Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment

Preface

The Committee on Mutagenicity (COM) provides advice on potential mutagenic activity of specific chemicals at the request of UK Government Departments and Agencies. Such requests generally relate to chemicals for which there are incomplete, non-standard or controversial data sets for which independent authoritative advice on potential mutagenic hazards and risks is required. Frequently recommendations for further studies are made.

During 2007, the Committee provided advice on a wide range of topics including genotoxicity of acrylamide, chemical mixtures, phenol and the assessment of mutagenic impurities in pesticides. A large proportion of COM business was devoted to the evaluation of acrylamide and its genotoxic metabolite glycidamide.

The COM initiated a revision of its guidance document (Guidance on a Strategy for Testing of Chemicals for Mutagenicity) which had been published in 2000, and initiated a review of the use of Toxicogenomics in genotoxicity evaluation.

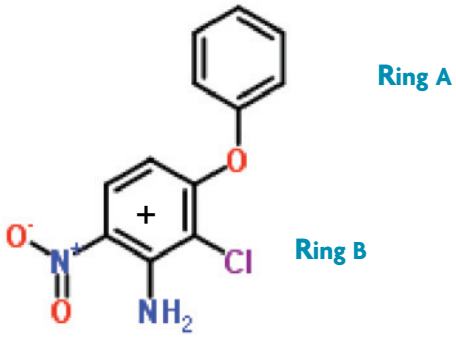
Professor P B Farmer Chair
MA DPhil CChem FRSC

COM evaluations

Aclonifen

2.1 The COM was asked for advice by the Pesticides Safety Directorate (PSD) on a pesticide active ingredient new to the U.K. which is undergoing evaluation through the independent Advisory Committee on Pesticides (ACP). The referral statement was as follows: 'ACP requested advice on the mutagenicity of Aclonifen and the genotoxicity risk assessment of the postulated metabolites hydroquinone and phenol. The referral does not include carcinogenicity data or the evaluation of mode of action for tumours in rodents observed in long-term carcinogenicity bioassays with Aclonifen'. 2. Aclonifen (2-chloro-6-nitro-3-phenoxyaniline) (figure 1.) is a selective systemic herbicide used for pre-emergence control of grass and broad leaved weeds in a range of crops.

Figure 1: Aclonifen



+ = position of uniformly radiolabelled phenoxyaniline ring

2.2 The COM considered a large amount of data on Aclonifen, which included an extract from the detailed record of ACP consideration of Aclonifen, extracts from draft EU assessment report on metabolism and genotoxicity of Aclonifen, which presented information on structure, use as a pesticide, ADME studies, toxicology, mutagenicity, carcinogenicity and reproduction, data from mutagenicity test reports on Aclonifen, copy of the report on the investigation of the potential for DNA-binding of Aclonifen and the revised position paper from the data holder on the cleavage of the diphenyl ether bond of Aclonifen. The data holder BayerCrop Science, submitted a presentation which was circulated to Members and in addition a revision to a report on cleavage of the diphenyl ether bond in the Aclonifen molecule. The COM considered Aclonifen at its 23 October 2008

2.3 The COM reached a number of conclusions as shown below;

i) The COM agreed that further data on Aclonifen metabolism was required. This could involve more *in vivo* tests with specific analysis for the formation of hydroquinone and phenol. Alternatively, it might be possible to undertake comparative *in vitro* studies using rodent and human tissues (with specific measurement of hydroquinone and phenol formation). It was considered this could provide evidence that exposure to Aclonifen was unlikely to be associated with significantly increased genotoxic risk, although this would not preclude the possible need for additional mutagenicity tests dependent on the outcome of the metabolism studies.

ii) The COM noted the approach to risk assessment had not been considered during the presentation, but that the data holder had included a proposed Margin of Exposure approach in the submission dated 13 August 2008. This would need to be considered further when appropriate metabolism data were available.

2.4 The COM agreed a statement which is reproduced at the end of this report.

Impurities

2.5 The COM had been informed of a published literature survey to evaluate the lowest detectable level of response in the Ames test for mutagens during the horizon scanning exercise for 2007. The approach adopted by the authors might have potential wider generic use which could be valuable for the review of the COM strategy and also for generic advice to Government Departments. (*Kenyon MO et al Regulatory Toxicol, Pharmacol, 48, 75-86, 2007.*) The COM reviewed this publication and also considered other recent publications which had considered a rationale for determining, testing, controlling specific impurities in pharmaceuticals that possess potential for genotoxicity.

Kenyon MO et al Regulatory Toxicol, Pharmacol, 48, 75-86, 2007

2.6 A literature survey of 454 mutagens tested in the Ames test was undertaken to estimate the lowest effective concentrations for a variety of classes of mutagens and to develop an understanding of the sensitivity of the test system. Overall for most representative classes, all compounds were detected at 2500 µg/plate. In a further analysis by class, the authors reported that only a small number of compounds had LECs that were greater than 250 µg/plate. Overall, the authors estimated that 85% of mutagenic impurities in an Active Pharmaceutical Ingredient (API) should be detected in Ames tests if present at ≥5% assuming the API is tested up to 5000 µg/plate. The literature review had been supported by a number of Ames tests of pharmaceutical agents undertaken in the presence of excess mannitol (to represent excess API) and verapamil and diltazem (two highly metabolised medicines). Members agreed that many impurities in APIs were present at less than 5% and it was likely such impurities would need to be isolated and tested separately in order to evaluate their potential mutagenic hazard. A negative result in Ames tests for a test material containing impurities below 5% would not provide reassurance that the impurity had been tested adequately.

