DRAFT

MUT/MIN/2009/3

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

Minutes of the meeting held at 10.30 am on Thursday 22nd October 2009 at Room 136/137B Skipton House, Department of Health, London SE1 6LH.

Present:

Chairman:	Professor P Farmer
Members:	Dr B Burlinson
	Dr G Clare
	Dr B Elliot
	Dr G Jenkins
	Dr D Gatehouse
	Mrs R Glazebrook
	Professor D Kirkland
	Dr D Lovell
	Dr E Parry
	Professor D Phillips
Secretariat:	Mr J Battershill (HPA secretariat)
	Dr L Hetherington (HPA secretariat)
	Dr D Mason (HPA secretariat)
	Mr S Robjohns (HPA minutes)
	Ms S Kennedy (HPA administration)
	Mr K Mistry (DH administration)
	Mr B Maycock (FSA)
Assessors:	Mr M Hawkins (CRD)
	Mr P Holley (DH)
	Dr R Shillaker (HSE)
	Dr A Smith (HSE)
In attendance:	D K Burnett (DH Tox unit)
	Dr P Edwards (HPA)
	Dr D Parker (FSA)
Observers:	Ms V Clayton (Chemtura)
	Mr S Creton (NC3RS)
	Ms C Moodley (Chemtura)
	Dr R Walmsley (University of Manchester & Genotronix Ltd.)

A G E N D A

Open session

	Paragraph
1. Announcements/Apologies for absence	1
2. Minutes of the meeting on 18th June 2009 (MUT/MIN/09/2)	4
3. Matters Arising (not covered by later agenda items)	5
4. Draft Discussion Paper: Genotoxicity of Para-chloroaniline (MUT/09/13)	6
5. GADD 45a Assay (MUT/09/14)	14
6. Draft Discussion Paper: Guidance series Risk Assessment Of *in vivo* mutagens (MUT/09/12)	21
7. Horizon Scanning Paper 2009 (MUT/09/11)	26
8. Any other business	32
9. Date of next meeting 4th March 2010	33

ITEM 1: ANNOUNCEMENTS/APOLOGIES FOR ABSENCE

1. The Chair welcomed Dr D Mason (HPA), Dr L Hetherington (HPA), Mr S Robjohns (HPA), Dr K Burnett (DH Tox Unit), Dr P Edwards (HPA), Mr B Maycock attending in the place of Dr D Benford (FSA secretariat), Mr M Hawkins (CRD), and Mr P Holley (DH). The Chair also welcomed Ms V Clayton (Chemtura) and Ms C Moodley (Chemtura) who would be attending for item 4. Additionally, the Chair welcomed Dr R Walmsley and Mr S Creton who would be attending for item 6.

2. Apologies for absence were received from the members Dr C Allen and Dr A Lynch. Apologies were also received from Dr D Benford (FSA secretariat), the assessors Mr Huw Brunt (Assembly for Wales) and Dr H Stemplewski (MHRA).

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

ITEM 2: MINUTES OF MEETING ON 18th June 2009 (MUT/MIN/09/2)

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

ITEM 3: MATTERS ARISING (NOT COVERED BY LATER AGENDA ITEMS)

3.1 Review of tobacco toxicology

5. The committee heard that a submission from Philip Morris had been received regarding the COM draft discussion paper on the mutagenicity testing of tobacco products. Members agreed that no changes to their evaluation of mutagenicity data were required in response to the Phillip Morris submission. The Chair noted a number of additional references needed to be obtained.

ITEM 4: DRAFT DISCUSSION PAPER: GENOTOXICITY OF PARACHLOROANILINE (MUT/09/13)

6. The Advisory Committee on Pesticides (ACP) asked the COM for its opinion on the available genotoxicity data on para-chloroaniline (4-CA). A copy of the referral letter was provided in Annex 1 for members' information. Members were informed that detailed written comments had been submitted by one COM member who was unable to attend the meeting.

