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**DRAFT**

**MUT/08/8**

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

**REVIEW OF GENOTOXICITY OF PHENOL**

**Referral to COM**

1. The HPA have asked for advice on the genotoxicity of phenol and specifically whether a threshold approach can be used with regard to the risk assessment of genotoxicity of phenol.

**Background to COM review**

2. HSE asked for advice from COM on phenol (along with hydroquinone) in 1994/95 and in 1999. A copy of the conclusions and the statement agreed in 1999 (published January 2000) are appended as Annex 1. [Hydroquinone is a metabolite of phenol, see section on metabolism below]

3. In brief, in 1994, the COM concluded the *in vitro* mutagenicity data on phenol were of poor quality and results difficult to interpret, but *in vivo* data showed phenol to be a somatic cell mutagen at very high dose levels. (COM noted negative results in long term carcinogenicity bioassays in rats and mice). The COM noted the potential for rapid conjugation and detoxication via the glutathione pathway and that the mutagenicity of phenol appeared to be predominantly related to peroxidase activity and catalase could have a protective role. The COM agreed there was a potential for a threshold mechanism by the oral route of exposure but could not reach a similar conclusion with regard to dermal or inhalation exposure.

4. In 1995, the COM considered a submission from industry which provided some metabolism data. Overall the COM concluded that appropriate studies to determine the extent of pre-systemic metabolism following either inhalation or dermal exposure had not been undertaken. The COM provided guidance on the approaches which could be used (including administration of hydroquinone or phenol via a bronchoscope with very early sampling for free and conjugated test substance in the blood.

5. In 1999, the COM considered a study on bioavailability and metabolism of hydroquinone after intratracheal instillation in male rats. The results showed free systemic hydroquinone in arterial blood 5-10 seconds after dosing. The COM considered the data suggested the potential for site of contact and systemic mutagenic effects after inhalation exposure. The COM considered an inhalation exposure transgenic Muta™ mouse study but were unable to draw any conclusions in view of unacceptable levels of DNA packaging in many of the trials in the experiment. The COM noted a small but consistent positive result in bone marrow micronucleus studies in mice given intraperitoneal

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doses of around 100-160 mg/kg bw. (relevant BMMN studies are reviewed in para below and appended in Annex 3).

6. The COM agreed a statement (00/S1) in January 2000 which appended in Annex 1. The conclusions reached with regard to phenol were similar to those reached in 1994.

7. In 2003, the COM considered a pre publication report from the Dow Chemical Company which provided results to suggest that the *in vivo* mutagenicity of phenol in the mouse bone marrow micronucleus assay originated from a transient hypothermia induced by high doses of phenol. The COM agreed the data supported a case for a threshold mechanism for the induction of MN in bone marrow of mice but considered publication of the study in a peer-review journal would be necessary before drawing any definite conclusions. A further COM statement was not published in 2003. The relevant study Spencer PJ et al Tox Sci, 97, 120-127, 2007) has now been published and was identified during the 2007 COM horizon scanning exercise. Members asked for a review of the paper during the COM horizon scanning exercise.

### Introduction to current COM review

8. The COM consideration of phenol covers a period from 1994-2003. The objectives of the current review is to i) produce an up to date COM statement on phenol, ii) to evaluate the Spencer study on hypothermia and also iii) to consider if any *in vivo* mutagenic effect of phenol can be considered as related to a threshold effect.

9. The COM have considered many of the key studies on phenol in full in the past but over quite a period of time. Thus in order to provide a comprehensive overview of the mutagenicity of phenol, the secretariat have submitted a draft EU risk assessment review which has been provided by HSE (Germany acting as rapporteur) dated 1/09/2005 (Annex 2 to this paper, for members use only). In addition extracts from important studies on phenol are appended in Annex 3. A copy of the Spencer paper is appended in Annex 4.

### Overview of phenol mutagenicity (Draft EU risk assessment report Annex 2. Relevant papers Annex 3)

10. Pages 95-160 of the draft EU risk assessment report have been provided for members. The information covers ADME studies (pages 95-98), mutagenicity (pages 128-154) and carcinogenicity (pages 155-157).