*Muller L et al Regulatory Toxicology and Pharmacology, 44, 198-211, 2006. and EMEA guidance (CHMP/SWP/*5199/02, 28 June 2006)

2.7 Members acknowledged that the approach suggested was specific to pharmaceuticals and provided guidance on assessing genotoxic impurities in APIs particularly in relation to decisions on safety in respect of clinical trials. The TTC approach was based on assessment of likely intakes of impurities (i.e. a de *minimus* risk value (Threshold of Toxicological Concern (TTC) (1.5 µg/person/day)) could be identified for any chemical, including those of unknown toxicity, taking chemical structure into consideration). The TTC was originally applied to foodstuffs (e.g. impurities present in flavour materials and food contact materials) was introduced as a way of prioritising action on those most likely to cause the greatest risk and there had also been proposals that the TTC could be used to inform on decisions of acceptability.

2.8 The COM agreed the proposed approach had an advantage in aiding assessment of risk/benefits from clinical trials. Members agreed that it was not possible to conclude that scaling intakes resulted in the same mutagenic risk. Members noted that the EMEA guideline limit for genotoxic impurities in APIs could exceed the TTC for life-threatening illnesses.

Chemical Mixtures

2.9 The COM expressed an interest in the evaluation of the mutagenicity of chemical mixtures during the 2005 and 2006 horizon scanning exercises. One recommendation from COM was to consider the possible occurrence of synergistic interactions regarding mutagenic effects of chemical mixtures, the possible mechanisms for any synergistic effects and the implications of such a finding for risk assessment. It is possible that if synergistic effects between two or more *in vivo* mutagens occurred then co-exposure to mixtures containing these chemicals might result in a significant increase in the risk of mutagenicity and cancer compared to the risks associated with exposure to the individual chemicals alone. The COM evaluation outlined was intended to build on the work of the COT work on Risk Assessment of Mixtures of Pesticides and similar substances (WiGRAMP)[1] http://www.food.gov.uk/science/ouradvisors/toxicity/cotwg/wigramp/ which was subsequently extended to encompass other types of chemicals in food (see 2004 COT Annual report http://www.food.gov.uk/multimedia/pdfs/cotsection.pdf) and the ongoing work of the Interdepartmental Group on Health Risks from Chemicals (IGHRC) on the risk assessment of chemical mixtures http://www.silsoe.cranfield.ac.uk/ieh/ighrc/mixtures_document.pdf. Thus the definitions and nomenclature used to describe interactions regarding mutagenicity induced by chemicals in this statement were taken from these reviews.

2.10 A number of strategies have been considered for the evaluation of chemical mixtures. These include testing whole mixtures (integrative), fractionation of mixtures to determine mutagenic components (dissective, topdown approach), and investigations of interactions by testing simple combinations, recombined fractions, and spiking of mixtures/fractions (synthetic, bottom up approach). All of these approaches were identified from literature searches with regard to mutagenicity testing, although relatively few studies of whole mixtures were identified. Approximately 110 research papers with potentially relevant information were identified for consideration during the COM review

COM Discussion and Conclusions

Whole mixtures

2.11 The COM considered mutagenicity testing of whole mixtures, and approaches to dissection (fractionation/concentration) of mixtures. The primary purpose of such studies is to monitor mutagenic response in tests of a wide variety of mixtures (for example foods, samples of pollution (air and water) condensates or particles from pyrotechnic mixtures (e.g. cigarette smoke or mixtures of known compounds), hazardous wastes including industrial process effluents and municipal sludges. The COM noted that there were comparatively few data on mutagenicity testing of whole mixtures. The COM agreed that testing whole mixtures first using an *in vitro* screen (such as the Ames test or SOS chromotest) would have the advantage of picking up evidence of potential interactions, such as

synergy, that could be missed by testing individual fractions. However, the failure to detect mutagenicity when complex mixtures (e.g. fried foods) or fractions (e.g. catalytically cracked clarified oil) are tested either *in vitro* or *in vivo* did not prove the absence of potentially mutagenic compounds.

Approach to dissection of mixtures

2.12 The COM agreed an outline proposal for a strategy for monitoring mutagenicity of chemical mixtures (in particular occupational and environmental mixtures), based on proposals for evaluating the mutagenicity of mixtures in the published literature but noted that this was only general guidance and a case-by-case approach was needed.

Preliminary considerations

A. Collect information on chemical composition, and mutagenicity of chemicals in the mixture. Define the purpose of the monitoring approach (is this to monitor overall mutagenic hazard of the mixture, or to monitor the mutagenicity of selected levels of chemicals or groups of chemicals within the mixture?).

B. Review the literature for appropriate data on sampling, extraction and testing of similar mixtures. Review the mutagenicity test data on the mixture or similar mixtures or the chemicals within the mixture selected for monitoring.

Mutagenicity testing

C. Define *in vitro* testing strategy, focusing on optimising and standardising the approach.

D. Undertake *in vitro* monitoring to validate approach and identify sources of variation and their impact.

E. Consider, if necessary on a case-by-case basis, developing an *in vivo* segment to strategy. (This might include studies to test whether chemical(s) selected for monitoring had *in vivo* mutagenic potential if this was not known. It is unlikely that chemical(s) within a mixture which were known to be *in vivo* mutagens would need to be routinely tested.

Review of strategy

F. Implement the strategy and use data to inform on risk reduction strategies. It is important to periodically review the results of a monitoring strategy, particularly if there is any evidence for a change in the results being reported. There are many potential sources of variation which could affect the results and it would be important to differentiate between a change in results due to composition of the mixture from a change due to variation in fractionation and/or testing procedures. The inclusion of spiked samples in a strategy for mutagenicity testing of mixtures may be valuable.

Approach to evaluation of studies to investigate interactions

2.13 The COM agreed the concept of the 'envelope of additivity' was a helpful approach in the presentation of the results of studies and in the identification of non-interaction (e.g. dose-response and effect additive responses) and interaction responses (e.g. synergy and antagonism). The COM noted the proposed unifying approach of Gennings and colleagues (see Gennings C *et al* (2005). A unifying concept for assessing toxicological interactions: changes in slope. Tox Sci, 88 (2), 287-297.) for application of statistical methods in chemical mixture research which is based on the shape of the dose response curve and changes in the slope of the dose-response curve in studies using two or more chemicals, and agreed that this could be of potential use in evaluating genotoxicity.

