NANOGENOTOX

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"Considerations to achieve a robust method for testing the genotoxicity of nanomaterials"

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Hazard Assessment Needs to study different exposure routes

In Nanogenotox : Target organ cells for exposure: the focus for research

- Target organs: Lung, Skin, and Gut
- Other secondary target organs?
- Endpoint under investigation: Genotoxicity

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Hypothesis: "There is one method (or there is a minimum battery of tests based on OECD TG) that can be applied to test the genotoxicity of manufactured nanomaterials"

Implication: The "genotoxicity test method" is suitable to be used on target organ cells *in vitro* to indicate the necessity of further tests on target organs *in vivo*

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Chemicals genotoxicity testing a reminder:

In chemicals genotoxicity testing, commonly a gene mutation assay (bacteria or mammalian cells) and an assay for chromosome mutations to detect clastogenic and aneugenic effects (Chromosome aberration, micronuclei) *in vitro,* are applied first

Usually for most chemicals (soluble, or well dispersed for those less soluble) genotoxicity is investigated by the *in vitro* micronucleus assay and the Ames test (bacteria)

The Micronucleus assay *in vitro* (TG487) is considered to be robust and effective in a variety of cell types, and in the presence or absence of cytochalasin B (actin polymerisation inhibitor)

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Nanomaterials specificity in genotoxicity testing:

The Ames test has been questioned because bacteria are not expected to sufficiently take up nanomaterials, if size is larger than 20 nm.

Do mammalian exposure target cells take up Nanomaterials?

YES, but to different extent, as often shown with different cell types *in vitro*

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In vitro Study design:

Study design is sound according to endpoint selection (genotoxicity: here DNA damage), test method selection (Micronucleus and Comet assay), and test system selection (exposure target cells)

Which target cells can be used specifically for the DNA damage detection with the Comet and Micronucleus test ?

➔ Preliminary Assumption: a statistically significant positive *in vitro* result may indicate genotoxicity

Answer from WP5:

All cell types that show sufficient proliferating activities can be used in the *in vitro* Micronucleus assay

To define a biological significance is unfortunately not so simple.

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In vitro Testing design and cell lines

Answer from WP5:

- Testing procedure of OECD TG 487 *in vitro* micronucleus assay is valid for use on nanomaterials
- The alkaline Comet Assay is a possible indicator test for DNA damage.
- The testing of MN genotoxic effects in different *in vitro* cell systems turned out to be very heterogeneous.

Recommendation : to choose the most sensitive and relevant cell type according to the route of exposure

- BEAS2B and Caco2 cells for both inhalation and oral exposure routes for genotoxicity tests showed sufficient sensitivity
- 3D reconstructed human skin model is a good candidate to estimate the barrier passage

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Round Robin test on Caco2 and BEAS2B cells

Results from WP5

"It is technically feasible to perform such genotoxicity assays with dispersed MN in cultured cells by different laboratories, but the predictive value of these *in vitro* tests in identifying MNs that are genotoxic *in vivo* or MNs that could be carcinogenic is presently unclear."

Why do statistical variances in Round Robin outcome occur for some particles and not for others?

- Difficulties in reproducing weak positive effects
- The dispersion stability in test systems?
- Post dispersion handling?
- Read out method in Comet assay (automatically versus manually) ?
- During set up of a validation study and, optimally also in a round robin study, participating laboratories should all be able to meet predefined acceptance criteria (range of response to positive and negative controls)

Recommendation: a strong technical harmonisation of test item preparation, dispersion, exposure, cell harvesting and scoring is required in case on MN safety testing

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Nanomaterials tested

Three MN families were investigated: TiO_2, SAS, MWCNTs

→ Necessity of a reliable and complete characterisation of each MN

Results from WP4 :

- Full characterisation of the raw material and MN dispersions
  - Notable difference of phys-chem. parameters per family
- General agreement between different methods
  - Primary particle size: TEM correlates with SAXS
  - Aggregates size: SAXS (and DLS) correlates with TEM
  - Specific surface area: SAXS correlates with BET
- Dustiness parameters (levels and size distribution in powder dust)
  - Enables comparison of relative exposure potentials
  - Enables identification of inhalation exposure characteristics

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MN and Culture media

Difference between culture media for cell culture

- ➔ Each test system has to be maintained in its optimized form to reduce artifactual "background" DNA damage or prevention
- ➔ Need for a stable dispersion medium that does not interfere with the test system and does not produce artifactual DNA damage

Results from WP4 :

- For all MNs a sufficiently metastable stock dispersion could be produced by a harmonized sonication procedure in a non genotoxic dispersion medium!
- The use of this stock dispersion with all culture media was ascertained
- Quality control of dispersions and agglomeration/sedimentation behaviour fate in exposure media is always required to ensure comparable exposures and knowledge on exposure characteristics.

