

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

Minutes of the meeting held at 10.30 am on Thursday 18th June 2009 at Room 137B Skipton House, Department of Health, London SE1 6LH.

Present:

Chairman: Professor P Farmer

Members: Dr B Burlinson
Dr G Clare
Dr G Jenkins
Mrs R Glazebrook
Professor D Kirkland
Dr D Lovell
Dr A Lynch
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 Sue Kennedy (HPA administration)
Mr K Mistry (DH administration)

Assessors: Mr B Maycock (FSA)
Dr R Shillaker (PSD)
Dr A Smith (HSE)
Dr H Stemplewski (MHRA)

In attendance: D K Burnett (DH Tox unit)
Dr R Fayokun (DH)

Observers: Dr E Massey (British American Tobacco)
Dr Mariner (British American Tobacco)
Mrs Dillon (British American Tobacco)
Mr J Pritchard (Imperial Tobacco)

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ITEM 1: ANNOUNCEMENTS/APOLOGIES FOR ABSENCE

1. The Chair welcomed the new members Dr G Jenkins, Professor D Kirkland and Dr A Lynch. All Members, assessors and secretariat present introduced themselves. The Chair also welcomed Dr D Mason (HPA), Dr L Hetherington (HPA), Mr S Robjohns (HPA), Dr R Fayokun (DH Tox Unit), Dr K Burnett (DH Tox unit), and Mr B Maycock attending in the place of Dr D Benford (FSA secretariat). Additionally, the Chair welcomed Dr E Massey, Dr D Mariner, Mrs D Dillon (British American Tobacco) and Mr J Pritchard (Imperial Tobacco) who would be attending for item 6.

2. Apologies for absence were received from the members Dr D Gatehouse, Dr B Elliot and Dr C Allen. Apologies were also received from Dr D Benford (FSA secretariat), the assessors Mr Huw Brunt (Assembly for Wales), Mr M Hosford (EA) and Mr A Browning (VMD).

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

ITEM 2: MINUTES OF MEETING ON 26th February 2009 (MUT/MIN/09/1)

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

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

3.1 Fumagillin dicyclohexylamine

6. The committee was informed that its statement on fumagillin dicyclohexylamine had been finalised through Chairman's action. The Veterinary Medicines Directorate (VMD) had agreed to publication on the COM internet site. VMD had set formal data requirements in accordance with COM advice which would have to be conducted within six months.

3.2 Toxicogenomics

7. Members heard that the International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/HESI) trial of inter-laboratory variation in transcriptomic studies for genotoxicity had been published. This would be brought to the COM October meeting.

ITEM 4: THRESHOLDS FOR IN-VIVO GENOTOXINS. DATA SUBMITTED BY ROCHE IN SUPPORT OF A THRESHOLD FOR ETHYL METHANESULPHONATE (MUT/09/08)

8. One member declared a direct interest in this item and was asked not to contribute to the discussion.

9. During 2007, several thousand HIV patients had ingested Viracept (Nelfinavir mesylate) tablets as an HIV protease inhibitor, which contained relatively high levels of the impurity ethyl methanesulphonate (EMS). The

estimated dose of EMS received by patients was around 0.055 mg/kg bw/day. The available *in-vitro* mutagenicity and toxicity data for EMS did not allow a full risk assessment of this incident to be undertaken. This led the manufacturer Roche, to undertake *in-vivo* mutagenicity studies in mice (i.e. bone marrow (BM) micronucleus (MN), *lacZ* gene mutation in BM, liver and small intestine). Roche employed a novel statistical analysis of the data and undertook investigations to allow a risk assessment based on toxicokinetic data (Annex 1). The relevant documents provided to the COM by Roche (Annexes 1 – 5) were in various stages of peer-review. Members were asked to consider these documents and the approach to risk assessment. The COM was reminded that the committee had previously considered a presentation from Dr G Jenkins at its February 2009 meeting, which referred to studies by Roche in support of a threshold for the mutagenicity of EMS.

