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Secretariat: K. Rothenbacher (EPMF)

LIST OF PARTICIPANTS

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MINUTES

1. Background

PMC commissioned the genotoxicity expert Prof D. Kirkland to review the PGM testing programme on genotoxicity testing and to advise on the best testing strategy going forward. Prof Kirkland was also consulted on some specific questions, such as the apparently conflicting genotoxicity test results on Rh (III) substances.
Prof Kirkland's interim report is provided in the appendix – it is scheduled that an evolution of this report will be provided early in 2012 with additional data and commentary. A brief summary of his main findings/ conclusions were summarized at the start of the meeting as follows:

2. General discussion

2.1. Suitability of the Ames test for metals genotoxicity assessment

- HERAG considers the Ames test not suitable for genotoxicity assessment of metals in most cases. DK's view is that this due to the fact that the Ames assay can produce false negative results for some metals (e.g. where direct or indirect genotoxic effects cause large DNA deletions). However this needs to be assessed on a case by case basis, and the HERAG strategy may not be applicable when genotoxic effects elicited by particular metals do not result in large DNA deletions and point mutations can be detected.
- DK's assessment of available Ames tests on PGMs (see full report) indicates that that the tests were valid (no obvious false negative findings) and were likely not influenced by extreme pH either.
- DK concludes that Ames is likely a suitable test system for PGMs if direct genotoxic effects could occur. As a matter of standard practice for PGM testing it would be prudent to commence with the Ames test. However, a 'screening test' should be conducted initially to determine if excessive toxicity to the bacterial cells is apparent that prevents exposure to at least a few hundred ug/plate. Such toxicity could affect the ability of the Ames test to detect mutational effects

2.2. Potential confounding influence of pH in in vitro tests

a. The available test reports on PGMs show no evidence that pH confounding effects occurred at the concentrations that were scored for mutations or micronuclei.
b. Ames tests are robust in terms of tolerance of low pH.
c. Tests based on mammalian cells can be affected by pH, more likely in the presence of S9. The exact reason for this is not fully known yet; but a hypothesis is that lipid peroxidation can occur within the S9 and lead to release reactive oxygen species (ROS) thus causing DNA damage and cytotoxicity. This effect is thus more likely in cells that have poorer ROS defense mechanisms.
d. Test substances with low pH can be handled via buffering to avoid confounding effects
    i. Most mammalian cell culture media has effective buffering capacity, but in any case

the pH indicator in the medium would change colour if there were even small pH changes, and tests with neutralization could then be performed, if desired.

ii. CROs usually carry out a pre-test on solubility, to evaluate the maximum possible concentration that can be achieved by use of various primary solvents followed by dilution in bacterial or cell culture media. pH changes seen on dilution into medium could be noted at this time, and the strategy for testing changed (e.g. addition of tests with neutralization) if necessary. It was strongly suggested that the PGM tests are preceded by such pre-tests. However, even if pH changes occur in the medium, the cells may not survive these concentrations, and so the concentrations tolerated by the test system may be effectively buffered.
Action:
WCA to ensure such pre-tests are included in the test protocol.
WCA to request CRO to flag any pH changes at that step

e. The genetic effects observed for PGMs do not seem to be caused by extreme pH but appear to be real effects.

2.3. In-vivo studies (in vivo micronucleus assay)

There is no evidence of pH effects causing false positive/negative effects in vivo:

a. However, if negative effects in vivo are reported, it is crucial to demonstrate actual target organ exposure has occurred (e.g., via toxicokinetic measurements and in particular blood concentration and AUC assessment). As an indication, this could be done at ca 5 time points with ca 4 animals from 2 dose groups: top dose and control = 5 x 4 x 2 = 40 samples, so the additional cost will be limited.

b. The route of dosing needs careful consideration: for substances with low pH (acidic), oral dosing might be preferable to intraperitonial (ip) dosing, since the stomach already has a low pH. There is a possibility that the substance might precipitate due to pH changes in the peritoneum. If both methods are difficult intravenous (iv) exposure should be considered, in particular if the test is carried out to clarify an in vitro positive result. As a standard choice, the oral route should be selected in precedence.

