auspices of the OECD in order to reduce the potential for duplication and to promote engagement of all relevant expertise.

PART II: AGENDA

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

ORGANISATION DE COOPÉRATION ET DE DÉVELOPPEMENT ÉCONOMIQUES

WPMN Workshop on the Genotoxicity of Manufactured Nanomaterials

18-19 November 2013

Ottawa, Canada

Agenda

Day 1 9h30-18h00		
Registration 9h00-9h30		
SESSION I. WELCOMING REMARKS AND SETTING THE SCENE		
9:30	**Welcoming Remarks**	Canada
9:45-10:00	**Objectives and Expected Outcomes from the Expert Meeting**	OECD Secretariat
10:00-10:45	**Plenary Lecture: Nano-Genotoxicity Testing for Safety Assessment**	Shareen Doak Swansea University, UK (via WebEx)
10:45-11:15	**Break**	
SESSION II. TEST METHODS AND TEST GUIDELINES ON GENOTOXICITY		
11:15-11:45	**OECD Test Guidelines on Genotoxicity**	Nathalie Delrue OECD Secretariat

	Presentation to outline the process of revision of the genotoxicity TGs, the work of the genotoxicity expert group, the current status of each revised TG, and the anticipated timelines and opportunities to feed into the revision process.	
11:45-12:15	***ENPRA project and FP7 ITS-NANO (Intelligent Testing Strategy for Nanoparticles)*** Presentation to highlight the outcomes of the ENPRA project and the FP7 ITS-NANO projects.	Vicki Stone Nano Safety Research Group, Heriot-Watt University, Edinburgh, UK (via WebEx)
12:15-13:15	**Lunch**	
13:15-13:45	**NANOGENOTOX Joint Action** Presentation to highlight the key findings of the EU NANOGENOTOX Joint Action. Deliverables and the final report are available at: http://www.nanogenotox.eu/	Hannu Norppa Finnish Institute of Occupational Health
13:45-14:15	**Genotoxicity test methods and assessment challenges – A regulatory perspective** This presentation will: (1) describe current challenges associated with nanomaterial risk assessments; and (2) what is required from test guidelines on genotoxicity from a nanomaterial risk assessment perspective.	Myriam Hill Health Canada
14:15-14:45	**Genotoxicity of Nanomaterials: Refining Strategies and Tests for Hazard Identification** Presentation to highlight the outcomes of the workshop held at the Environmental Mutagen Society annual meeting in October 2010.	Stefan Pfuhler Procter and Gamble, USA
14:45-15:15	**Break**	
15:15-16:30	***Plenary Discussion:*** • Needs for revision of existing OECD Test Guidelines and guidance documents • Opportunities for development of new or revised test guidelines and guidance documents: what is feasible given our current knowledge Preparation for breakout sessions, including review of charge questions and consideration of data from testing programs	Tim Singer Health Canada

SESSION III. BREAK OUT GROUPS			
16:30-18:00	**Group A: *In vitro* assays** Consideration of charge questions. (See Annex I)	**Group B: *In vivo* assays** Consideration of charge questions. (See Annex II)	
18:00	**End of day 1**		

Day 2 9h00-17h00			
9:00-9:15	**Summary of major issues from the 1st day**		Tim Singer Health Canada
BREAK OUT GROUPS (CONT.)			
9:15-13:00	**Group A: *In vitro* assays**: Consideration of charge questions. (See Annex I)	**Group B: *In vivo* assays**: Consideration of charge questions. (See Annex II)	
Lunch Break **(time at discretion of groups)**			
SESSION IV. REPORTS FROM BREAK-OUT GROUPS			
13:00-14:30	**Each group will report on the outcomes and results of the break out session with an aim to highlight the recommendations made. Plenary discussion of these views.** **Group A: *In Vitro* Assays** **Group B: *In Vivo* Assays**		Break-out group chairs - TBD
14:30-14:45	**Coffee Break**		
SESSION V. CONCLUSIONS AND RECOMMENDATIONS			
14:45-15:45	**Discussion on testing strategies** Considering all of the test guidelines, the typical composition of test batteries, test performance, testing gaps, and on the basis of published studies, the results of the Nanogenotox project, the OECD testing programme and other available data: 1. Are the existing test batteries sufficient to detect genotoxicity caused by nanomaterials? 2. Given the technical and other limitations of the various assays and mechanistic considerations, what is the optimal combination of tests? 3. Are there any new test methods that could play a role in		Tim Singer Health Canada

