

MUT/07/11

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

**DEVELOPMENT OF OECD GUIDELINE ON THE IN-VITRO MICRONUCLEUS TEST.**

**CONSULTATION FOR COM INPUT FOR AN OECD MEETING TO PRECEDE EMS MEETING IN ATLANTA 20-24 OCTOBER 2007**

**Introduction**

1. COM members have contributed to the UK input to the development of an OECD guideline for the in-vitro micronucleus test. US EPA outlined in a memorandum to the US national coordinator a number of remaining topics for discussion. In addition an OECD discussion document for a meeting to be held in Paris on 28-30 March 2007 has been provided to the secretariat. This document is approximately 89 pages in length and details a large number of comments which are to be discussed in brief at the Paris meeting. This document has not be appended in full, although the introductory pages which set out the main areas for further consideration are outlined in para 3, page 3 of this document. (Relevant documents are included in Annex 1 to this discussion paper.)
2. The COM is asked to provide comments which will aid in developing a UK position for discussions to be held in Atlanta USA in October 2007. Of predominant concern raised by EPA is the need for confirmatory testing, the use of cytokinesis block in tests using mammalian cell lines, and the appropriate level of cytotoxicity to determine maximum concentrations for testing.
3. Other areas identified in the OECD discussion document for the Paris meeting in the introductory section include areas include use of exogenous metabolic activation, repeat testing when the first assay is negative, and exposure times and also requests where the guidance needs further redrafting including differentiating between tests using lymphocytes and cell lines and clearer methodological descriptions.
4. The current draft guideline are appended for member's information as Annex 2 to this draft discussion paper.
5. The secretariat note that it wont be possible in the time available at a full COM meeting to have a detailed discussion of all the areas where there is need for further clarification. In this regard the OECD secretariat have suggested a number of ways forward (as outlined on page 3 of the OECD discussion document for the Paris meeting , Annex 1 to this paper). The COM is asked to focus its discussions on practical options relating to the need for confirmatory testing, the use of cytokinesis block in tests using mammalian cell lines, and the appropriate level of cytotoxicity to determine maximum concentrations for testing.

## **Background to development of a protocol for the in-vitro micronucleus test (IVMNT)**

6. COM members will be aware of most of the background regarding the development of the IVMNT. The secretariat have outlined a number of comments regarding the areas for further consideration and have appended a selection of the most relevant published papers to aid discussion.<sup>1-5</sup> (Annex 3 to this discussion paper) An overview of the international consensus on the IVMNT was published by Kirsch-Volders M et al in 2003<sup>1</sup> (Annex 3). This reported on an initial review of validation studies at the 3<sup>rd</sup> International Workshop on Genotoxicity Testing, 28-29 June 2002 (IWGT). The previous 2<sup>nd</sup> IWGT meeting held in Washington, USA on 25-26 March 1999 had reported on current methodologies and available data at that time.
7. The 3<sup>rd</sup> IWGT report<sup>1</sup> produced consensus statements on cell proliferation, assessment of toxicity and dose-range finding, treatment schedules for cell lines, treatment schedule for lymphocytes, choice of positive controls, numbers of cells to be scored, repeat experiments and statistics. A position statement on use of cytokinesis block (CB) with cell lines was agreed noting it should be added during the first cell cycle following treatment and cell harvested prior to the second mitosis. No particular protocol was established for CB in cell lines but reference was made to preliminary data from a number of published papers which are included in Annex 3. (This includes Lorge E et al 2006<sup>2</sup>, Garriott et al 2002<sup>3</sup> and Phelps et al 2002<sup>4</sup>). At the time of publication of the 3<sup>rd</sup> IWGT report there was some evidence to suggest that CB improved detectability in cell lines. However one key argument advanced by Kirsch-Volders M et al for using CB in cell lines was the provision of additional data allowing the mode of action of chemicals in formation of micronuclei and in particular identification of aneugens. This aspect is further commented on, including a strategy for assessment of aneugens, in an editorial by Parry JM and Parry EM 2006<sup>5</sup>

## **Comments on areas for further consideration**

### *The need for confirmatory testing,*

8. With regard to cell lines, the 3<sup>rd</sup> IWGT recommended a confirmatory test for negative and equivocal first tests (3-6 h exposure) using continuous exposure (2-2.5 cell cycles or possibly up to 3 cell cycles for certain groups of chemicals such as nucleoside analogues) and a repeat of the first study with modified exogenous metabolic activation (and in some instances justified modifications to the first test).
9. In the case of assays using lymphocytes which all use CB, the 3<sup>rd</sup> IWGT recommended, the first test involves 20 h exposure to the test compound in the absence of exogenous metabolic activation. A test with exogenous metabolic activation may follow this test (if protocols without metabolic activation are negative or equivocal) or be carried out at the same time. If the test with exogenous metabolic activation is negative then the repeat

test involves similar testing conditions but with a longer PHA stimulation of lymphocytes (cf 48 h for repeat test compared to 24 h for initial test in presence of exogenous metabolic activation).

10. The draft IVMNT guideline is consistent with the 3<sup>rd</sup> IWGT report and there are no published data cited in this discussion paper which suggest a further modification for confirmatory testing is required. One comment in the detailed comments on the draft IVMNT guideline is that the requirement for repeat testing in the presence of exogenous metabolic activation exceeds the requirements for other in-vitro mammalian guidelines. What are member's views?