10. The Chair proposed that the Committee discuss Annexes 2-5 first before considering the overall approach adopted by Roche (Annex 1 to MUT/09/08). He asked for any general comments on the approach taken.

11. Members considered the approach Roche had used for testing and risk assessment of mutagenicity of EMS was consistent with other chemicals and pharmaceuticals. It was agreed a case-by-case approach was needed with careful selection of doses above and below the NOEL dose. The COM considered that the use of a fractionated dose compared to single dose regime was particularly helpful. There was a need to provide data on the underlying mechanism for *in vivo* mutagenic effects. In this instance the risk assessment concerned exposures to EMS which had occurred and members considered the approach would be different for risk assessment of prospective likely exposures to a mutagen. Overall members considered the approach taken by Roche should be commended and most comments concerned technical aspects of the studies and risk assessment process.

Annex 2 In-vivo studies on EMS/ENU

12. The committee considered comparative dose-response studies using 7 day treatment for BM MN test in mice and a 28 day (or single) treatment of MutaTM mouse for LacZ mutations in BM, liver and small intestine. The authors reported no increases in BM MN at dose up to 80 mg/kg bw/day EMS with evidence for saturation of MN induction at a cytotoxic dose of 260 mg/kg bw/day. No evidence for a NOEL was reported with the referent compound ENU which indicated a clear dose response relationship for MN induction. The authors also reported daily doses up to 25 mg/kg bw/day (BM and intestine) and 50 mg/kg bw/day (liver) did not induce mutations in the LacZ gene in the three tissues examined. The genotoxicity of EMS only became apparent at higher dose levels. Dose fractionation of EMS (28 times 12.5 mg/kg versus a single high dose of 350 mg/kg) provided further evidence for a threshold dose response relationship for EMS and showed no accumulation of gene mutations below the threshold. In contrast, no threshold was apparent for ENU and dose fractionation indicated full additivity of individual dose effects.

13. Members noted that the mouse repeat dosing (7 day) oral bone marrow micronucleus test had been conducted with a sufficient number of doses and that the ethylvaline adducts indicated exposure of the test tissue. Members also noted that 4,000 PCE had been analysed, which was double the normal number. Members agreed that it would have been impractical to investigate all of the dose levels needed to evaluate dose-response in one experiment. It was commented that a higher sensitivity might have been expected using peripheral blood erythrocyte MN in a repeat dosing schedule and that a power calculation could have been conducted to determine whether the assay was sufficiently sensitive to measure the NOEL. Power calculations could be done retrospectively. However, overall the COM agreed that this study was adequately conducted and showed a NOEL.

14. Regarding the MutaTM mouse oral 28 day and single treatment studies the COM agreed that this provided evidence for a threshold. It was noted that omitting the 25 mg/kg bw dose from assessment would have suggested a linear dose response for EMS in this test system and there had been no evidence for a mutagenic effect for five out of the seven doses used. Thus dose selection may not have been optimal. Overall members agreed the data provided evidence for a NOEL of 25 mg/kg bw.

15. Members agreed the mechanism for a threshold mutagenic effect of EMS was exceeding the capacity for error free DNA repair and metabolic overload.

Annex 3 In-vivo studies on EMS/ENU

16. This paper reported predominantly the same data presented in annex 2, but with some additional information on the approach to the risk assessment and threshold considerations. This included more dose response data for ENU (re-evaluation of a study by Consentino and Heddle *Env Mol Mut*, 34, 208-215, 1999) and the application of a dose-response model proposed by Lutz and Lutz (2009, accepted for *Mutation Research*). Members agreed that the presentation of the data was consistent with that reported in annex 2. There was discussion of the predicted threshold for ENU based on the assumption of a ten-fold higher formation of O⁶-guanine-adducts with ENU compared to EMS. Members felt there was a need to undertake appropriate experiments to demonstrate an *in vivo* NOEL.