#### *ADME studies*

11. Phenol is well absorbed via the gastrointestinal (ca 90% in rats) and respiratory tract (ca 60-88% in humans) and the dermal route (ca 75% in rats). Absorbed phenol is rapidly distributed in body tissues. It is extensively

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metabolised predominantly to sulphate and/or glucuronide conjugates predominantly in the liver, gastrointestinal tract and kidneys. The ratio of sulphation compared to glucurination varies with species and dose level and route of administration. Extrahepatic metabolism in rats increased at doses of 5 mg/kg bw and above. Phenol is also metabolised to hydroquinone (with a lower proportion metabolised via this route following oral administration to mice compared to intravenous administration, cf 8% v 17%). Other metabolites reported in mice included hydroquinone sulphate and mercapturic acid conjugates of hydroquinone. The proportion of these metabolites in mice was also reduced following oral administration compared to intravenous administration (cf 1% v 11%). After oral administration only traces of conjugated hydroquinone were detected in the metabolic profiles of rats and humans. The metabolic profile after dermal administration in humans is not known. Evidence for covalent binding to proteins and DNA has been reported *in vitro*, but not in one *in vivo* study in rats given oral doses of 75 mg/kg bw. Absorbed phenol is rapidly excreted as metabolites in the urine irrespective of route of administration.

### *In vitro* mutagenicity studies

#### Bacteria

12. Phenol was not mutagenic in standard bacterial tests for mutagenicity in *Salmonella typhimurium* strains. A weak positive result was reported in one assay in *S. typhimurium* TA98 which was dependent on the medium used.
13. Do members agree phenol is negative in bacterial mutagenicity tests?

#### Mammalian cell; gene mutation tests

14. The EU risk assessment report describes positive results in mammalian cell mutagenicity tests using the *hprt* locus in V79 cells and SHE cells in the absence of exogenous metabolic activation and at the Na/K locus in SHE cells in the absence of exogenous metabolic activation. A positive result in the presence of S-9 mix was reported in two separate mouse lymphoma assays. A positive was reported in one assay in the absence of exogenous metabolic activation. It was noted that evidence of cytotoxicity was reported in these assays. (Tabulated data from these assays is presented on pages 136-139 of the EU risk assessment report. Data from the relevant references are also appended in Annex 3, Paschin and Bahitova Mut Res, 104, 381-393, 1982, Glatt et al EHP, 82, 81-89, 1989, Tsuitsui et al Mut Res, 373, 113-123, 1997, Wagenheim and Bolcsfoldi Mutagenesis, 3, 193-205, 1988, McGregor Env Mol Mut, 12, 88-154, 1988).
15. Appended data from the individual studies is appended in Annex 3. Overall there is evidence for a positive result in Paschin and Bahitova 1982 but the remaining assays (i.e. Glatt, Tsuitsui, Wagenheim and the study by McGregor) would seem to report at most equivocal results, i.e. relatively small

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increases in mutation frequency at doses inducing significant reductions (ca 80%) in cell growth in the absence of any evidence for a dose-response.

16. What are members views of mammalian cell mutagenicity tests with phenol? Would it be more appropriate to consider the data as equivocal rather than 'weak' positive as reported in the UE risk assessment report?

### Mammalian cell; chromosomal/aneugenic tests

17. Phenol gave a positive result for chromosomal aberrations in CHO cells in the presence and absence of exogenous metabolic activation (tabulated data in EU risk assessment report on page 140, Ivett et al 1989 in Annex 3). Positive results were also reported in a number of micronucleus tests in CHO cells (both in presence and absence of exogenous metabolic activation page 141, Miller et al 1995), in V79 cells and human PBLs (both in the absence of exogenous metabolic activation, page 141 of EU risk assessment report, Glatt et al 1989, Yager et al 1990 in Annex 3). Tsuitsui et al scored chromosome number in metaphase spreads and reported no evidence for an aneugenic effect of phenol (positive results were reported for benzene in the same experiment but a known aneugenic positive control was not used). Yager reported a moderate increase in both kinetochore positive and negative micronuclei in PBLs indicating some evidence for both clastogenic and aneugenic activity with phenol.