2.4. Recommended testing strategy

a. An Ames assay followed by in-vitro micronucleus test is recommended as a general strategy. This is in-line with Reach requirements and this testing strategy has also been approved by EFSA and the UK Committee on Mutagenicity. It is recommended that the in-vitro micronucleus test is preferably performed in p53-competent human lymphocytes to minimize the risk of false positive results.

b. If an Ames negative is obtained at reasonably high plate concentrations (1000 – 5000 mcg/plate) there is no need to follow with confirmatory MLA assay.

c. The mouse lymphoma assay tk mutation assay (MLA) is not recommended for routine replacement of the Ames test. Although there is no evidence that existing MLA test results on PGMs have been affected by extreme pH, the MLA test is considered to be sensitive to pH (see also point 2b) above. If the Ames test does not seem appropriate (e.g. because the test substance is very toxic and the Ames test may therefore be deemed insensitive), it may be possible to carry out an HPRT gene mutation test in mammalian cells. This could be done instead of the MLA, where tk mutations detect both chromosomal and gene mutation events, because the chromosome damaging potential will already have been measured in the in-vitro micronucleus test. Thus it would only be necessary to measure gene mutations. The HPRT

endpoint is less susceptible than the tk endpoint to false positives. However, it should be noted that the best cells in which to measure HPRT mutations are mouse lymphoma L5178Y cells because they do not suffer the sensitivity limitations of trying to measure HPRT mutations in monolayer cells such as CHO or V79.

3. Detailed discussions

3.1. Interpretation of test results for Rhodium (III) compounds and proposed testing strategy - review of available data on Rhodium chloride and Rhodium nitrate:

- General points, mechanism of action
  - All tests were considered valid
  - The data indicate DNA effects probably via frameshift mutation rather than point mutation or oxidative damage (based on TA98 and TA100 reversion).
  - DK was informed that a review of Rh genotoxicity is available from the Dutch Expert Committee on Occupational Standards (DECOS) at http://www.gezondheidsraad.nl/en/publications/rhodium-and-compounds-evalaution-carcinogenicity-and-genotoxicity
  - Positive Ames data are reported for Rh(III) iodide and -acetate[1]. **Action -reports should be provided by PMC so they can be considered in the revisions to DKs expert report (done)**
- The purity of the test substances was not specifically reported, and it is thus difficult to judge whether mutations could have been due to impurities
- In vivo MN testing protocol: the tests carried out at Bioservice used flow cytometry to measure MN in reticulocytes in their evaluation rather than measuring MN in polychromatic erythrocytes of bone marrow using visual scoring. Flow cytometry has more power due to the larger number of cells examined and allows for better quantification of the results. DK recommended to consider this method also in future tests, otherwise future tests might be seen as following lower standards
- Conclusions
  - Rhodium chloride clearly shows a genotoxic potential while the data on Rhodium trinitrate indicate negative results
  - Available Ames studies and an in vivo micronucleus assayfor Rhodium trinitrate are negative —the reason for this disparity is unclear (but see below)
  - In summary, the weight of evidence suggests a genotoxic potential for Rh(III) ion/compounds on a class basis (frameshift mutagen and clastogen).
  - **Action discuss situation/ next steps with PGM WG to better understand the seemingly contradictory results.**
    - Several options are possible to further evaluate the situation: foremost there is a need to better understand the chemistry of the tested compound
      - Is Rh(NO3)3 a nitrate at all or will it undergo speciation changes?
      - Is it possible that the trinitrate will precipitate at pH changes (e.g. if injected into the peritoneum of test animals)?, etc)
    - However, DK's current opinion is that there is a sufficient basis to classify Rh(III) as mutagenic, and that Rh(III) nitrate represents an outlier for reasons still to be determined

Action: PMC to set up PGM WG call in January 2012

[1]Positive in TA 98 and TA 100 strains, with and without S9 (effect particularly clear with TA100).