Review of published studies on interaction between chemicals with regard to mutagenicity.

2.14 The COM noted that the available published literature presented a number of examples where interaction between chemicals with regard to mutagenicity had been reported. However, there was essentially no appropriate independent confirmation of the results in separate tests, or within an appropriate mutagenicity testing strategy for the identification of interactions and therefore no definite conclusions could be reached.

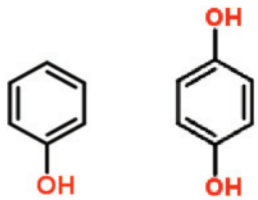
2.15 The COM agreed that the available studies had raised a number of potential hypotheses for interaction (see statement enclosed at the end of this annual report). There was a need for further research regarding such mechanisms, which if confirmed in an appropriate mutagenicity testing strategy might be of potential significance for public health.

2.16 The COM agreed a statement which is reproduced at the end of this report.

Phenol

2.17 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, COM/00S1). [Hydroquinone is a metabolite of phenol, see figure 1 below]

Figure 1:



Phenol Hydroquinone

2.18 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.

2.19 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.

2.20 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 a 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 doses of around 100-160 mg/kg bw.

2.21 The COM agreed a statement (COM/00/S1; http://www.iacom.org.uk/statements/COM00S1.htm) in January 2000. The conclusions reached with regard to phenol were similar to those reached in 1994

2.22 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 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. In addition the HPA 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.

Introduction to current COM review

2.23 The COM current consideration of phenol covered the period from 1994-2003. The objectives of the current review were i) produce an updated 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

2.24 The COM agreed with the conclusions reached on phenol in its previous statement (COM/00/S1). (The COM agreed the overall conclusions reached in the draft EU Risk Assessment report which had been provided for members' information.)

2.25 The following overall conclusions were agreed.

a. Phenol is mutagenic *in vitro* in mammalian cells giving rise to gene mutation and chromosomal damage in the presence and absence of exogenous metabolic activation. The mode(s) of action had not been fully elucidated although there was evidence that effects were in part due to oxidative DNA damage

b. Phenol should be regarded as an *in vivo* somatic cell mutagen. The COM confirmed that there was consistent evidence for a small effect at doses below the i.p. LD50.

c. The COM agreed that the published study by Spencer *et al* 2007 had been well conducted but considered a dose level of 200mg/kg bw i.p would have been valuable. The dose level used in the study of 300 mg/kg bw clearly exceeded the maximum tolerated dose level. The committee considered that the degree and duration of hypothermia reported with phenol was severe and prolonged. Members concurred with the conclusion reached by the study authors and reported in the publication '.. overall, these studies suggest a role, but not necessarily a causality, for phenol-induced hypothermia in the formation of MN.'

d. The COM concluded that the additional 'in confidence' data on thermoregulatory support in phenol treated animals provided inconclusive evidence regarding the role of hypothermia in phenol-induced micronuclei in mice. Thus for phenol-treated animals there was evidence of impaired capacity to modulate temperature compared to controls and a transient hypothermia. It was possible that the application of thermoregulatory control could influence the formation of MN in control and phenol treated mice.

e. The COM concluded that all the available data on phenol suggested phenol should be regarded as a non-threshold *in vivo* systemic mutagen. There is insufficient evidence to support a threshold approach to risk assessment of systemic phenol.

2.26 The COM agreed a statement which is reproduced at the end of this report.

Horizon Scanning

2.27 The annual horizon scanning exercise was intended to provide an opportunity for members and advisers from Government Department/Agencies to discuss and suggest topics for further work. Considerable progress on the items identified in the 2006 horizon scanning exercise had been made, although it was noted that review on mutational spectra had not been initiated and this would be carried over to next years work programme. The primary objective of the 2008/9 horizon scanning exercise was to provide information to aid members' consideration of the scope and format of the revision of the COM guidance. The committee agreed that the following topics should be considered and could be included in the COM guidance; aneuploidy, mutational fingerprints/spectra, GADD 45 assay, and risk assessment.

2.28 Other suggestions for potential consideration included tissue concentrations in relation to lowest effect dose in carcinogenicity studies (the International Life Sciences Institute (ILSI) was doing some work on this), the Pig A assay (Bryce SM *et al* Envion Mol Mut, 49, 256-264, 2008), pesticide impurities and nanomaterials.

Test Strategies and Evaluation
Review of COM Guidance 2000

2.29 The current COM mutagenicity testing strategy (2000) was developed to update the strategy document published in 1989 (Report on Health and Social Subject No 35) which had been based on a strategy agreed in 1981. The COM guidance document published in 1989 contained a number of chapters on the basic science of mutations and their significance for human health as well as a testing strategy. The current COM strategy was a scientifically based approach to mutagenicity testing which updated the 1989 guidance, for example, with incorporation of the *in vitro* micronucleus assay as a test for clastogenicity/aneuploidy and the inclusion of newer approaches to *in vivo* testing such as use of transgenic animal models. The need to periodically reflect on developments was recognised by COM in 1981 and in 1989. The current COM guidance was not developed in response to a specific regulatory request but reflected the desire of COM members to update their guidance.

2.30 The guidance should produce a scientifically based strategy which can be used for screening compounds (not limited to one sector such as pharmaceuticals), evaluating genotoxicity of existing chemicals (such as contaminants) and providing case-by-case guidance in specific circumstances where specific questions regarding a compound arise (e.g. evaluating genotoxicity mode of action in rodent carcinogen target and non target tissues).

2.31 The Committee held two wide ranging scoping discussions during 2008 and during consideration of horizon scanning (see paragraph 2.24 for examples of areas to be considered during revision of the COM guidance). In addition several options for disseminating the COM review were explored including publication of a further booklet on a strategy for genotoxicity testing, a peer review publication and publication of a series of guidance documents on the COM Internet site. The advantage of a series of general guidance statements would be that these could be more readily updated when significant advances in genotoxicity testing and evaluation became available (e.g on identifying thresholds for genotoxicity or the assessment of *in vivo* mutagenic potency). It was agreed that all three options should be explored.

