

DRAFT

MUT/MIN/2010/2

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Minutes of the meeting held at 10.30 am on Thursday 17th June 2010 at Room 136/137B Skipton House, Department of Health, London SE1 6LH.

Present:

Chairman: Professor P Farmer

Members: Dr G Clare
Mrs R Glazebrook
Professor G Jenkins
Professor D Kirkland
Dr D Lovell
Dr A Lynch
Professor D Phillips

Secretariat: Mr J Battershill (HPA secretariat)
Dr D Mason (HPA secretariat)
Mr S Robjohns (HPA minutes)
Ms S Kennedy (HPA administration)
Dr D Parker (FSA)

Assessors: Dr R Shillaker (HSE CRD)
Ms C Pease (EA)

In attendance: D K Burnett (DH Tox unit)
Dr P Edwards (HPA)
Ms F Pollitt (HPA)
Dr O Sepai (HPA)

A G E N D A

Open session

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ITEM 1: ANNOUNCEMENTS/APOLOGIES FOR ABSENCE

1. The Chair welcomed Dr D Mason (HPA), Dr D Parker (FSA secretariat attending for Dr D Benford), Dr K Burnett (DH Tox unit), Mr S Robjohns (HPA), Dr O Sepai (HPA), Ms F Pollitt (HPA) and Dr P Edwards (HPA). The Chair also welcomed a new assessor Ms C Pease (EA).

2. Apologies for absence were received from the members Dr B Burlinson, Dr B Elliot, Dr D Gatehouse, Dr E Parry and Dr C Allen. Apologies were also received from Dr D Benford, Dr A Smith (HSE), Dr H Stemplewski (MHRA) and Mr Huw Brunt (Assembly for Wales).

3. Members were reminded of the need to declare any interests before discussion of items.

ITEM 2: MINUTES OF MEETING ON 4th March 2010 (MUT/MIN/09/3)

4. Members agreed the minutes subject to some minor editorial changes.

ITEM 3: MATTERS ARISING NOT COVERED BY LATER AGENDA ITEMS

5. The committee was informed that its statement on thresholds had been published on the COM website. The secretariat also reported progress regarding the genotoxicity testing recommended by COM with Fumigillin.

ITEM 4: PRESENTATION; PROFESSOR KIRKLAND (MUT/2010/08)

6. Professor Kirkland had provided a short paper for the March 2010 meeting entitled 'Is an *in vitro* battery of Ames plus micronucleus sufficient?' Subsequently, Professor Kirkland agreed to make a presentation to the COM to update the committee with additional analyses of rodent carcinogens and *in vivo* genotoxins. **[Please see post meeting note below paragraph 27 for information on amended analyses of rodent carcinogens and *in vivo* genotoxins.]**

7. Professor Kirkland outlined that most genotoxicity test guidelines recommended three *in vitro* genotoxicity tests i.e. gene mutations in bacteria; a test for induction of gene mutations in mammalian cells (usually the mouse lymphoma assay (MLA); and chromosomal aberration (CA) or micronucleus test (MN).

8. It was agreed in various genotoxicity guidelines that there was a requirement for bacterial and mammalian tests and that the endpoints of gene mutation; chromosomal damage; and aneuploidy needed to be investigated. However, Professor Kirkland examined whether two mammalian cell tests were necessary to achieve this aim i.e. whether both bacterial and mammalian cell tests were required to investigate the endpoint of gene mutation. It was suggested that the *in vitro* micronucleus test (MNvit) included

in a test battery would be sufficient to detect both chromosomal aberrations and aneuploidy.

9. Published carcinogenicity data were analysed to address two questions: 1) Are there Ames negative rodent carcinogens that are not positive in the mouse lymphoma assay (MLA) that are not detected in the MNvit or CA assays?; and 2) Are there rodent carcinogens that are not detected in either Ames or MNvit, but might be uniquely positive in the MLA?

10. Regarding question 1), out of 757 rodent carcinogens in Kirkland *et al* (2005), 562 had published Ames results. Of the 215 that were Ames negative, 65 were positive in the MLA. Of these 65, 16 were also tested in the MNvit, and 14/16 were positive. Cadmium sulphate was equivocal for MNvit, but positive for CA. Toluene was negative in both MNvit and CA.

11. Additional analysis of question 1) showed that 65 Ames negative carcinogens were positive in the MLA: 22 were positive in CA but were not tested in the MNvit. 19 were negative in CA, but not tested in MNvit. Most of these chemicals had been tested as part of the NTP programme using short treatments and early sampling times and might be positive for CA (and therefore for MN) if using a modern protocol. The remainder were either equivocal, inadequate (technically compromised), inconclusive or not tested.