Annex 4 Statistical assessments of in-vivo studies on EMS and ENU

17. This paper undertook a statistical analysis of the dose –response data for the genotoxicity of EMS i.e. both the MN and lacZ data. In addition members considered the publication from Lutz and Lutz 2009 which had been circulated prior to the COM meeting. Members noted the step-wise approach used to exclude a linear dose-response over the whole dose-range and accept a linear dose-response below the observed NOEL and application of the ‘hockey-stick’ software developed by Lutz and Lutz. In this paper different statistical models were used to try to better fit the dose-response relationships, for example the ‘hockey stick’ approach to dose-response

modelling developed by Lutz and Lutz (2009). Members commented that several models could fit the entire data set and thus the process was acceptance of a more complex model than linear rather than rejection of a linear dose-response. It was also conceptually difficult to accept a linear dose-response below the NOEL when data suggested no-dose-response. With regard to a comment on detail, members noted the wide confidence interval reported by Lutz and Lutz including the value of zero and queried that accuracy of this estimate. Members agreed that the additional statistical analysis did not affect the conclusions reached with regard to Annexes 2 and 3 and the approach reported an upper confidence limit at which there was no effect for mutagenicity.

Annex 5 Pharmacokinetic modelling of exposure to EMS

18. The effects observed in *in vivo* genotoxicity studies with mice were correlated to pharmacokinetic parameters of EMS and then extrapolated to predict likely human exposure from the ingestion of the contaminated Viracept tablets. The AUC and C_{max} values in mice at the threshold dose for mutagenicity of 25 mg/kg were calculated to be 350 µM.h and 315µM respectively, and 13 µM.h and 0.85 µM for patients that had ingested the most contaminated Viracept tablets. The margins of exposure (MOEs) based on AUC was estimated to be 28 and 370 based on C_{max}. The pharmacokinetic model was extended to include EMS interaction with haemoglobin to form ethylvaline adducts in mice, rats and monkeys to provide confidence in the model simulations of EMS exposures.

19. The committee considered that both of these pharmacokinetic parameters had been estimated by a sufficiently conservative method and that both parameters could be important in a MOE risk assessment of genotoxicity. AUC was considered important as adducts can form continuously over time and that C_{max} could also be important as saturation of DNA repair can be both concentration and time dependent.

Annex 1 Overall risk assessment of EMS in Viracept

20. This provided an overview of the of the proposed risk assessment approach involving the use of MOE based on a comparison of a clear NOEL (with doses above and below this level) for mutagenicity and estimated C_{max} and AUC for the highest likely human exposure. Overall, the COM agreed that a threshold had been demonstrated for EMS mutagenicity and that there was an adequate MOE between the NOEL for mutagenicity and likely maximum exposures in patients who ingested the EMS contaminated Viracept tablets. The COM agreed with MHRA that the work on EMS contamination of Viracept had been well designed and was a successful piece of work. The Chairman would write to Roche outlining the COM comments.

21. Members briefly discussed the hypothetical argument of the one-hit hypothesis of mutagenicity and noted this still applied even though there was a great deal of redundancy in DNA.

ITEM 5: DRAFT GUIDANCE PAPER: RISK ASSESSMENT OF *IN VIVO* MUTAGENS (MUT/09/09)

22. A draft guidance document on the risk assessment of *in vivo* mutagens was provided to members. This was intended to be the first of a series of guidance documents that would be published on the COM website. The approach was based on the current COM statement COM/01/S3. The general advice from COM when considering the risk assessment of chemicals which are mutagenic *in vivo* has been that it is prudent to assume a linear, non-threshold dose response. Thus, it is assumed that any exposure to an *in vivo* mutagen is associated with some damage to DNA and consequently an increased risk of mutation leading to an increased risk of adverse health effects, although at low doses this may be small. In such instances the committee has recommended that exposure should be as low as is reasonably practicable. The COM has previously considered specific chemicals on a case-by-case basis, with regard to deviations from this general approach to *in vivo* mutagens. Examples are outlined in terms of both generic chemical groups and mechanism of action and individual chemicals. The examples included aneugens acting by tubulin inhibition, such as benomyl, carbendazim and thiophanate-methyl belonging to the methyl benzimidazole carbamates class of chemicals. Topoisomerase inhibitors, compounds rapidly detoxified e.g. hydroquinone and phenol, and small molecular weight DNA alkylating agents whose adducts are rapidly repaired were other examples. Acrylamide was also presented as an example compound that had a number of genotoxic endpoints, some with thresholds and others without. The committee was asked for its view on the draft guidance document.