2.32 The COM agreed to consider the subject of potential thresholds for genotoxins at its February 2009 meeting.

Ongoing Reviews

Acrylamide

2.33 In 2007, the HSE requested a further evaluation from the COM regarding the information cited by the Polyelectrolyte Producers Group (PPG) in a letter to the chair of COM (dated 8 May 2007, COM statement 07/02). In view of the widespread dietary exposure to acrylamide, the Food Standards Agency requested that such a review should consider all available genotoxicity data on acrylamide. In 2007, the COM agreed that the EU risk assessment review completed by HSE (EU Risk Assessment report 2002) could be used as a basis for the review, and agreed a strategy for this to be extended with a systematic review of the scientific literature available subsequent to the EU report.

2.34 In 2008 Members reviewed the findings of the EU Risk Assessment Report and were presented with the systematic review of data relating to the genotoxicity of acrylamide and glycidamide published after 1995, and other pre 1995 references that had not been included in the EU risk assessment report. This systematic review, together with several presentations and submissions from the PPG, formed the basis of extensive discussions at each meeting in 2008. This has enabled a detailed statement to be drafted. The Committee expect to receive final comments on the fourth draft of the statement from the PPG in January 2009; with publication of the statement expected soon after, subject to any revisions in light of the submitted comments.

Toxicogenomics

2.35 The COT/COC/COM held a joint symposium on the issue of genomics and proteomics in October 2001 and published a joint statement in December 2004 on the use of toxicogenomics in toxicology. This was based on literature review of 50 studies and included information from the International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/HESI) collaborative programme of research. This topic was identified during the 2006 horizon scanning exercise for an updated review. The DH Toxicology unit drafted a short overview of a number of new relevant *in vitro* studies, which included data on gene expression changes in studies on DNA adducts and mutagenicity for the October 2007 meeting. A large number of papers had been retrieved, but those selected for review were specifically chosen with the aim of identifying any advancement in the field, which may affect the conclusions drawn in the last statement. The COM considered a draft discussion paper at its October 2008 meeting. A further discussion paper is to be considered in 2009 reporting on the results of the ongoing ILSI/HESI trials.

Statements of the COM

Statement on mutagenicity assessment of chemical mixtures

COM/08/S1- March 2008

Introduction

1 The COM expressed an interest in the evaluation of the mutagenicity of chemical mixtures during the 2005 and 2006 horizon scanning exercises. One recommendation from COM was to consider the possible occurrence of synergistic interactions regarding mutagenic effects of chemical mixtures, the possible mechanisms for any synergistic effects and the implications of such a finding for risk assessment. It is possible that if synergistic effects between two or more *in vivo* mutagens occurred then co-exposure to mixtures containing these chemicals might result in a significant increase in the risk of mutagenicity and cancer compared to the risks associated with exposure to the individual chemicals alone. The COM evaluation outlined in this statement is intended to build on the work of the COT work on Risk Assessment of Mixtures of Pesticides and similar substances (WiGRAMP)[1] http://www.food.gov.uk/science/ouradvisors/toxicity/cotwg/wigramp/ which was subsequently extended to encompass other types of chemicals in food (see 2004 COT Annual report http://www.food.gov.uk/multimedia/pdfs/cotsection.pdf) and the ongoing work of the Interdepartmental Group on Health Risks from Chemicals (IGHRC) on the risk assessment of chemical mixtures http://www.silsoe.cranfield.ac.uk/ieh/ighrc/mixtures_document.pdf. Thus the definitions and nomenclature used to describe interactions regarding mutagenicity induced by chemicals in this statement have been taken from these reviews and are briefly commented on in paragraph 2 of this introduction.

2 The COT had noted that although there were a large number of studies on mixtures relatively few had appropriate data on the nature of the interactions between chemicals. The general principle reached from substantive consideration by the COT of data on pesticides across all toxicological end points was that in absence of data to the contrary, substances with similar modes of action could be assumed to act by dose-additivity, and substances with dissimilar modes of action could be assumed to act by effect additivity. The term interaction could imply a range of effects such as synergism, potentiation, supra-additivity, or sub-additivity. The COT had not specifically considered the most appropriate approaches to mutagenicity testing of mixtures or development of mutagenicity testing approaches to identify interactions with regard to mutagenicity.

Introduction to approaches to evaluation of mutagenicity of mixtures

3 A number of strategies have been considered for the evaluation of chemical mixtures.[3] These include testing whole mixtures (integrative), fractionation of mixtures to determine mutagenic components (dissective, top-down approach), and investigations of interactions by testing simple combinations, recombined fractions, and spiking of mixtures/fractions (synthetic, bottom up approach). All of these approaches have been identified from literature searches with regard to mutagenicity testing, although relatively few studies of whole mixtures were identified. Approximately 110 research papers with potentially relevant information were identified for consideration during the COM review.

4 A discussion paper on the mutagenicity testing of whole mixtures, approaches to dissection (fractionation/concentration) of mixtures regarding mutagenicity, and the presentation of a draft strategy for mutagenicity evaluation of mixtures was considered at the February 2007 meeting. http://www.iacom.org.uk/papers/documents/muto703.pdf

5 A discussion paper which presented a systematic review of published literature (up to the beginning of June 2007) of studies which had examined the potential interaction between chemicals regarding mutagenicity was considered at the October 2007 meeting. The Committee also briefly discussed approaches to design and evaluation of 'synthetic' studies investigating interaction between chemicals regarding genotoxicity. The COM considered the 'envelope of additivity' approach could be a useful approach to presenting data from studies designed to investigate potential interaction between chemicals with regard to mutagenicity and genotoxicity (outlined in paragraph 17). http://www.iacom.org.uk/papers/documents/muto715.pdf

6 This statement summarises the information contained in these discussion papers and the conclusions reached by COM.

Mutagenicity testing of whole mixtures, approaches to dissection (fractionation/concentration)