18. What are members views of the mammalian cell chromosomal/aneugenicity data? Does the evidence suggest a direct clastogenic effect of phenol but is less convincing regarding the effect of metabolic activation and with respect to potential for aneugenicity?

### Mammalian cell; DNA damage

19. A positive result for UDS was reported in SHE cells in the absence of exogenous metabolic activation using a liquid scintillation method (page 147 of EU risk assessment report, Tsuitsui et al in Annex 3).

20. A positive result for DNA strand breaks in mouse lymphoma cells in the presence of exogenous metabolic activation was reported (page 147, EU risk assessment report, Garberg et al 1988 in Annex 3). These authors used a statistically significant increase in ssDNA of 6.5% in the presence of relative toxicity of 5% or less as the criteria for a positive result. Negative results were reported in the absence of exogenous metabolic activation.

21. Positive results for DNA damage (8-hydroxy-2-deoxyguanosine 8-OHdGua formation ) were reported in HL60 cells (which contain myeloperoxidase activity) in the absence of exogenous metabolic activation. (page 148 of EU risk assessment report, Kolanchana P et al 1993 in Annex 3)

22. It is noted the study by Pellak and Walker had not been received at the time of writing this covering paper.

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23. What are members views of these studies? Can any conclusion be reached regarding the mode of action for positive results reported, and in particular the potential for free radical formation following the metabolism of phenol?

### Mammalian cell; DNA adducts

24. A positive result for DNA adducts was reported using calf thymus DNA in the presence of horseradish peroxidase and hydrogen peroxide. (page 148 of EU risk assessment report. Subrahmanyam V and O'Brien 1985 in Annex 3). It is noted that there was no evidence for DNA binding in the absence of peroxidase.

25. What are members views of this study. Do the data indicate DNA binding due to oxidative metabolism of phenol?

### Conclusion; In vitro mutagenicity, DNA damage, DNA adducts.

26. The draft EU risk assessment report presents a conclusion that phenol is a mammalian cell mutagen inducing gene mutation, chromosomal damage and micronuclei. Phenol induces UDS in mammalian cells and DNA strand breaks and DNA adducts. It is also concluded in the draft EU risk assessment report that phenol is not a bacterial mutagen and does not induce aneuploidy in mammalian cells.

27. From the review undertaken for the COM, it is suggested that the evidence for gene mutations in mammalian cells is at best equivocal and the mechanism for the reported results is unclear. In addition there is some limited evidence from kinetochore staining in micronuclei tests for an aneugenic effect in addition to chromosomal damage. The evidence that DNA damage and DNA binding is dependent in peroxidase activity in the test system suggests that free radical metabolism of phenol is required for the mutagenic effects of phenol.

### *In vivo mutagenicity studies*

#### Bone Marrow MN

28. The results of available studies are tabulated on page 131 of the EU risk assessment report. Evidence for a 2-2.5 fold induction of BMMN has been reported in some studies at oral and i.p. doses which equate to or exceed the relevant LD50 in mice. (Additional details are tabulated on pages 150/151, but some details appear to have been omitted.) The relevant data from these studies are appended in Annex 3 (Barale R Mutat Res, 244, 15-20, 1990, Ciranni R Mutat Res, 208, 61-67, 1988, Gad El Karim M et al Am J Ind Med, 7, 475-484, 1985, McFee A Mutat Res, 260, 387-391, 1991,)

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29. An important conclusion reached by COM during its previous consideration of phenol related to the evidence for a small but consistent *in vivo* BM MN positive effect at dose levels below the i.p. LD50 in mice. In brief there were three key studies supporting this conclusion. Chen and Eastmond (Carcinogenesis, 16, 1963-1969, 1995) used 3 doses of 160 mg/kg phenol i.p. followed by BM sampling 24h after the last dose. There was no discernable effect on the PCE/NCE ratio but signs of toxicity, if observed were not reported. FISH analysis indicated that the positive results were due to chromosome breakage. Mazzarini A et al Mutat Res, 341, 29-46, 1994 reported a significant positive effect following a single i.p. dose of 120 mg/kg bw to a group of 3 CD-1 mice followed by bone marrow sampling 18h after treatment. There was no apparent effect on the PCE/NCE ratio but signs of toxicity, if observed were not reported. Shelby M et al Env Mol Mutagen, 21, 160-179, 1993 reported a positive trend test for BM MN induction in two separate studies where male B6C3F1 mice were given i.p. doses of 0, 45, 90 or 180 mg/kg bw phenol on three consecutive days with bone marrow sampling 48 h after the last dose. All animals survived and there was no apparent effect on percent PCEs. However signs of toxicity, if observed, were not reported.