12. Regarding question 2), analysis of the database published by Kirkland *et al* (2005) identified 87 rodent carcinogens for which MNvit studies had given clear positive (70 carcinogens) or clear negative (17 carcinogens) results. Many of the 17 carcinogens, negative in the MNvit were also negative in the Ames test and the MLA. Many of these were accepted non-genotoxic carcinogens e.g. clofibrate and di(2-ethylhexyl)adipate. There were three carcinogens negative in the MNvit but positive in the MLA. Two of these, dichloroacetic acid and phenobarbital, were positive in the Ames test. Toluene was the only rodent carcinogen that was negative in both the Ames test and MNvit, but positive in the MLA. However, the published positive MLA result has not been confirmed in a modern robust assay at doses up to 80% toxicity. The overall conclusions on rodent carcinogens was that the Ames test plus the MNvit (or CA in the absence of MNvit data) detects 410/557 (73.6%) of rodent carcinogens with available *in vitro* data. The remainder were negative; negative but technically compromised; weak; equivocal or inconclusive/with insufficient detail. By adding the MLA to this battery of two tests an additional 24 carcinogens were detected (434/557 = 77.9%). It was noted that of these, only 1 was tested in the MNvit (i.e. toluene, a non-reproducible MLA positive); 4 were not tested either in MNvit or CA; 17 were negative but technically compromised in CA; 3 were apparently real negatives (malonaldehyde, methyl tertbutyl ether, sodium o-phenylphenol) in CA but not tested in MNvit;. The committee were made aware of a reanalysis of the NTP MLA database by Moore and Gallapudi in which many of the positive results were considered to be of a questionable significance.

13. Since not all hazardous chemicals are carcinogens, or have been tested for carcinogenicity, the analysis was extended to include 464 *in vivo*

genotoxins not present in the Kirkland *et al* (2005) carcinogens database. The literature was searched for positive results for *in vivo* MN, CA, UDS, transgenic mutation and comet assays. The same *in vitro* data was searched as for rodent carcinogens. Two questions were posed: 1) Are there Ames negative *in vivo* genotoxins that are positive in the MLA that are not detected in the MNvit or CA assays?; 2) Are there *in vivo* genotoxins that are not detected in either Ames or MNvit, but might be uniquely positive in the MLA?

14. Out of 464 *in vivo* genotoxins: 55 had not been tested for *in vitro* genotoxicity. Therefore, 409 chemicals had results in at least 1 *in vitro* test. Most of these were for the Ames test (369 results). Of the 369 Ames test results, 202 (55%) were clearly positive. 38 were 'weak', equivocal, inconclusive, or with insufficient detail, 63 were negative not to current standards, and 66 were convincing negatives.

15. Of the 66 Ames negative *in vivo* genotoxins, 14 were clearly positive in the MLA (or HPRT). Only morphine/morphine sulphate was positive in the HPRT assay and not clearly detected in MNvit or CA. The MNvit with morphine was a non-standard assay (splenic lymphocytes, only treated in absence of S9 for 21h) and may have been positive in a standard assay.

16. Of 63 Ames negative (but technically compromised) *in vivo* genotoxins, 13 were clearly positive in the MLA (or HPRT). However, no chemicals in this group were positive in the MLA and clearly not detected in the MNvit or CA.

17. Of 38 weak, equivocal or inconclusive Ames test results for *in vivo* genotoxins, 14 were assumed to be clearly positive in the MLA (or HPRT). No chemicals in this subset were positive in the MLA and clearly not detected in the MNvit or CA.

18. Regarding question 1), 167 *in vivo* genotoxins were either clearly negative, or negative but technically compromised, or were weak, equivocal or inconclusive in the Ames test. None of the 41 that gave clearly positive results in the MLA (or HPRT) was uniquely positive in this test. All that were tested in MNvit or CA either gave clearly positive results in 1 or both test, or testing was inadequate.

19. Regarding question 2), of the 464 *in vivo* genotoxins, 127 had published results in the MNvit. 102 were clearly positive.

20. 25 *in vivo* genotoxins that were clearly negative or negative but inadequate; or equivocal in MNvit. It was noted that of these; 6 were not tested in the Ames test; 11 were clearly positive in the Ames; 1 was equivocal in Ames; 1 had insufficient detail; 2 were negative but inadequate; and 4 were clearly negative in Ames.