	an amended integrated testing strategy for the genotoxicity of nanomaterials?	
15:45-16:45	**Recommendations to the WPMN and WNT** How to act upon the workshop outcomes considering the current test guideline revision process and Mutual Acceptance of Data. What should be taken up by the Genotoxicity Expert Group into the draft test guidelines and what should be incorporated into (a) guidance document(s)? **Next Steps**	Tim Singer and OECD Secretariat
16:45-17:00	**Final Conclusions and Remarks**	Tim Singer Health Canada
17:00	**End of meeting**	

ANNEX I: CHARGE QUESTIONS TO BREAKOUT GROUPS
GROUP A*: IN VITRO* ASSAYS

Considering the following *in vitro* test guidelines:

TG 471, 473, 476, 487 and the draft revised test guidelines posted for commenting and on the basis of published studies, the results of the Nanogenotox project, the OECD testing programme and other available data:

1. For which tests are there sufficient data to address questions about their applicability and reliability for testing nanomaterials?

2. Considering the tests for which one can make conclusions, which are appropriate and produce reliable results for the genetic toxicity testing of nanomaterials?

3. For the tests deemed appropriate, are there methodological considerations or modifications that could be made to the test procedures to improve the applicability or reliability for testing nanomaterials, including (but not limited to) the following areas:

    a. Adoption of standard dispersion protocols.

    b. Selection of appropriate positive controls (nano and non-nanoscale).

    c. Characterisation of cellular uptake: Is it required and if so, how should it be done?

    d. Ensuring the intra- and interlaboratory reproducibility of the tests.

    e. Selection of appropriate cell lines.

    f. Nanomaterial incompatibilities with test conditions, including such issues as the impact of cytochalasin-B in the binucleate version of TG 487, precipitating concentrations, interference caused by test components and the impact of whether degradation of nanomaterials in cell culture systems mimics *in vivo* conditions and if this is important.

2. Are there particular training needs for laboratories conducting nanomaterial genetic toxicity tests using the OECD test guidelines?

3. Are there *in vitro* testing gaps; for example, assays to detect genotoxicity caused by secondary mechanisms?

4. Are there new *in vitro* tests that should be further developed that may be particularly suitable for testing nanomaterials?

5. Are there currently any knowledge gaps that prevent the development of further guidance for users of the *in vitro* test guidelines who are testing nanomaterials? If so, what are they and is there a practical means by which they can be filled?

ANNEX II: CHARGE *QUESTIONS TO BREAKOUT GROUPS* *GROUP B: IN VIVO* ASSAYS

Considering the following *in vivo* test guidelines:

TG 474, 475, 478, 483, 488 and the draft revised test guidelines posted for commenting

and on the basis of published studies, the results of the Nanogenotox project, the OECD testing programme and other available data:

1. For which tests are there sufficient data to address questions about their applicability and reliability for testing nanomaterials?

2. Of those identified in Q1, which tests are appropriate and produce reliable results for the genetic toxicity testing of nanomaterials?

3. For the tests deemed appropriate, are there methodological considerations or modifications that could be made to the test procedures to improve the applicability or reliability for testing nanomaterials, including (but not limited to) the following areas:

    a. Adoption of standard dispersion protocols

    b. Selection of appropriate positive controls (nano and non-nanoscale)

    c. Tissues to examine (where applicable)

    d. Ensuring the intra- and interlaboratory reproducibility of the tests

    e. Characterisation of uptake: Is it required and if so, how should it be done?

    f. Appropriate route(s) of exposure and its influence

    g. Selection of dosing levels and sampling times

4. Are there particular training needs for laboratories conducting nanomaterial genetic toxicity tests using the OECD test guidelines?

5. Are there any testing gaps, those being areas not covered by the existing assays?

6. Are there new tests that should be further developed that may be particularly suitable for testing nanomaterials?

7. Are there currently any knowledge gaps that prevent the development of further guidance for users of the *in vivo* test guidelines who are testing nanomaterials? If so, what are they and is there a practical means by which they can be filled?

PART III:PARTICIPANTS LIST FOR WPMN WORKSHOP ON THE GENOTOXICITY OF MANUFACTURED NANOMATERIALS OTTAWA, CANADA

18/11/2013 - 19/11/2013

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