23. Members felt that it was important to distinguish between thresholds where there is no biological effect or where the biological effect is present but undetectable, and to generally provide more information in relation to definition of terms and explanation of abbreviations. It was also suggested that there was a need to include some discussion for the concept of threshold of toxicological concern (TTC). It was noted that a number of organisations were currently considering a TTC for mutagens, such as ILSI and EFSA. There was a need to note other DNA repair mechanisms in addition to MGMT.

24. Other suggestions for expanding the document included discussion of the approach taken with regard to risk assessment of aneugens, consideration of statistical analysis e.g. Lutz and Lutz 1999 'hockey stick model', redundant non-DNA targets (which would include topoisomerase inhibitors), acknowledgement of endogenous formation of mutations, and the importance of understanding the biological mechanism for identified thresholds. Further examples of compounds with a threshold for genotoxicity were requested such as paracetamol following glutathione depletion and the antimetabolite methotrexate. Members agreed a bibliography was required

25. Members made a number of initial comments on the conclusions. A revised draft would be made available for the October COM meeting.

ITEM 6: REVIEW OF TOBACCO TOXICOLOGY

6.1 Draft discussion paper: Genotoxicity of Tobacco Products (MUT/09/05)

6.2 Regulatory aspects surrounding the toxicological Testing of Tobacco Products (MUT/09)

6.3 Draft COT scoping paper on Toxicology of Tobacco Products (MUT/09/10) (for information)

26. One member declared a direct interest in this item involving payment for one meeting on generic aspects of mutagenicity testing of tobacco products and was asked not to contribute to the discussion but to answer queries from members at the end of the discussion.

27. The Chairman asked observers to briefly introduce themselves. He asked observers not to comment unless specific questions were raised by members through the Chair.

28. MUT/09/05 was a draft discussion paper on the genotoxicity of tobacco products. This was provided in conjunction with MUT/09/07, a short discussion paper on the regulatory aspects relating to the toxicity testing of tobacco products and a scoping paper on the toxicology of tobacco products (MUT/09/06). MUT/09/05 followed-up the 2004 joint COM/COC/COT statement on the toxicological testing of tobacco products.

29. The Department of Health (DH) had specifically requested an update of the 2004 joint COM/COC/COT statement. Dossiers on the toxicological testing of tobacco product ingredients in their burnt and unburnt forms are submitted to DH. As there are no internationally agreed approaches to the hazard assessment of these products, scientific advice was sought from the COM on the suitability of mutagenicity tests for the evaluation of these products. Another reason for the DH request was that there are a number of new products purporting to reduce harm to users (i.e. by reducing exposure to harmful chemicals), for which the Department had no means of evaluating toxicity. New tobacco products that potentially reduce exposure to harmful chemicals, such as electrically heated tobacco products, were known as PREPS. There was a contention that existing tests do not give sufficient information to draw meaningful conclusions.

30. Members were also provided with a copy of a letter to the Secretariat from Dr Justine Williamson of BAT, outlining their approach to the toxicology of tobacco products and an additional paper on whole smoke exposure of human pulmonary carcinoma cells. Additionally, members were provided with an email from Professor Martin Jarvis on compensatory smoking. The overall aim was to produce a statement from the COM.