30. Do members concur with the previous COM conclusion on these studies?

31. The EU risk assessment report contains a short section on the potential for phenol induced hypothermia as a mode of action for the observed result of BMMN tests. This is considered in detail in paragraphs 38-43 below.

### Bone Marrow chromosomal aberrations.

32. A negative result was reported in a study where groups of 3 Sprague-Dawley rats were dosed orally or by i.p. administration with a dose equivalent to the LD30. Only 30 metaphase spreads/animal were examined. Information from this study on page 152 of the EU risk assessment report. (Reference not obtained for Annex 3 at the time of writing).

### Other *In vivo* studies

33. No evidence for oxidative DNA damage (8OHdGua) in bone marrow was reported in a group of three mice given an i.p dose of 75 mg/kg bw (EU risk assessment report 133 and 153, Kolachana et al Cancer Research, 53, 1023-1026, 1993 Annex 3). Data reported in this reference are the mean of three separate experiments.

34. No evidence for DNA adducts was reported in a <sup>32</sup>P-postlabelling study in rats five oral doses of 75 mg/kg bw on four successive days and tissues (bone marrow, zymbal gland and liver) sampled 24 hours after the last dose (EU risk assessment report pages 133, 154). (Reference not obtained for Annex 3 at the time of writing).

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35. Negative results were reported in a test for single strand breaks in rat testes following a single i.p dose of 7.9-79 mg/kg bw or five daily doses of 4-39.5 mg/kg bw i.p. (EU risk assessment report page 133, 152.153). (Reference not obtained for Annex 3 at the time of writing).

### Conclusion *In vivo* mutagenicity, DNA damage, DNA adduct studies

36. The EU risk assessment report concluded (page 134) that negative results had been found for chromosomal aberrations, DNA strand breaks and DNA adducts. A negative result was also reported for *Drosophilla* tests (Data from such tests are generally not used by COM in reaching conclusions.) Results from *in vivo* BMMN tests were considered weakly positive or negative. The magnitude of the response to phenol was low even at doses which correspond to the LD50. The induction of MN may be based on an indirect mode of action. A classification of category 3 was suggested. It was noted that hypothermia was a plausible mode of action but no definite conclusions on the proposal could be drawn.

37. From the review undertaken for COM, it is suggested that the EU risk assessment report presents accurate conclusions based on the studies reviewed in that document. However there would seem to be evidence for small consistent increases in BM MN at doses below the LD50. The mode of action is discussed below.

### **Induction of micronuclei by phenol in mouse bone marrow. Association with chemically induced hypothermia. (Spencer et al *Tox Sci*, 97, 120-127, 2007, Annex 4)**

38. Groups of four male and four female CD-1 mice were dosed i.p with 0, 50, 150, 200, 300, 400, or 500 mg/kg bw phenol (Hypothermia test) . The relative Body Temperature was monitored subcutaneously using programmable transponders (also used for animal identification) prior to dosing, 5, 30, 60, 90 min and 2h, 3,4,5,6,24 and 48h after dosing. Clinical signs of toxicity were recorded. In the MN test groups of 6 animals/sex were dosed at 30, 100 or 300 mg/kg (separate group dosed p.o. with 120 mg/kg cyclophosphamide, 24 h sampling). BT was measured prior to dosing, and 2,5,24 and 48 h. Animals were killed at 24 or 48h post does and bone marrow collected. For kinetochore evaluation a group of 6 males was dosed with 300 mg/kg bw phenol (CP (p.o 120 mg/kg bw) and vinblastine (4 mg/kg bw i.p) used as positive controls with 24 h sampling). For MN evaluation 2000 PCEs were scored blind to dosing status. Data were transformed by adding one and taking natural log of adjusted number. Pairwise comparison of data used Dunnett t-test. Kinetochore positive MN-PCEs were compared using Fisher exact test.