Whole mixtures

7 There were comparatively few studies where whole mixtures had been subjected to mutagenicity evaluation retrieved. An *in vivo* approach to the mutagenicity testing of cooked meats was considered.[4] The primary purpose for mutagenicity testing of whole mixtures outlined in the literature was the development of monitoring approaches to inform on risk reduction strategies. The studies need to be interpreted in terms of the overall mutagenic potency of the mixture and the sensitivity of the assay used to detect an effect, but it was noted that the data from such studies provided no information on the relative contribution of mutagenic chemicals present in the food or the interactions between chemicals regarding mutagenicity. A number of investigators have suggested that, where there is evidence that components of a mixture do interact and, in particular, where there is evidence of mutagenic synergy, then it might be prudent to evaluate whole mixtures as they exist to obtain appropriate information on mutagenic hazard.[5] Anwar (1993) proposed the term 'total mutagenic burden' for whole mixtures[6] However the failure to detect mutagenicity when complex mixtures (e.g. fried foods) or fractions (e.g. catalytically cracked clarified oil) are tested either *in vitro* or *in vivo* did not prove the absence of potentially mutagenic compounds.[7,8] The COM agreed that testing whole mixtures first using an *in vitro* screen (such as the Ames test or SOS chromotest) would have the advantage of picking up evidence of potential interactions, such as synergy that could be missed by testing individual fractions or chemicals isolated from a mixture.

Approaches to dissection (fractionation/concentration)

8 The key elements to approaches that might be potentially used are shown below in figure 1;

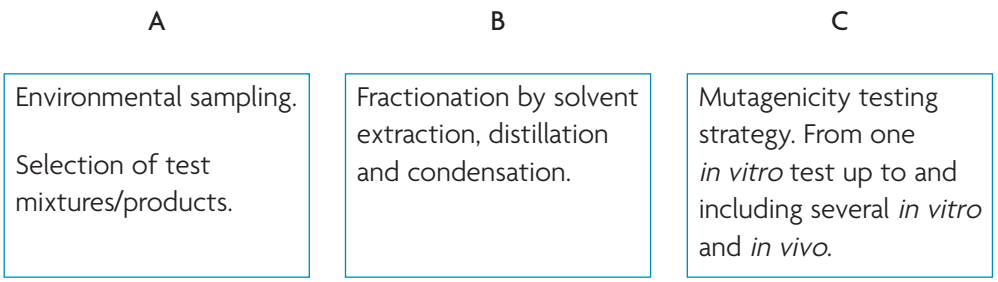


Figure 1; outline approach which could be used to evaluation of mutagenicity of chemical mixtures.

9 The COM considered published studies on the approach outlined in figure 1.[9-26] The COM agreed that a detailed review of environmental sampling for mutagenicity evaluation of mixtures was beyond the scope of the COM review. There were a wide range of factors which might affect the chemical mixture in samples recovered for mutagenicity testing including those affecting the emission of mixtures to the environment including variation in sources of release, distribution and degradation in the environment, the sampling procedure used (e.g. mass and volume of sample collected, the size distribution of particles in samples, the potential for reaction of sample with adsorbents/filters used in collection), and storage of samples prior to mutagenicity testing.[9] Overall, it was concluded sampling strategies can significantly influence the estimation of mutagenicity of chemical mixtures and there is thus a need for a careful case-by-case approach to a sampling strategy with consistency of sampling procedure attained in order to generate mutagenicity data that are comparable.

10 The COM reviewed fractionation procedures using solvent extraction, distillation and condensation for a number of mixtures samples (diesel particles collected occupationally or environmentally[13,14,15] or directly from exhausts[20,22] or from fumes e.g coke oven, roofing tar)[17], oil based liquids[16,23], condensates or particles from pyrotechnic mixtures (e.g cigarette smoke[9,12,19] or mixtures of known compounds[19]), hazardous wastes including industrial process effluents and municipal sludges18 and water samples taken from various points in the distribution system[21]). Most approaches used a single step extraction procedure. One particular difficulty in developing a strategy was optimising mutagenic response whilst avoiding excessive toxicity to the mutagenicity test indicator organisms used (e.g. bacteria). Multi step procedures can result in loss or modification of mutagenic components. In an WHO International Programme on Chemical Safety (IPCS) led collaborative study of the mutagenicity of mixtures (urban air samples, diesel particles and coal tar solution) significant interlaboratory and intralaboratory variance in the results of *Salmonella typhimurium* TA98 and TA100 with or without exogenous metabolic activation was noted, which was partly due to the method of extraction (either soxhlet or ultrasonication) using dichloromethane as a solvent as well as the mutagenicity test procedures used.[10,11] The final step in the fractionation procedure usually involved evaporation of extracts and resuspension in a solvent (usually DMSO) which is compatible with cell cultures used in mutagenicity tests and *in vivo* mutagenicity test systems. This final step may also introduce a potential source of variation regarding mutagenicity test data.

11 The COM considered that general guidance could not be provided regarding fractionation procedures, and that the testing strategy would need to be considered on a case by case basis. Both the top down and bottom up approaches to mutagenicity testing of mixtures were considered to have potential applications in different circumstances.

12 The primary objective of the mutagenicity testing strategy for chemical mixtures should be to identify hazard in the tested material or mixture. A comparison of the mutagenicity test data for test mixtures derived from the same sources and subject to the same extraction and fractionation procedures may provide information for monitoring hazard of environmental samples, commercial products, pyrolysis products and hazardous wastes. The IPCS collaborative study also reported considerable variation in results with regard to strain of *Salmonella* used, the activation conditions and between replicate mutagenicity tests within the same laboratory.[11,12] It is therefore likely that any successful approach to monitoring mutagenic hazard in chemical mixtures over a period of time would need to use well established sampling, extraction and fractionation procedures, and mutagenicity testing procedures with a high degree of quality control for each step. Additional procedures could include spiking mixtures with compounds of known structure and mutagenic potential to investigate procedures used (e.g. extraction[14] or pyrolysis[12]). Most studies are conducted to monitor the mutagenicity of chemical mixtures (e.g. serial samples from one potential source or batch to batch sampling of a product) but it is possible to use them in an investigative approach to study potential sources of mutagen release (e.g. the effect of agricultural run off on mutagenicity of water samples by timing and positioning sample collection from water courses[21]).