31. The Chairman asked for general comments on the Regulatory aspects surrounding the toxicological Testing of Tobacco Products (MUT/09/07)

32. Members agreed with the statements abstracted from WHO Technical Series Report 945 that the rate limiting steps in the mechanistic pathways leading to tobacco product induced disease were not understood and hence this limited the value which could be attributed to the available data on biomarkers. The available data on biomarkers of mutagenicity would inform on overall exposure to mutagens. Members commented that the available test strategies for evaluation of mutagenicity of tobacco products had been largely dependent on the practicality of deriving cigarette smoke condensate (CSC) or total particulate matter (TPM) which could be easily obtained, stored frozen and transported between laboratories and the observation that there was a correlation between potency in skin painting bioassays of tumourigenicity in mice and potency in *Salmonella typhimurium* mutagenicity tests. COM members encouraged the development of whole smoke exposure procedures which were likely to provide more relevant data on mutagenic activity of tobacco smoke, but noted none of the test systems had been adequately validated and there was no agreement on what reference material would be used for comparative purposes.

33. The Chair asked for comments on the draft discussion paper on the genotoxicity of tobacco products.

Validity of genotoxicity tests

34. Members reaffirmed that any ranking of mutagenicity of tobacco products could not be extrapolated to in vivo exposure to chemicals in tobacco smoke. Thus tobacco smoke was a multi-site carcinogen in humans and it was not possible to evaluate which exposures were relevant for each of the fifteen different target organ cancers induced by tobacco smoke. Members commented that data on appropriate reference materials were needed for comparative data on whole smoke methods currently under development, and that the smoking regimes used did not necessarily reflect human exposure to tobacco smoke. It was also noted that mutagenic effects *in vivo* would also be influenced by target organ inflammation. The Secretariat noted that the study by De Marini from the US EPA (Mutation Research, 650, 15-29, 2008) suggested that it was not possible to rank tobacco products using CSC tests with *Salmonella typhimurium*. Members agreed and noted that potency rankings were also dependent on the smoking regime used. Members commented that there were advantages to reporting mutagenicity data on a per cigarette basis and on a per mg nicotine basis. The former gave an easy measure on mutagenic potency per unit consumed whereas reporting data in terms of mutagenicity/mg nicotine more closely reflected the behaviour of smokers who adjusted cigarette consumption to maintain nicotine exposure.

35. The COM agreed that there was a need for international harmonisation to reach a consensus on mutagenicity test procedures, use of reference materials, and cigarette smoke generation regimes. In this regard it was

noted that there were proposals to establish a group of experts to consider tobacco toxicology within the EU.

Potential effects of ingredients, additives and flavours.

36. Members felt that the mutagenicity evaluation of ingredients, additives and flavours by adding test materials to tobacco products pyrolysing and then testing CSC in *in vitro* mutagenicity tests with *Salmonella typhimurium* would not provide any useful information on the mutagenic properties of the pyrolysed ingredients, additives and flavours.

Biomarkers of effect

37. Members agreed that biomonitoring of urinary mutagenicity using *Salmonella typhimurium* TA98 and TA 100 in the presence of exogenous metabolic activation using rat liver S-9 from Aroclor 1254 treated rats might inform on potential risks of bladder carcinogenesis but not for other tobacco related cancer target organs. Thus urinary mutagenicity was essentially a biomarker of exposure to absorbed mutagens that were sensitive to the mutagenicity testing regime used. Members noted the standard deviation for urinary mutagenicity in the paper submitted (Mendes P et al *Regulatory Toxicology and Pharmacology*, 51, 295-305, 2008) suggested there were large inter individual differences in absorbed mutagens. There was discussion relating to the possibility of developing biomarkers for potential inflammation induced by tobacco smoke.

Available information on PREPS

38. The available data on mutagenicity suggested significant reductions in both *in vitro* mutagenic activity in *Salmonella typhimurium* TA98 and TA 100 in the presence of exogenous metabolic activation using rat liver S-9 from Aroclor 1254 treated rats and urinary mutagenicity in biomonitoring studies using a number of acceptable study designs. These data indicated that mutagenicity was not reduced to background levels. The data were consistent with a substantial reduction in exposure to mutagenic effects of aromatic amines in tobacco smoke. Overall the COM agreed the data supported the approaches used to reduce exposure to mutagens, notwithstanding the primary advice not to smoke tobacco products, but cautioned that the association between chemical mixtures present in tobacco smoke and disease outcomes was very complex and no conclusions regarding risk of mutagenicity could be reached from the available data.