39. All mice dosed at 400 mg/kg bw or 500 mg/kg bw died within 24h of dosing. A single male and female in the 300 mg/kg bw group died prior to the 48 h observation time point. No deaths at 200 mg/kg bw and elwo. Signs of

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toxicity included reduced activity (200 mg/kg bw and above) and twitching and tremors (at 100 mg/kg bw and above) which were noted shortly after dosing. Surviving mice appeared normal 1h post dose. Males appeared to be more sensitive with a more rapid onset of signs of toxicity and shorter period to death. Predose mean body temperatures in males and females were 36.7°C and 37 °C respectively. Thirty minutes post dose at 300 mg/kg bw mean BT reduced to 32 °C with mean BT as low as 28 °C 5h post dose in both sexes. BT did not return to baseline within the 48h observation period and was depressed 4-5 °C at the end of the experiment. BT reductions of up to 8 °C were recorded at 400 and 500 mg/kg bw (at up to 6h post dose). Smaller transient reductions in BT were reported at 100, 150 and 200 mg/kg bw. From the information presented in figure 1 of the published paper, the reduction at 100 mg/kg bw appears to be around 2 °C with a return to baseline around 2-3h post dose. At 200 mg/kg bw the decrease in BT appears to be around 2-3 °C with a return to baseline at around 4-6h. No evidence for an effect on BT was reported at 50 mg/kg bw.

40. In the MN test one animal dosed at 30 mg/kg bw died (not related to treatment). The authors report phenol related signs of toxicity in about one third of males and one half of females dosed at 300 mg/kg bw (table 1 of paper). Signs of toxicity appeared within minutes and had subsided about 1h post dose. There was evidence for very transient signs in animals dosed at 100 mg/kg bw (lasting only several minutes). No treatment related signs of toxicity were reported at 30 mg/kg bw. BT was reported at 24 and 48 h post dose. A 4-5 °C reduction was evident at 24h post dose in both males and females. By 48h the decrease was approximately 7 °C in males and 6 °C in females. BT at these time points was unaffected at 100 mg/kg bw and 30 mg/kg bw. BT was unaffected in CP positive control animals.

41. A statistically significant increase in MN-PCE/1000 PCE was recorded at 300 mg/kg bw at 24 h sampling (male 10.8 cf 2.1 in control and 11.3 in females cf 2.5 in controls). At 48 h the mean frequency of MN-PCE/1000 PCEs was 18.3 in males and 17.8 in females. The mean percent PCE values was reduced at 24h (all doses) and 48h (in males/females at 300 mg/kg bw). The frequency of MN-PCEs/1000 PCEs was not increased at 30 and 100 mg/kg bw. CP gave the expected positive result.

42. The authors conclude that phenol induced MN formation occurred only in the presence of marked hypothermia.

43. In the kinetochore experiment, a statistically significant increase in the proportion of kinetochore positive MN was observed in phenol treated mice at 300 mg/kg bw. VB gave the expected positive result. The proportion of kinetochore positive MN was substantially higher in VB treated mice.

44. In their discussion the authors note the finding of phenol induced hypothermia at doses at or above the MTD was a novel finding. The induction of hypothermia was associated with a NOEL for MN formation and thus phenol induced MN by a secondary mechanism associated with regulation of

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BT in mice. It was noted that in part, it was possible to speculate that BT affected spindle function thus resulting kinetochore positive MN. However a proportion of phenol induced MN were clastogenic and might have been due to an effect of phenol, hydroquinone (a metabolite of phenol) or a combination of phenol/hydroquinone. It is noted that the available data on phenol suggest that any direct genotoxic activity is likely to be mediated by oxidative DNA damage and hence would be presumed to have a potential threshold for activity. Overall the authors suggested a role for hypothermia but didn't prove causality. The authors suggest further studies to investigate the role of physically induced changes in BT on the induction of MN in phenol treated animals would be an appropriate way forward.