*The use of cytokinesis block in tests using mammalian cell lines,*

11. The consensus view reported from the 3<sup>rd</sup> IWGT included CB in the IVMNT for both cell lines and lymphocytes. Additional recommendations from the 3<sup>rd</sup> IWGT noted the value of CB for identifying mode of action of genotoxicants. Lorge E et al 2006 subsequently published the SFTG (French Branch of European Environmental Mutagen Society) which specifically investigated the role of CB in the IVMNT in cell lines. Thus it was noted that CB application to lymphocytes ensures that cells that have undergone one cell division are counted for micronuclei. With regard to unsynchronised mammalian cell lines these are continuously undergoing cell division. It was also noted that there was evidence that the concentration of cytochalasin B might impact on the conduct of tests in cell lines. (The secretariat note that the investigation by Surrallés J et al Mutagenesis, 9, 347-353, 1994 was considered in the recent evaluation by COM on background incidence of MN formation biomonitoring studies using peripheral blood lymphocytes. Higher MN frequencies are found in lymphocytes at 3ug/ml cytochalasin B compared to 6 ug/ml cytochalasin B). Lorge E et al also noted that cytochalasin B had been reported in one communication to induce DNA fragmentation.
12. The study undertaken involved 38 participating laboratories from Europe, Japan and America with tests in four cell types (lymphocytes, CHO, CHL and L5178Y) in presence and absence of cytochalasin B (details of concentrations not reported). A range of chemicals (n=11) were selected including clastogens, base and nucleoside analogs, aneugens and/or polyploidy inducers and non-genotoxic compounds (n=2 out of the 11). The authors noted that MN frequencies in the presence of CB counted MN in binucleate cells whereas counts for MN frequency in the absence of CB counted mononucleated cells. Thus the expected background frequency of MN in binucleated cells in the presence of CB would be double that for the same cell line in the absence of CB. The background frequency of MN in fibroblast cell lines was increased in BN cells but did not double indicating lowered efficiency of CB. With L5178Y cells a doubling of MN frequency was noted in control cultures in presence of CB. It was also noted that DMSO affected the frequency of MN formation in these cells. The authors reported that scoring both mononucleated and binucleated cells was the preferred approach for identification of aneugens. However overall the use of CB made no impact on the detection of MN in cell lines exposed to genotoxicants.

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13. Garriott M et al<sup>3</sup> and Phelps et al<sup>4</sup> published data which were consistent with the conclusions reached by Lorge et al.
14. Parry and Parry<sup>5</sup> note the application of the assay using CB offered a strategy for distinguishing clastogens from aneugens and providing information on the mechanism responsible for micronucleus inducing activity. Reference is made to the COM guidance which indicates the value of the aneugen detection in genotoxicity testing. One important element to the proposed strategy is to use CB to aid MN identification and the use of centromere probes to provide information the nature of MN and on possible mode of action. (see appended paper in Annex 3).
15. The current draft OECD guideline (Annex 2) mentions use of CB in a number of sections including introduction (para 1), principle of test (para 10), there is a section on the use of the CB (para 20-22), procedure (cell lines, para 38/9, lymphocytes para 40), analysis (paras 47-50), and treatment of results (paras 51-52). The draft guideline is generally consistent with the 3<sup>rd</sup> IWGT report but doesn't take into account the interpretation placed by Lorge et al<sup>2</sup> on the use of CB in cell lines to identify MN inducing chemicals. The strategy suggested by Parry and Parry is not specifically cited in the current draft OECD guideline.
16. The COM is asked to consider the broad aspects of using CB in the IVMNT and to advise where sections of the current draft OECD guideline might be amended. It is noted that there are a large number of detailed comments which will be presented to OECD Paris meeting which refer to the use of the CB technique.

*The appropriate level of cytotoxicity to determine maximum concentrations for testing.*

17. The 3<sup>rd</sup> IWGT reported recommended 50-60% toxicity based on reduction in CBPI. In part this recommendation was based on a personal communication (see ref 33 of appended Kirsch-Volders paper Annex 3), There are no data available in the appended published papers to suggest that 70% toxicity is required.
18. Members are asked to provide advice on the most appropriate approaches to measuring cytotoxicity for all possible approaches which could be used for the IVMNT (i.e. separately for lymphocyte and cell lines either with or without CB).

#### **COM discussion**

19. Members will be aware that there is a long history of use and development of the IVMNT and considerable interest on behalf of researchers, industry and regulators at attaining a unified OECD guideline. COM advice will be used to inform on a UK line for each aspect considered to help facilitate the final stages of the drafting of the OECD guideline.
20. The areas to consider are i) the need for confirmatory testing, ii) the use of cytokinesis block in tests using mammalian cell lines, and iii) the

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appropriate level of cytotoxicity to determine maximum concentrations for testing.

**Secretariat March 2007**

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## References

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2. Lorge E et al (2006). SFTG international collaborative study on in-vitro micronucleus test 1. General conditions and overall conclusions of the study. *Mutation Research*, 607, 13-26.
3. Garriott ML et al (2002). A protocol for the in-vitro micronucleus test 1. Contributions to the development of a protocol from an examination of 16 chemicals with different mechanisms of action and different levels of activity. *Mutation Research*, 517, 123-134.
4. Phelps JB et al (2002). A protocol for the in-vitro micronucleus test II. Contributions to the validation of a protocol suitable for regulatory submission from an examination of 10 chemicals with different mechanisms of action and different levels of activity. *Mutation Research*, 521, 102-112.
5. Parry JM and parry EM (2006). Editorial. The use of the in-vitro micronucleus assay to detect and assess aneugenic activity of chemicals.