7. 4-CA is a potential mammalian metabolite of the pesticide diflubenzuron. The ACP had reviewed the available information from a World Health Organization (WHO) Concise International Chemical Assessment Document (CICAD 48); summary information on the metabolism of diflubenzuron from a draft EU Risk Assessment Document; a published study on the metabolism of diflubenzuron in swine; a draft risk assessment of the

carcinogenicity of 4-CA in diflubenzuron produced by the EU Rapporteur Member State (Sweden) and a submission from the approval holder for diflubenzuron regarding the potential for metabolism to 4-CA. Based on the available data the ACP considered the need for additional studies to investigate the metabolism of diflubenzuron to 4-CA and an *in vivo* comet assay to investigate potential genotoxicity in tumour target organs.

8. Additionally, the secretariat had conducted a review of relevant available published information on 4-CA. There were published data to suggest that 4-CA has genotoxic potential both *in vitro* in bacteria and mammalian cells, and *in vivo* in rodents. NTP bioassays for potential carcinogenicity provided clear evidence for carcinogenicity in male rats (splenic sarcoma, osteosarcoma and adrenal phaeochromocytoma). There was equivocal evidence for tumours in the spleen in female rats. There was some evidence for liver tumours in male mice and no evidence for carcinogenicity in female mice. It is notable that increased haemangiosarcomas were seen in both rats and mice (in spleen and/or liver).

9. The COM noted that the lack of sulphotransferase activity in exogenous metabolic activation systems used in the available *in vitro* mutagenicity tests systems may have limited the ability of the systems to convert the agent (an aromatic amine) to mutagenic species, thereby underestimating its *in vitro* mutagenic potential. Members agreed that absorbed 4-CA is widely distributed with specific binding to erythrocytes reported. 4-CA is rapidly metabolised with the predominant route in most mammalian species being hydroxylation at the *ortho-* position followed by conjugation with sulphate. A minor pathway involves N-hydroxylation to form 4-chloro-N-phenyl-hydroxylamine which can undergo oxidation to 4-chloronitrosobenzene in erythrocytes. Oxidation to 4-chloronitrosobenzene and its subsequent binding to oxy-haemoglobin are thought to be involved in MetHb formation. Excretion as metabolites predominantly via the urine is rapid in rodents.

10. Members agreed that positive *in vitro* results had been seen in a number of test systems. This included the Ames test with *Salmonella typhimurium* TA98 in the presence of exogenous metabolic activation, where a small dose-response was consistently reported at doses levels of ≥1000 µg/plate. Members considered that the available mouse lymphoma studies were limited and were not adequate as assessed using the International Working Group on Genotoxicity Testing (IWGT) Global Evaluation Factor. A positive result was reported in mouse lymphoma cells L5178Y TK (+/-) without exogenous metabolic activation at dose levels where cytotoxicity was reported. Clastogenicity in Chinese Hamster Ovary (CHO) cells had been reported both with and without exogenous metabolic activation, but the results showed considerable inter study variation in the magnitude of response that was possibly influenced by cytotoxicity. Negative results had been reported in one micronucleus assay using a Chinese Hamster lung cell line but the selection of the dose levels used had not been explained and no assessment

of cytotoxicity had been provided. A positive result had been obtained in a UDS assay with rat hepatocytes. No evidence of DNA strand breaks was reported in L5178Y TK ($^{+/-}$) cells in the absence of exogenous metabolic activation. Mutagenic effects in mammalian cells were reported, particularly after exposure to what appeared to be cytotoxic concentrations.

11. Regarding *in vivo* mutagenicity, the committee agreed that a positive result had been reported in *Drosophila meloganaster*, but noted that the significance for human health hazard assessment was unclear.

12. The committee agreed that there was no clear evidence for DNA binding in the liver of rats given an oral dose of 64 mg/kg bw 4-CA. The study had limitations, such as the number of potential DNA adducts included in the analysis and the chosen dose level may have been insufficient (a relatively low dose compared with the maximum tolerated dose in the rat). There was evidence for a positive result in a comet assay in a number of tissues in mice given an oral dose of 200 mg/kg bw 4-CA. However, there were limitations in the conduct and reporting of this investigation, which reduced the weight that could be placed on this study. A positive response was also reported in an NTP oral bone marrow micronucleus (MN) assay in mice given three daily doses of 300 mg/kg bw/day with sampling 24 hours after the final dose. The assessment of toxicity in this study was inadequate (i.e. only up to 24 hours after the last dose) and it is possible that the dose level used may have induced significant toxicity. Members discussed the potential influence of methaemoglobin formation, which was likely to have occurred at the dose level used in this study, on the micronuclei formation. It was noted that no change in the percentage of PCEs had been reported in the study and the investigators had used fluorescent staining which adequately identified micronuclei containing DNA. Thus, although a positive result was reported there is uncertainty over its biological significance. The COM were aware of two abstracts that reported negative results for bone marrow MN studies in mice, but sufficient evaluation of these studies was not possible.