### **Additional data submitted by Dow: Study 011064 (In confidence data Annex 5)**

45. A full report of the studies undertaken by DOW has been submitted as an in-confidence document. Essentially phase 1 and phase 2 of the study were published in Spencer et al Tox Sci, 97, 120-127, 2007. Additional studies were undertaken to investigate the approach to thermoregulatory control (i.e. applying external heat to prevent hypothermia) in mice dosed with phenol (phase 3) and a rescue experiment was undertaken (phase 3). The objective of the rescue experiment was to obliterate phenol induced MN formation in mice by appropriate thermoregulatory control. This was not achieved (a statistical increase in MN formation was reported at 24h post dose). The investigators also noted that the application of external heat to control mice also resulted in a statistically significant increase in MN formation at 24h post dose. Overall the results of the rescue study were considered to be inconclusive. A further Telemetry experiment (phase 4) was undertaken to monitor body temperature in phenol dosed and control animals under normal and thermoregulatory control conditions at 5 minute intervals to provide more comprehensive data on the effectiveness of thermoregulatory support. Overall thermoregulatory control in control mice resulted in an overall elevation of body temperature compared to animals maintained under normal environmental conditions. For phenol-treated animals there was evidence of impaired capacity to modulate temperature compared to controls and a transient hypothermia. Overall it was possible that the application of thermoregulatory control could influence the formation of MN in control and phenol-treated mice. In phase 5, the results of kinetochore staining experiments were reported. These data have been published in Spencer et al Tox Sci, 97, 120-127, 2007.

### **COM Discussion and questions**

46. The secretariat have prepared a short paper on generic aspects of BT effects on MN formation in rodents. (MUT/08/09). The overall comment from this draft discussion paper is reproduced in paragraph 47.

47. The data support the observation that chemical induced hypothermia in mice and hyperthermia in rats and mice may be potential modes of induction

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of MN in bone marrow. Experimental evidence needed to support hypothermia or hyperthermia as a mode of action for an unknown chemical would include a time course showing the association between core body temperature and MN induction and evidence for reversibility of the chemical induced MN formation by adjusting core body temperature. The mechanism by which hypothermia can result in the induction of MN in mice is unclear. Thus single doses of ethanol can induce significant hypothermia in susceptible rats and mice but no evidence for *in vivo* mutagenicity was reported in rodents (COM statement 00/S5, December 2000). The assessment of hypothermic induction of MN for a specific chemical also requires evaluation for evidence regarding other modes of genotoxicity. A clear negative *in vitro* package of genotoxicity tests would rule out other modes of genotoxicity when deriving conclusions regarding the role of hypothermia in any observed *in vivo* MN formation. Evidence for positive *in vitro* genotoxicity would suggest other potential modes of genotoxic action which need to be taken into account in the overall assessment.

48. The COM is asked to consider the following questions
- i) Do members agree phenol is negative in bacterial mutagenicity tests?
  - ii) What are members views of mammalian cell mutagenicity tests with phenol? Would it be more appropriate to consider the data as equivocal rather than 'weak' positive as reported in the UE risk assessment report?
  - iii) What are members views of the mammalian cell chromosomal/aneugenicity data? Does the evidence suggest a direct clastogenic effect of phenol but is less convincing regarding the effect of metabolic activation and with respect to potential for aneugenicity?
  - iv) What are members views of the *in vivo* genotoxicity data on phenol. Does the COM concur with its previous conclusion that there is evidence for small increases in BMMN in mice at dose levels below the LD50.?
  - v) What are members views regarding the recently published study on the potential role of hypothermia on the induction of BMMN in mice dosed with phenol (Spencer et al Toxicolo Sci, 97, 120127, 2007). What additional data, if any, are required to further support the hypothesis proposed by these authors regarding the mode of action of phenol induced MN in mice.
  - vi) What are members views on the additional unpublished information submitted by DOW. Was thermoregulatory control adequate?

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- vi) What are members view on the generic approach to evaluating the potential role of hypothermia in the induction of chemical mediated MN in mouse BM as set out in paragraph 47 of this discussion paper.

**Secretariat April 2008**