21. Only morphine and thiabendazole were not clearly detected in the Ames test plus MNvit, yet were positive in MLA (or HPRT). Thiabendazole has given mixed results in the MNvit.

22. The overall conclusions on *in vivo* genotoxins were that a combination of Ames plus MNvit (or CA where no MNvit data) clearly detects 317/409 (77.5%) *in vivo* genotoxins with available *in vitro* data. By adding the MLA to this battery of two tests, only an additional six *in vivo* genotoxins are detected (323/409 – 78.9%). Four of these six have not been tested in either MNvit or CA. It was suggested that it was not advisable for an *in vitro* battery to contain more tests than necessary (to reduce the number of misleading positives). From this analysis it was concluded that there is no convincing evidence that any rodent carcinogen or *in vivo* genotoxins would be ‘missed’ by using an *in vitro* battery consisting of Ames plus MNvit.

23. The Chair thanked Professor Kirkland for his presentation which was of considerable value to the revision of the COM strategy for genotoxicity testing.

24. Members heard that the reanalysis of NTP MLA assays was likely to report that a substantial number were inadequate (technically compromised). It was possible that future analyses of MLA assays undertaken to modern standards could report improved sensitivity and specificity for rodent carcinogen and *in vivo* genotoxin detection.

25. The COM noted that the analysis suggested that the Ames test plus the MNvit test detected about 77% of rodent carcinogens and 75% of *in vivo* genotoxins. Members felt that the remaining 25% could be accounted for by a number of reasons such as negative, but technically compromised results; insufficient metabolic activation; and too short a sampling time i.e. many studies were inadequate or could not be interpreted. The potential for biases in the evaluated database was raised. These potentially included selection of chemicals included in the datasets used for assessment of genotoxicity assay performance. It was also suggested that robust negative *in vivo* genotoxicity studies are less likely to be published compared to studies reporting positive results and therefore this could introduce bias into the published literature used in the analyses. The committee was aware that there was no published available database of chemicals clearly shown to be negative *in vivo* genotoxins.

26. Members acknowledged that there would always be some degree of uncertainty over the use of the proposed battery of two *in vitro* tests. Thus there would always be a small number of *in vivo* genotoxic chemicals that would be undetected whatever *in vitro* testing strategy was used. It was also accepted that a larger number of MNvit tests in the analysis would have been desirable.

27. The committee agreed that minimising the number of essential *in vitro* tests to a battery of two would optimise the sensitivity and specificity of genotoxin and rodent carcinogen detection. Overall the COM agreed that the analysis presented by Professor Kirkland provided convincing evidence that the use of the Ames plus the MNvit would be sufficient for *in vitro* genotoxicity testing.

[Post meeting note: Professor Kirkland has revised his paper which now takes into account the re-evaluation of the NTP MLA studies. Thus the numerical assessment given in paragraphs 6-27 above supporting the use of Ames and MNvit as a test battery has altered. The revised analysis fully supports the conclusions reached by COM in paragraph 27. A copy of the revised analysis can be found in the published COM papers as MUT/2010/14. <http://www.iacom.org.uk/papers/index.htm>]

ITEM 5: REVISION OF COM GUIDANCE ON A STRATEGY FOR TESTING CHEMICALS FOR GENOTOXICITY

5.1 Does the MLA detect aneugens? (MUT/2010/11)

28. Dr G Clare declared an interest as a co-author of the paper submitted to COM. The Chair concluded this declaration did not affect participation in COM discussion.

29. The MLA is currently recommended as a third *in vitro* assay in stage 1 of the COM strategy for genotoxicity testing. Some authors have reported that the MLA can detect information on recombination, deletion and aneuploidy, in addition to gene mutations and clastogenicity.

30. A COM member had provided one pre-publication paper and a pre-publication poster as in-confidence documents to the committee. The poster by O'Donovan MR *et al.*, presented results from a number of laboratories which independently reported negative results for the MLA with colchicine using a 24 hour exposure, but concurrent positive results with carbendazim and griseofulvin. The authors concluded that the MLA does not detect all aneugens. A pre-publication paper by Fellows MD *et al.*, investigated the mode of action of a number of aneugens in the MLA (taxol, carbendazim, noscapine, econazole, chloral hydrate, diazepam and colchicine) using a 24 hour exposure time. Increased mutant frequencies were reported at cytotoxic concentrations for econazole, taxol, carbendazim, and chloral hydrate. No increases in mutant frequency were reported for noscapine, diazepam and colchicine. Further investigations using loss of heterozygosity and FISH were undertaken for taxol and carbendazim. The pattern of results suggested chromosome 11 loss with duplication of the remaining chromosome 11 for these two aneugens. Overall the data suggested that aneugens are only positive in the MLA when tested up to highly toxic doses. Members were asked how these results might affect the use of the MLA within the COM genotoxicity testing strategy.