13 The majority of mutagenicity studies of chemical mixtures identified for the COM review used *Salmonella typhimurium* test strains as the only mutagenicity test.[3,9,13,14,18,20,23,25] These studies may include exogenous metabolic activation systems selected to increase the number of revertant colonies formed for a particular tested mixture or to test for the mutagenicity of particular groups of compounds within a mixture (e.g. use of hamster S-9)[23] or selection of particular *Salmonella* strains (e.g. use of nitroreductase (NR) deficient strains[20], and NR and O-acetyltransferase deficient strains[25]) or treatments (use of ROS scavengers such as α-tocopherol and/or ascorbate[15]) to monitor the mutagenicity of particular groups of mutagenic chemicals within the mixture. Additional *in vitro* tests (e.g. using mammalian cells) can extend the potential for monitoring mutagenic hazard over a wider range of chemicals present in the mixture.

14 Relatively fewer studies use additional *in vitro* and *in vivo* tests.[15-17] *in vivo* mutagenicity tests are usually incorporated into testing strategies for single chemicals to confirm the potential for a compound of unknown mutagenic potential to induce effects *in vivo*. The COM agreed that the inclusion of *in vivo* tests would have a confirmatory role only for monitoring of chemical mixtures, rather than being used routinely. This would be the case particularly when the environmental monitoring procedures were being carried out on mixtures containing known *in vivo* mutagens, but possibly at levels below the level of detection in *in vivo* assays. One potentially useful approach regarding the inclusion of *in vivo* tests in a strategy for monitoring complex mixtures was provided by Williams and Lewtas 1985[17] who correlated the mutagenic response (slope of the dose-response curve) to organic extracts from diesel, coke oven, roofing tar and cigarette smoke emissions in *in vitro* tests (*Salmonella typhimurium* TA98 +S-9 (rat or hamster), and mouse lymphoma mutagenicity) with the

response in mouse skin tumour initiation assays. Having correlated mutagenic potency *in vitro* and *in vivo* (in this case between different mixtures) it would therefore be possible to continue monitoring and undertake comparative ranking of different samples of these mixtures using an *in vitro* mutagenicity test strategy. It is possible to reach this conclusion as there was relatively good knowledge of the chemical composition of the mixtures included in the study, and a key hypothesis under test was the investigation of mixtures of PAHs which helped to define the *in vitro* and *in vivo* parts of the testing strategy.

15 The COM agreed an outline proposal for a strategy for monitoring mutagenicity of chemical mixtures (in particular occupational and environmental mixtures such as described in paragraph 10 of this statement), based on proposals for evaluating the mutagenicity of mixtures in the published literature[24,26] but noted that this was only general guidance and a case-by case approach was needed.

Preliminary considerations

A. Collect information on chemical composition, and mutagenicity of chemicals in the mixture. Define the purpose of the monitoring approach (is this to monitor overall mutagenic hazard of the mixture, or to monitor the mutagenicity of selected levels of chemicals or groups of chemicals within the mixture?).

B. Review the literature for appropriate data on sampling, extraction and testing of similar mixtures. Review the mutagenicity test data on the mixture or similar mixtures or the chemicals within the mixture selected for monitoring.

With regard to mutagenicity testing

C. Define *in vitro* testing strategy, focusing on optimising and standardising the approach.

D. Undertake *in vitro* monitoring to validate approach and identify sources of variation and their impact.

E. Consider, if necessary on a case-by-case basis, developing an *in vivo* segment to strategy. (This might include studies to test whether chemical(s) selected for monitoring had *in vivo* mutagenic potential if this was not known. It is unlikely that chemical(s) within a mixture which were known to be *in vivo* mutagens would need to be routinely tested.)

Review of strategy

F. Implement the strategy and use data to inform on risk reduction strategies. It is important to periodically review the results of a monitoring strategy, particularly if there is any evidence for a change in the results being reported. There are many potential sources of variation which could affect the results and it would be important to differentiate between a change in results due to composition of the mixture from a change due to variation in fractionation and/or testing procedures. The inclusion of spiked samples in a strategy for mutagenicity testing of mixtures may be valuable.

Approaches to evaluating mutagenic interaction between chemicals

16 The design of synthetic studies to investigate the potential for interaction between chemicals, fractions or after spiking mixtures with chemicals is particularly complex. A number of factors to include, illustrated in the studies identified for review (for example[3,12,25]) included the need for consideration of expected patterns of mutagenic response in bacterial tester strains used, the design of a testing strategy to limit the number of combinations tested to a minimum required to evaluate the nature of any interactions in mutagenicity tests (by selecting concentrations of test materials taking into account the dose-response of individual compounds or fractions in the tester strains, the consideration of the need for replicate experiments), and the consideration of the most appropriate approach to statistical analysis of data. The data could be analysed by a number of methods including the projections to latent structures (PLS) approach which overcomes many of the problems inherent in inter-correlated (dependent) predictor variables and produces results which are easily viewed.[13]

17 The COM agreed the concept of the envelope of additivity was potentially a helpful approach to graphically presenting the results of studies and to help identify non-interaction (e.g. dose-response and effect additive responses) and interaction responses (e.g. synergy and antagonism)[27]. The COM noted the proposed unifying approach for application of statistical methods in chemical mixture research based on the shape of the dose response curve and changes in the slope of the dose-response in studies using two or more chemicals.[28] The approach suggested by Gennings *et al* linked the traditional statistical models of interaction (as found in the general linear model / factorial ANOVA models) to the different concepts of joint toxic action. The unification of the approaches is achieved by showing that there is no interaction if the dose-response relationship of one chemical is not changed by the presence of other chemicals. An interaction exists if there is a change in the slope of the response. This concept of interaction related to underlying statistical models of additivity. Members agreed that the approach suggested by Gennings *et al* 2005 could be potentially helpful when assessing mutagenicity studies of interaction between chemicals.