13. Overall, the COM concluded that 4-CA was an *in vitro* mutagen, but that no definite conclusions on the *in vivo* mutagenicity could be drawn. Regarding further testing for the assessment of *in vivo* genotoxicity, the committee agreed that as a first step a repeat MN test in mice conducted to internationally acceptable standards to include sampling of the bone marrow and peripheral blood for reticulocytes. Members noted that in previous studies of a substituted aniline, peripheral blood sampling had been more sensitive than bone marrow sampling and hence assessment of micronuclei in reticulocytes should be included in the repeat MN test to be undertaken with 4-CA. If this study was positive, then 4-CA should be regarded as an *in vivo* mutagen. If this study was negative or equivocal then a second *in vivo* study in rats should be undertaken. This second study should be a rat liver UDS assay with a concurrent rat comet assay to investigate DNA damage in the spleen, liver and other tissue (not considered to be a rat tumour target organ).

If this second study were positive, then 4-CA should be regarded as an *in vivo* mutagen. If this study was negative, then 4-CA would not be regarded as an *in vivo* mutagen. If the results were equivocal, then further consideration of testing and further advice would be required. [Post meeting note; The COM agreed that the proposed comet investigations would be adequate to assess for other tumour target organs in rats (e.g. adrenal). Members agreed that normal sampling procedures for the rat liver UDS and mouse liver comet assays would capture both parenchymal and endothelial cells]

ITEM 5: GADD 45a-GFP 'GreenScreen' HC ASSAY (MUT/09/14)

14. This item was considered before item 5 as listed on the published agenda. Two members declared a direct interest involving either consultancy work or use of the GADD 45a assay under licence from Gentronix Ltd. The Chair commented that they could not take part in the COM discussion but could answer questions from members at the end of the discussion.

15. The committee had been introduced to the TK6 GADD 45a assay in 2007 when Professor Walmsley had given a comprehensive talk on this newly developed high-throughput *in vitro* genotoxicity assay. The assay utilises GADD45a, a gene considered to play a role in DNA repair, cell cycle control and apoptosis in response to genotoxicity. Induction of GADD45a has been identified in early gene expression in microarray experiments in response to a wide range of genotoxins (e.g. direct DNA damaging, topoisomerase inhibitors, nucleotide synthesis inhibitors, aneugens and generators of reactive oxygen species) in various cell types. The increase in GADD45a gene expression suggested that it could be used as a marker for genotoxic stress. In the test system GADD45a is fused to a green fluorescent protein (GFP) gene. The plasmid construct is transfected into P53 proficient human lymphoblastoid cell line (TK6) and the assay is conducted in microplates. After incubation with test compounds GFP reporter fluorescence and cell culture absorbance are measured.

16. Since the original presentation, a number of significant studies have been conducted to further validate the assay and introduce modifications. Most importantly, a protocol using metabolic activation and a higher throughput schedule had been outlined. A written overview detailing these developments by Professor Walmsley had been appended to MUT/09/14 for members' use. A number of papers were also made available to members covering areas such as: inter-laboratory validation; metabolic activation; further general validation; a trial of ECVAM recommended chemicals as part of a project to reduce the number of false positives; and a higher throughput protocol. Generally, the assay appeared to perform robustly and had been shown to have high specificity (correct identification of negatives) and sensitivity (correct identification of positives. However, one study by Olaharski A *et al* (Mutation Research, 672, 10-16, 2009) suggested a lower sensitivity. Members were asked for their views on the data presented.

17. The COM agreed that there was a lot of new data conducted to acceptable standards that provided evidence of a high degree of sensitivity and specificity. Regarding the inter-laboratory trial by Billinton N *et al.*, (Mutation Research, 653, 23-33, 2008), members noted that this study had included a number of genotoxic and non-genotoxic compounds, but none of the genotoxic chemicals required metabolic activation.