31. The committee agreed that the MLA produced inconsistent results for the detection aneugens and there was marked inter-laboratory variation in the performance of the assay. One possible explanation for the inter-laboratory variation was evidence that some cell lines used in the MLA were trisomic for chromosome 11. It was felt that there was a potential to get different results with aneugens due to multiple possible mechanisms for aneugenicity (e.g. effects on microtubules, effects on kinetochores, cell cycle etc.). The data provided suggested chromosome duplication with subsequent loss of the TK⁺

locus bearing chromosome. Given the wide range for potential mechanisms for aneugenicity it was unlikely that the MLA could detect these mechanisms.

32. Overall, the COM agreed that the available data showed that the MLA would not detect all aneugens. The Chair thanked members for their comments and noted these were in accordance with the written comments from COM members who had not been able to attend the meeting.

5.2 Revised format for guidance documents and short paper on significance of mutation for human health (MUT/2010/12)

33. The COM considered a draft discussion paper (Annex 1 to MUT/2010/01) at the March 2010 meeting outlining a proposed format for the publication of COM guidance documents. Members suggested a number of revisions. The committee was informed that the draft Guidance had been amended to include an expanded introduction and a structure that allowed for the development of statements on individual genotoxicity tests and the overall testing strategy and components of the of the testing strategy. A short summary of the content for each guidance document was intended to provide preliminary information prior to assessment of the whole guidance document. The 2nd draft strategy for genotoxicity testing was divided into a summary statement (G01), stage 0 (G02), stage 1 (G03) and stage 2 (G04).

34. Members were asked to first consider the format of the proposed COM Guidance and then to go through and comment on the whole Guidance.

35. It was suggested that an executive summary for the strategy for genotoxicity testing replace the current summary statement (G01). The background information on the Guidance statement internet page would be best placed on the COM Home page.

36. Members agreed with the format of separating the Guidance into individual statements and with the proposed system for navigating between documents via links and cross-referencing. The committee considered that the titles of some of the proposed guidance statements were confusing and agreed there was a need for consistency between the overall Guidance and individual statements. Additionally the numbering of the statements did not appear to match the numbering of the stages of the COM strategy for the assessment of genotoxicity.

37. Regarding the Guidance statement on the significance of mutations to public health, members felt that discussion of mutagenic effects on carcinogenesis should be expanded including examples of the food contaminant aflatoxin and smoking and lung. Members agreed that it would

be helpful to provide some explanation for why it was difficult to obtain convincing evidence for a role of chemical induced mutation in adverse heritable effects following human exposure. Furthermore, it would be useful to indicate that this is potentially important and to include evidence for the suggested role of mutation on reduced male fertility. There was also a need to say something about epigenetic effects.

5.3 Revised 2nd draft strategy for genotoxicity testing (MUT/2010/09)

38. Amendments to the 2nd draft included a brief overview of the significance of mutation for human health; a section on additional considerations for genotoxicity testing of chemicals with limited or inadequate genotoxicity data; pre-screening was now termed stage 0; a revised simplified flow diagram was provided; the QSAR section had been expanded to include information on publically available databases; references to both three and two test approaches to stage 1 were included; data on sensitivity and specificity had been included; and the suggested default was no requirement for independent confirmatory mammalian cell tests, provided a number of criteria were fulfilled; the strategy referred to core tests within stage 1 and 2, which had a higher value than others cited in the text; stage 2 referred to approaches that could reduce the number of animals required; and a glossary of terms had been incorporated.

39. Members were asked to consider the 2nd draft Guidance document in light of the previous item i.e. the presentation by Professor Kirkland on which mammalian cell tests best complement the Ames test in terms of detecting rodent carcinogens and *in vivo* mutagens. Regarding genotoxicity testing, the committee was informed that specific guidance statements on individual genotoxicity assays would be developed in the future with the emphasis on interpretation/assessment of genotoxicity test data.