Review of studies investigating the potential interaction between chemicals regarding mutagenicity

18 A total of 91 research papers were identified by literature searches up to June 2007. A quality scoring approach was used to select the best quality studies for further review by COM. The quality screening approach was based on Borgert *et al* 2001[29] for evaluating interaction studies in terms of the quality of design, data and interpretations. Reliable interaction studies were considered to be those that are interpretable without making assumptions about untested and unanalysed parameters. (An overview of the quality scoring criteria is given in Annex 1 to this statement.) Very few (n=15) published studies met all five of the criteria and these were considered in detail.[30-44] Brief summaries of other papers not meeting all of the quality screening criteria were also provided for the COM.

19 The COM agreed that the well-conducted studies of defined mixtures of mutagenic chemicals did not provide a consistent picture of combination effects being predictable on the basis of the single agent dose-response information. In the majority of cases, substances tested in these studies are mutagens with relatively well understood mechanisms of action (e.g. B[a]P, and the alkylating agents EMS, MMS, MNU). In only one instance was the same combination of chemicals tested (EMS and ENU) in two

different tests (Ames[36] and in an *in vivo* mouse micronucleus test[42]). Kawazoe and colleagues showed that in the Ames assay EMS and ENU induced linear dose-responses and that using dose addition it was possible to model the combined effect of these chemicals.[36] In the mouse micronucleus assay, these chemicals induced non-linear dose response curves, but mixture effects were consistent with dose addition predictions.[42] For other combinations of alkylating agents, however, it is not clear why additivity is not observed. In many of these cases, observed mixture effects appear to fall within the additivity envelope and as some investigators do not estimate confidence 'belts' for the additivity predictions, it is possible the observations are not truly statistically significantly different from the non-interaction predictions.

20 The COM considered that an important part of the assessment of genotoxicity studies of interaction between chemicals would be reproducing results seen in one test system with other appropriate genotoxicity tests (e.g. confirming results seen in bacterial gene mutation assays in mammalian cell gene mutation assays). This could be used in a weight of evidence assessment of interactions and would be particularly important for assessment of interactive effects such as synergy or antagonism. The strategy for assessment of interaction with regard to mutagenicity would also need to include *in vivo* tests with appropriate consideration of toxicokinetics and exposure of sampled tissues. Members commented that the available published literature presented a number of examples[35,37,38,40] where interaction had been reported, but there was essentially no appropriate independent confirmation of the results in separate tests, or within an appropriate mutagenicity testing strategy for the identification of interactions and no definite conclusions could be reached.

21 The COM considered the four available published studies which reported the best evidence for interaction in detail to provide advice on possible mechanisms of mutagenicity might be associated with interaction.

22 Homme M *et al* (2000)[35] had documented synergistic DNA damage using UDS assays in human fibroblasts between 4-nitroquinoline-1-oxide (4-NQO) and non-effective methyl methanesulfonate (MMS). The authors had proposed that the ultimate DNA reactive metabolites formed from 4-NQO resulted in unwinding of super helical DNA so that more molecules of MMS could reach the bases of DNA resulting in increased methylation and mutation. The COM considered that a viable hypothesis had been proposed. It would be necessary to undertake independent confirmation of the results and to include additional combinations of mutagens with and without 4-NQO to provide further data to investigate the proposed mechanism. At present no definite conclusions could be reached on this specific example of an interaction.

23 Kojima H *et al* (1992)[37] had investigated the potential for interaction between MMS and EMS in Chinese hamster V79 cells using cell killing, induction of 6-thioguanine mutants (6TG resistant mutants) and chromosome aberrations. These authors had reported evidence for synergistic interactions for both cell killing and 6TG mutation and evidence for additivity with regard to chromosome aberrations. The authors had suggested that the DNA damage produced by one alkylating agent could be increased in the presence of a small amount of another alkylating agent. The COM noted the predominant SN2 mechanism of MMS and the SN1 mechanism of EMS and considered that these differences could form the basis for a hypothesis of interactive effects with regard to genotoxicity.

However the COM considered there was a need for independent confirmation of these results and further investigations of other alkylating agents before any definite conclusions could be reached.

24 Lutz WK *et al* (2005)[38] had reported evidence for antagonism using a combination of N-methyl-N-nitrosourea (MNU) and the topoisomerase-II inhibitor genistein (GEN) in the mouse lymphoma assay in LY5178Y cells. In separate tests when MMS was combined with GEN an additive response (reported to be within the envelope of additivity) was reported. The authors hypothesised that the profile of DNA methylation and or epigenetic effects were responsible for the different responses reported for the binary combinations tested. The COM considered these investigations raised interesting hypotheses for further testing but no definite conclusions could be reached on these data.

25 Marrazzini A *et al* (1994)[40] had undertaken *in vivo* mouse bone marrow MN tests in mice using intraperitoneal administration of binary combinations of hydroquinone, catechol and phenol. Mixtures of hydroquinone and phenol and catechol and phenol were reported to result in synergistic induction of micronuclei. Members noted that it was not possible to discern a potential mechanism of interaction from these studies which could be used to support hypotheses for further testing.

26 The COM was aware of the different interpretations of the term synergy was used by the research groups and the limitations in the available data made it difficult to reach any definite conclusions. However, overall there was insufficient evidence to conclude that the studies reviewed provided conclusive evidence for interaction effects (either synergy or antagonism). However, a number of the studies provided evidence to suggest hypotheses for interaction (see paragraphs 22-25) which could be further examined in appropriately designed mutagenicity testing strategies. These included the interaction between ultimate DNA reactive chemicals and DNA structure, (e.g. different mechanisms of DNA alkylation), the effect of covalent binding to DNA of one chemical on the potential for other reactive metabolites and chemicals to bind to DNA, and possible epigenetic mechanisms which could potentially result in a mutagenic response that resulted from an interactive effect between chemicals (i.e. synergistic or antagonistic). The COM agreed that the potential for interactions between chemicals with regard to genotoxicity needed to be studied on a case-by-case basis.