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Toxicokinetics : Identification of the target organs

Biopersistence results from WP7 following oral administration

- Limited if any uptake from the GI-tract, but some uptake is possible

Biopersistence results from WP7 following intravenous administration

- Main target organs: liver, spleen, and to lesser extent lung, and kidney
- SAS: Clearance from all organs. TiO_2: Some clearance from organs for one TiO_2 while for others there is only a minimal decrease. CNT: in general persistence but also for one CNT some clearance from liver and spleen.
- Bioaccumulation after 90 days for certain MNs in some organs (some of CNTs in liver and spleen, and TiO_2 in liver and spleen)

New analytical protocol from WP7

- Sample preparation and ICP-MS analysis protocols for the purpose of silica determination in organs following gavage exposure have been created.

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Tests done by WP6

- Not only OECD TG 474 on bone marrow, but non-OECD assays such as Comet assay, Micronucleus on colon, and lacZ assays were performed
- Comet and Micronucleus tests were performed on the same animals and on various organs, systemic and from the contact sites

Results from WP6

- At low doses with some MNs genotoxic effects were detected occasionally
- Effects are different even in the same MN "chemical family"
- Further need to receive some last results to complete the *in vivo* genotoxicity database
- Critical issues observed within WP6 should be confirmed by new experiments
- Importance of new *in vivo* assays apart from Micronucleus on bone marrow especially in organs of contact sites for the investigation of genotoxicity !

Recommendation: A sufficient level of skill is required to perform testing and analysis

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Even if all steps were harmonized, would *in vitro* genotoxicity testing become predictive for the *in vivo* genotoxic response ?

This question leads us back to a comparison with the results in target cells investigated in WP6 *in vivo*:

In WP6 no strong indications for micronuclei induction were observed following gavage, i.v., or instillation.

There is some indication that high doses of selected MNs could elicit DNA damaging effects in BAL fluid cells after instillation (Comet assay), pointing to inflammatory modes of action after instillation of high doses.

Following gavage, negative results have been detected on bone marrow but some effects were detected at low doses within colon cells with some MNs

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In most cases *in vitro* models are overpredictive and show the worst case for small soluble chemicals. Caution: only true if the test substance reaches its biological target !

The *in vivo* results emphasize the necessity to investigate target organ cells (lung and gut) in MNs genotoxicity testing *in vitro* **and** *in vivo*!

If not human primary culture systems can be used (i.e. human 3 D lung and gut models), the *in vitro* model systems would have to be characterized with respect to cellular particle or fibres/tubes -uptake, DNA repair systems, differentiation state, and barrier function.

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If the *in vitro* Micronucleus test is negative, could we then renounce *in vivo* testing ?

For soluble chemicals mostly: YES
For particles and fibres: at present, NOT YET !

Caco2 and BEAS2B cells are good test systems that can be chosen to identify a primary genotoxic effect !
More doses in the low dose range are required.

These systems do not mimic the secondary genotoxicity that can occur due to inflammation or due to unrealistically high doses.

Organs accumulating MNs, like CNTs in lung or TiO_2 in liver and spleen, should be investigated for adverse effects in priority, and especially in long-term low dose studies!

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Conclusions

- No possibility of classifying as "monosubstance" the families of MNs studied as differences have been observed in:

  - Phys-chem characterisation
  - *In vivo* and *in vitro* genotoxicity
  - Toxicokinetic behavior

- Several physico-chemical characterization methods are available. To determine the primary MN size-distribution, TEM is strongly recommended. SAXS may be applied for average size. DLS may be useful for determination of hydrodynamic aggregate size, but strict protocols must be established. DLS is very useful for evaluation of dispersion quality and stability in MN suspensions. SSA may be determined by BET, SAXS, (and TEM), but SAXS is also applicable for in situ SSA (and average size) analysis in MN dispersions.

- According to preliminary scientific results, the MNs investigated in Nanogenotox did not so far show strong genotoxicity *in vivo* and *in vitro;* neither in exposed target cells following gavage (colon), nor after instillation (lung), nor *in vitro* on 3 D reconstructed human skin models. However, in several cases, even at the lowest tested doses of MNs, some genotoxic effects were detectable *in vitro* and *in vivo*.

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Conclusions

- For hazard identification of substance related genotoxicity: the OECD *in vitro* mammalian cell micronucleus test guideline TG 487 can be used but with different target cells than usually investigated. However, particle uptake into the cells of the chosen test system should be proven, otherwise negative results might occur due to lack of exposure, hence not describing the potential hazard.

- The *in vivo* mammalian erythrocyte micronucleus test (TG 474) can be used, however again, the test item must reach the target cells *in vivo*.

- Other *in vivo* tests might be applicable for genotoxicity investigation of MNs like for example the *in vivo* micronucleus assay on lung, intestine and colon as some genotoxic effects were observed *in vivo* on those organs.

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Recommendations for a Test Method

- **In general and according to the behavior of MNs and their specificities, any genotoxic TG should :**
  - **be amended to include some toxicokinetic testing:** there is a critical need to ALWAYS investigate whether the nanomaterials reach the target cells and not just rely on genotoxicity methods commonly used.
  - include historical data or criteria of acceptability and reproducibility of testing, especially for non-OECD tests and with cell models which are not commonly used for genotoxicity investigations

- In addition any genotoxicity assessment *in vitro* should provide :
  - dispersion protocol and characterisation of the resulting dispersion, the uptake proof, positive and negative controls
  - the most sensitive and relevant cell type according to exposure

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