40. Members went through the 2nd draft Guidance paragraph by paragraph and made detailed comments.

41. The COM confirmed that its guidance on a testing strategy was not intended to be applied retrospectively. Members agreed there was a need for consistency in the use of the terms 'mutagenicity' and 'genotoxicity'. The COM had a number of comments on the pre-screening stage (stage 0). These included correct referencing for the various computer models for structure activity relationships (SAR) used to predict structural alerts for potential mutagenicity and the need to emphasise that pre-screening cell tests (e.g. the Green Screen) could provide useful information for prioritisation, but were not acceptable as replacement tests for genotoxicity testing.

42. The committee agreed that a two *in vitro* test battery consisting of the Ames test and the *in vitro* micronucleus test in mammalian cells could be used with a higher degree of confidence and was the preferred Stage 1 battery of tests, but acknowledged that mutagenicity could also be assessed using other adequate *in vitro* genotoxicity assays (i.e. it was important to indicate in the COM Guidance that adequate data from studies other than those recommended in the core testing strategy could be used in the assessment of mutagenicity).

43. Members confirmed there should not be a requirement to routinely provide toxicokinetic data as part of the dose-selection procedure. s during dose selection for *in vivo* studies. Regarding confirmatory evidence for target tissue exposure to test chemical/metabolite, the committee felt that supporting toxicokinetic data was important to confirm exposure but evidence from other toxicity studies could be used as alternative data supporting exposure of target tissues.

44. The COM discussed the rationale for genotoxicity testing chemicals with existing, possibly limited or inadequate data and proposed it was important to stress that robust evidence to assess mutagenic potential could be derived from 'core' and non 'core' stage 1 tests. A flow diagram would be produced for the next draft document.

45. The Chair noted that the COM had devoted a lot of time to the consideration of the draft strategy document at this meeting but had not reached consideration of Stage 2 testing. He proposed that the next meeting of COM in October might need to be extended so that the full strategy could be discussed by members including those members who had been unable to attend this meeting.

ITEM 6: THE DEVELOPMENT AND VALIDATION OF A MUTATION ASSAY USING THE PIG A GENE (MUT/2010/13)

46. Paper MUT/2010/07 provided an overview of the development of the PIG-A mutagenicity assay including the proof of concept studies and method development studies. The PIG-A gene codes for one subunit of a glycosylphosphatidyl inositol (GPI) anchor protein. Mutation of GPI (+) to GPI (-) results in loss of protein anchorage which can be evaluated using immunohistochemical approaches. The PIG-A assay has been shown to work in a number of experimental animals using a variety of blood cells and splenocytes. The method is easily adapted to flow cytometry approaches. It can potentially be used as an adjunct investigation in conventional rodent toxicology studies and could potentially be developed for use in biomonitoring investigations.

47. Some members queried the role that this assay might play in a genotoxicity testing strategy and suggested that this should be better defined before validation. There were already transgenic assays that also detected gene mutation *in vivo*, which had been extensively validated and could be used to assess mutagenicity in a wide range of tissues. Furthermore, the haematopoietic system was already a target tissue in the bone marrow MN *in vivo* assay. Other members considered that the *in vivo* PIG-A assay had potential to be an alternative to transgenic *in vivo* gene mutation assays in the future as it had the advantage of easy access; simpler method; a relative quick response time; and potentially could be used for more species and standard animal strains.

48. One member referred the committee to a Health and Environmental Sciences Institute (HESI) presentation on the ongoing approaches to validation of the PIG-A assay. The PIG-A assay was an *in vivo* mutation assay using easily accessible sampling of blood which might potentially be incorporated into routine toxicology studies. Participants in the HESI validation study were initially investigating the dynamics of PIG-A response with known *in vivo* mutagens. There was evidence to suggest that the mutagenic response in the assay accumulated with repeated exposure to *in vivo* mutagens and that inter-laboratory response was good. A number of participants in the HESI project were considering investigation of PIG-A response in the liver and gastrointestinal tract. One possible approach would be to undertake immunohistochemistry of tissue slices.

49. Members agreed there were many aspects of the PIG-A assay which needed investigation including identification of the optimum GPI-linked protein to use and the need for confirmatory DNA sequencing to confirm mutations.

50. Overall members felt that the PIG-A mutagenicity assay was an interesting development in genotoxicity testing, but that further work would be required before validation and there was a need to identify its role within a genotoxicity testing strategy.

ITEM 7: ANY OTHER BUSINESS

51. There were no other items of business raised.

ITEM 8: DATE OF NEXT MEETING

52. 21st October 2010.

Item	Actions	Responsibility
Item 5: Revision of COM Guidance: Draft discussion paper: Overview of strategy for testing of chemicals for genotoxicity	Revise draft in light of comments	Secretariat

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