18. The GADD45a assay had been adapted to use S9 exogenous metabolic activation by Jagger C *et al.*, (Mutagenesis, 24, 35-50, 2009) but it was felt that this aspect of the assay was less well validated. Members asked about the transferability of this method between laboratories and were informed that an inter-laboratory trial was being conducted with non-genotoxic, pro-genotoxic and genotoxic compounds, which would be published when completed. In answer to a question from the Chair, Professor Walmsley agreed to provide the secretariat with an evaluation of the transfer of S-9 flow cytometric assay between laboratories and further data from tests using exogenous metabolic activation in addition to those cited in the submitted papers. Professor Walmsley reported that information on the gating procedures used in the flow cytometric assay had been reported in the Jagger *et al* paper. In answer to a question from one member on the five pro-genotoxins which had not been correctly identified in this study, Professor Walmsley indicated this was most likely due to exogenous metabolic fractions being suboptimal in these particular tests. Overall, members felt that the assay was as good as any other *in vitro* genotoxicity test without metabolic activation. However, they wished to see more data with metabolic activation.

19. An evaluation of the GADD45a assay with proprietary and non-proprietary compounds by Olaharski A *et al.* (Mutation Research, 672, 10-16, 2009) had produced far less favourable validation results regarding sensitivity than other studies. The authors reported sensitivity and specificity for genotoxicity was 30% and 97% respectively (17/57 & 33/34). For rodent carcinogenicity, sensitivity and specificity was found to be 30% and 88% respectively (10/33 & 15/17). Members noted that the definitions of sensitivity and specificity used in this study were inconsistent with other reports and that the presentation of the data complicated the assessment of the results of this study. Members felt that the evaluation of the results for the Roche proprietary compounds was important to deriving the overall estimate of sensitivity in this study. In answer to a question from the chair, Professor Walmsley reported that Genotrix did not have access to the identity of the Roche proprietary compounds. The secretariat was asked to obtain any information Roche were willing to provide on an in-confidence basis for COM members only. In answer to further questions from COM members, Professor Walmsley agreed to provide a further commentary on the correlation between results from the GADD45a-GFP assay and results from other *in vitro* genotoxicity tests for the proprietary compounds published by Olarhski *et al* to supplement the commentary on the non-proprietary compounds published as a letter to the editor in Mutation Research, 672, 17-19, 2009.

20. The COM agreed that the GADD45a-GFP assay might be useful as a pre-screening tool similar to DEREK, but that it could not be used in a regulatory mutagenicity testing strategy at present. More data on the use of the GADD45a assay with metabolic activation and further analysis of the low sensitivity reported by the study by Olaharski *et al.*, 2009 would be required before the committee could produce a statement on the use of this assay.

ITEM 6: DRAFT DISCUSSION PAPER: GUIDANCE SERIES. RISK ASSESSMENT OF *IN VIVO* MUTAGENS (MUT/09/12)

21. The COM considered a draft guidance document at the June 2009 meeting. Members had asked for a number of topics to be further considered including: nomenclature definitions; further example of genotoxic chemicals with evidence of threshold mechanisms (e.g. paracetamol and methotrexate); information on EMS as discussed at the February 2009 meeting; stratification of examples into non-DNA target and protective mechanisms; information on examples where threshold toxicity had been used in risk assessments and further information on an approach to investigate potential thresholds including dose-response analysis. The COM secretariat had also added information from other relevant generic statements e.g. the COM statement on assessment of high dose positive bone marrow assays. A revised draft document was provided to members. The structure of the document was based on the logic outlined by Kirsch-Volders M *et al*. (Mutation Research, 678, 72-5, 2009). The structure of the Guidance section of the COM website had yet to be designed. It was intended that members would see the draft structure when available. One suggestion was that the Guidance notes should be divided into areas that could be updated more quickly. It was also suggested that the draft Guidance document on the risk assessment of *in vivo* mutagens could be split into two sections i.e. a section on thresholds for mutagenicity and a section on the risk assessment of *in vivo* mutagens. The chair asked members for their general comments on the revised draft guidance document.