39. The COM were informed that a discussion paper would be taken to a future COC meeting and COM conclusions would be forwarded to the COC. Finalised COM and COC conclusions would be forwarded to the COT.

ITEM 7: MOUSE LYMPHOMA ASSAY (Wang J et al., Toxicological Sciences 2009, 109 (1), 96-105) (MUT/09/10)

40. The committee was provided with a paper which reported data on detailed genetic alterations in L5178Y TK^{+/-} mutants with either small or large colony growth characteristics from studies investigating the mutagenicity of 3'-azido-3-deoxythymidine (AZT), mitomycin C (clastogens) and taxol (aneugen). Colonies that exhibited significant loss of heterozygosity in chromosome 11 were selected for further investigation. The increased mutation frequency in studies ensured that a high proportion of the mutants selected were due to chemical treatment. TK gene dosage, G-banding analysis for chromosomal changes, and FISH for detection of chromosome 11 numerical changes were undertaken. The results showed complex genetic changes with all three test substances, with evidence for deletion, recombination and aneuploidy. The absence of a functional P53 gene in L5178Y TK^{+/-} cells was in part responsible for survival of cells with larger scale DNA damage. The authors suggested that these new data provide evidence for the utility of the mouse lymphoma assay (MLA) in a mechanistically based genotoxicity hazard identification battery. The COM strategy suggests that the MLA is suitable for regulatory use for the detection of gene mutations and provides complimentary rather than equivalent data to metaphase analysis. The COM was aware that it recommended the MLA in its guidance on a strategy for mutagenicity testing (or an alternative of equivalent statistical power) as the third *in vitro* test in stage 1. More recently the COM has seen data to suggest that the MLA can detect clastogens and in some instances aneugens (the latter only at high doses resulting in cytotoxicity). The committee was asked its views on the proposed use of the MLA as part of a mechanistically based genotoxicity hazard identification battery.

41. Members agreed that this was an interesting paper. However, it was felt that the method outlined in the paper is dependent on a selective growth mechanism and thus will only detect the loss of chromosome 11 TK⁺. This meant that only a limited analysis could be conducted and that non-disjunction could not be detected. The test might be useful as an indicator of aneuploidy, but would not permit exact measurement. It was noted that chromosomal aberration studies had indicated that cells with structural chromosomal aberrations and aneuploidy do not survive cell division when changes represented a balanced event and genetic gain is better tolerated than loss and thus would not go on to form a colony. Colonies that were found would represent potentially toxicological relevant events. Most colonies analysed had acquired a duplicate chromosome 11. This may indicate that cells with a loss of chromosome 11 do not survive.

42. Members also noted the importance of the lack of p53 gene in L5178Y TK^{+/-} cells leading to genomic instability. The COM agreed that small and large colonies related to different mutagenic events, but that this was not demonstrated by the data presented. Members also felt that it was probably better to use the micronucleus test to analyse for aneuploidy. Overall, the committee agreed that MLA was a useful assay when used as part of a

battery of tests for mutagenicity, but could not be used in isolation of other tests.

ITEM 8: ANY OTHER BUSINESS

43. Members were asked to check that their details listed on the COM website were correct and up to date.

ITEM 9: DATE OF NEXT MEETING

44. 22nd October 2009.

Item	Actions	Responsibility
4. Thresholds for <i>in vivo</i> genotoxins. Data submitted by Roche in support of a threshold for mutagenicity for ethyl methanesulphonate.	Draft letter to Roche	Secretariat/Chair
5. Draft Guidance document: Risk assessment for <i>in vivo</i> mutagens.	Amend and expand the draft Guidance document in light of members' suggestions.	Secretariat
6. Review of Tobacco toxicology	Draft advice to the COC and COT on the mutagenicity testing of tobacco products	Secretariat