3.2. Platinum genotoxicity data

a. As explained by PMC participants, platinum dinitrate displays a complex chemistry and may undergo changes in speciation in physiological conditions. It is therefore perhaps not a good example to generalize to other Platinum compounds
   Action: provide DK additional details on above point (KR, DB)
b. As also described by PMC participants, There is evidence that chloroplatinate complexes may be reactive toward DMSO. For example, Cisplatin (cis-Diamminedichloroplatinum) and DMSO form adducts (see attached paper). The adducts then have less ability to bind to DNA; this could explain the weak positive Ames test effects seen with Hexachloroplatinic acid (CPA) when solubilised in DMSO versus the more clear mutagenic effects seen when dissolved in water. DK considered this a plausible reason for the test outcome difference.
   i. **Action: evaluate if this may have been the case for any relevant platinum compound test data (WCA/bibra)**
   ii. **Action: recommendation to use alternate vehicle for new testing on Pt series compounds (WCA to flag to CROs)**
c. Hexachloroplatinic acid is clearly cytotoxic in mutagenicity assays, but the genotoxic effects are considered real, despite the substance's cytotoxicity
d. An IRIS report by the US EPA is available on halogenated Pt compounds. The report concludes that
   i. Soluble tetrachloroplatinate compounds are mutagenic in Ames assays
   ii. Diammonium hexachloroplatinate is considered mutagenic
   iii. Tetraammine Platinum compounds are much less reactive due to leaving group behaviour and give negative results
e. The US EPA is currently working on an IRIS report on platinum compounds and halogenated platinum salts. The final report has not been published yet but a draft version can be downloaded here http://oaspub.epa.gov/eims/eimscomm.getfile?p_download_id=486407

3.3. In vitro micronucleus test (IVMN)

a. If this test should be required, DK recommends human lymphocytes rather than other cell line alternatives. This is due to the fact that some cell lines give high false positive results due to p53 deficiency versus lymphocytes (providing a potentially spurious damaged survivor effect). This phenomenon is now described via inter-laboratory test outcome and is forming part of the latest recommendations on genotoxicity testing.

4. Conclusions

- The Ames test is a central piece in regulatory evaluations. This test should only be waived/substituted if there are clear indications that the test is unsuitable for mechanism of action (MOA) reasons. Our data on PGMs suggest that there is no reason not to use it, so consequently it should be used.
- This conclusion applies in general also to Palladium and Gold compounds unless there is a MOA rationale not to do so. A toxicity screening test should however be conducted first (see above).
- DK will update his report in light of the discussions above and include the additional data mentioned
- REACH: if Ames and MN are negative, REACH normally calls for a confirmatory assay in mammalian cells. This can include mouse lymphoma *tk* assay, the MLA, but alternatives can be considered and may be preferred, e.g. *tk* mutation in TK6 cells, HPRT in mouse lymphoma cells etc.). The HPRT assay is recommended.
- Palladium: it remains to be confirmed if the Ames test is applicable to Pd-compounds if mutagenicity

testing is planned. It was noted that the EHC 226 review of Pd had identified some DNA reactivity via use of an isolated DNA test system. It is recommended to perform a screening-test as described under point A, 2d.

Action: PMC to urgently review testing programme for Diamminedichloro palladium and change work scope with Covance accordingly (i.e. put MLA on hold, and carry out Ames and IVMN tests in first phase). Any other evidence (even tentative) of Pd DNA reactivity, e.g. from literature should be identified if possible.

Annexes:

1. D. Kirkland report "Review of the effect of pH on the integrity of in vitro and in vivo genotoxicity assays" dated 1st December 2011
2. Publication by Fischer et al "Cisplatin and dimethyl sulfoxide react to form an adducted compound with reduced cytotoxicity and neurotoxicity"