22. The COM agreed that it would be helpful to split the Guidance into two separate documents with one document on thresholds and a separate document on aspects of risk assessment. Members heard that COM had historically not included expertise on exposure assessment. However, some aspects of exposure would need to be considered for example, in relation to Threshold of Toxicological Concern (TTC) and margin of exposure (MOE). Members requested further clarification of the definitions section. Thus the term mode of action was more applicable than mechanisms when referring to the biological plausibility of a threshold.

23. A number of specific comments were made. Members agreed that a clear statement should be included to avoid the use of logarithmic plots to

determine a threshold. Members noted a number of difficulties in assessing the study of MNU and MMS in L5178Y cells published by Pottenger and colleagues and agreed to delete this paragraph. Members agreed that the use of the phrase ‘redundant targets’ should be omitted. Members asked for clarification that the assessment of DNA adducts data was consistent throughout the document.

24. One member commented on the assessment of high dose positive mutagenic effects and noted that high levels of toxicity to animals might be a reason to question the biological significance of the results but this would be a case-by case assessment. One member offered to assist in the drafting of sections on experimental approaches to the identification of threshold doses and NOELs. One member who was not present had submitted written comments including a suggestion for a short presentation on the TTC. Members agreed this would be helpful in considering the draft document on some aspects of risk assessment of mutagens. One member requested that any such presentation should cover the adequacy of the TTC data set for *in vivo* mutagens. One member suggested it would be useful to consider if some aspects of carcinogen risk assessment, such as Mode-of-Action assessment and potency assessment, could be transferred to the assessment of *in vivo* mutagens.

25. Members heard that a revised document on thresholds for *in vivo* mutagens would be circulated by post for comment. It was hoped the document could be finalised by chairman’s action.

ITEM 7: HORIZON SCANNING 2009 (MUT/09/11)

26. The horizon scanning exercise provides information which can be used by Government Departments/Regulatory Agencies etc. to identify important areas for future work. Regarding progress on topics raised in the 2008 horizon scanning exercise, members were informed that a large amount of committee time had been spent undertaking reviews of aclonifien, fumagillin and tobacco products. Progress had been made on thresholds (and a draft guidance document on the risk assessment of *in vivo* mutagens), toxicogenomics and a draft outline proposal for a testing strategy. No progress had been made on mutational finger prints or mitochondrial mutagenicity.

27. The COM was asked to consider suggested horizon scanning topics for the upcoming year that had been identified from a literature search of PUBMED. Suggested topics included: mutagenicity testing approaches for nanomaterials; cigarette smoke exposure; PAH mixtures in soil; drinking-water disinfection by-products; a selection of chemicals of potential interest; mutational spectra; QSAR/*in vitro* metabolism; a number of *in vivo* tests, and biomonitoring.

28. The committee agreed that the main priority for COM work in 2010 would be to consider a revision of the mutagenicity testing strategy. Members agreed that a review of the mutagenicity of nanomaterials would be important and that the consideration of mutational spectra to investigate the role of chemicals in mutagenicity and carcinogenesis could be useful.

29. Regarding mutagenicity testing, the COM made some suggestions, which included consideration of the PIG A assay, the potential integration of genotoxicity tests into standard toxicity studies; measures for cytotoxicity in genotoxicity tests; top doses; reliability of cell types; and the use of oncogene/tumour suppressor gene arrays.

30. Members also suggested epigenetics as a potentially important topic, and consideration of repeat exposure.

31. The Committee agreed that the highest priority should be to review COM guidance on testing strategy and undertake a specific review of nanomaterial genotoxicity testing. One member noted that his group had recently published a review of nanomaterial genotoxicity and this would be a useful starting point for any review.

ITEM 8: ANY OTHER BUSINESS

32. The COM was informed that a Joint meeting with the COC on thresholds for mutagens and carcinogens and risk assessment had been proposed. Members were also informed that the International Life Sciences Institute (ILSI) would be holding a workshop in December 2010 on short-term and intermittent exposures.

ITEM 9: DATE OF NEXT MEETING

33. 4th March 2010.

1

Item	Actions	Responsibility
4. Genotoxicity of para-chloroaniline.	Draft conclusion paper and statement for the ACP.	Secretariat
5. Draft Guidance document: Risk assessment for *in vivo* mutagens.	Revise draft discussion document and split into sections.	Secretariat
6. GADD45a assay.	Further data on results with metabolic activation and analysis of the Olaharski A et al., results	Secretariat

2