COM Discussion and Conclusions

Whole mixtures

27 The COM considered mutagenicity testing of whole mixtures, and approaches to dissection (fractionation/concentration) of mixtures. The primary purpose of such studies is to monitor mutagenic response in tests of a wide variety of mixtures for example foods, samples of pollution (air and water) condensates or particles from pyrotechnic mixtures (e.g. cigarette smoke or mixtures of known compounds), hazardous wastes including industrial process effluents and municipal sludges. The COM noted that there were comparatively few data on mutagenicity testing of whole mixtures. The COM agreed that testing whole mixtures first using an *in vitro* screen (such as the Ames test or SOS chromotest) would have the advantage of picking up evidence of potential interactions, such as synergy, that could be missed by testing individual fractions. However, the failure to detect mutagenicity when complex mixtures (e.g. fried foods) or fractions (e.g. catalytically cracked clarified oil) are tested either *in vitro* or *in vivo* did not prove the absence of potentially mutagenic compounds.

Approach to dissection of mixtures

28 The COM agreed an outline proposal for a strategy for the fractionation and monitoring of the mutagenicity of chemical mixtures (as outlined in paragraphs 10-15 of this statement) but noted that this was only general guidance and a case-by case approach was needed.

Approach to evaluation of studies to investigate interactions

29 The COM agreed the concept of the 'envelope of additivity' was a helpful approach in the presentation of the results of studies and in the identification of non-interaction (e.g. dose-response and effect additive responses) and interaction responses (e.g. synergy and antagonism). The COM noted the proposed unifying approach of Gennings and colleagues (see reference 28) for application of statistical methods in chemical mixture research which is based on the shape of the dose response curve and changes in the slope of the dose-response curve in studies using two or more chemicals, and agreed that this could be of potential use in evaluating genotoxicity.

Review of published studies on interaction between chemicals with regard to mutagenicity.

30 The COM noted that the available published literature presented a number of examples where interaction between chemicals with regard to mutagenicity had been reported. However, there was essentially no appropriate independent confirmation of the results in separate tests, or within an appropriate mutagenicity testing strategy for the identification of interactions and therefore no definite conclusions could be reached.

31 The COM agreed that the available studies had raised a number of potential hypotheses for interaction (see paragraph 26). There was a need for further research regarding such mechanisms, which if confirmed in an appropriate mutagenicity testing strategy might be of potential significance for public health.

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COM/07/S1

Statement on Mutagenicity Evaluation of Mixtures Annex 1

Approach to quality screening of published papers on interaction studies: Sumerised from Borget CJ *et al* (2001) Hum Ecol Risk Assess, 7, 259-306

1 In 2001, Borgert and colleagues (*Hum Ecol Risk Assess* **7**(2): 259-306, 2001) proposed a set of criteria for evaluating interaction studies in terms of the quality of design, data and interpretations. Reliable interaction studies are those that are interpretable without making assumptions about untested and unanalysed parameters. Although there is debate among experts regarding which models of non-interaction, which methods of combination analysis, and which statistical tests are most appropriate, it was still possible to apply the principles outlined by Borgert *et al* to assist in data interpretation. The criteria proposed were designed to assist risk assessors in identifying studies that can be used in component-based mixture risk assessments as well as those studies that are less useful due to inadequacies in design or interpretation. The aim was for them to apply broadly to interaction data for all effects of drugs, pesticides, industrial chemicals, food additives and natural products.

2 These criteria appear to provide a useful basis on which to evaluate the studies identified on mutagenic interactions. The five criteria set out below have been refined where necessary to facilitate their specific application to genetic toxicology studies and then used to evaluate the 91 retrieved articles.

I. Dose-response relationship for the individual mixture components are adequately characterised

Without adequate dose-response relationship characterisation for the individual components, it is not possible to determine whether a biological effect of a mixture is due to interactions between the components.

Ideally, single agent dose-response characterisation should enable slope, inflection points, and maximum and minimum effects to be estimated. Most importantly, key to being able to decide the appropriate 'no interaction' hypothesis (Criterion II, below) is whether the individual components of the mixtures have linear or non-linear dose-response curves and whether they have similar slopes. Inadequate characterisation of the dose-response relationship can lead to erroneous conclusions of interactions and this might be compounded further if the mixture components have significantly different shaped dose-response relationships.

For the purposes of this COM review, it was decided to focus, in the first instance, on mixtures of chemicals where all components are mutagenic. That is, evidence of "potentiation" from mixtures of mutagens with co-mutagens has not been considered at this point. Therefore, it is assumed that each mixture component alone induces a measurable genotoxic effect and detailed dose-response data are available.

II. An appropriate 'non-interaction' or 'additivity' hypothesis should be, *a priori*, explicitly stated and used as the basis for assessing combination effects.

Interactions are inferred when a mixture of chemicals produces a biological response greater or less than expected based on mathematical concepts of additivity (non-interaction). Two models of non-interaction have been well-developed in the pharmacological and toxicological literature and are appropriate as the basis for non-interaction hypotheses. Dose addition is based on the concept that an agent cannot interact with itself, and predicts that two non-interacting compounds will behave as dilutions of one another when combined. The second model is response addition, and expresses probabilistic independence between two compounds. In this case, independence implies functional independence between two chemicals such that the incremental effect of one compound is unchanged in the presence of a second.

In the literature, dose addition largely assumes a strictly similar mechanism of action of all mixture components, while response addition is based on the idea of completely dissimilar mechanisms of action of the mixture components. Therefore, if mechanisms of action are well-enough understood, this may suggest the most appropriate non-interaction model to assume. However, in most cases adequately detailed understanding of the toxicological mechanisms of action for the individual mixture components is not available. Therefore it may be useful to compare observed combination responses with both models of non-interaction. In so doing, applying both models will generate a range of effects delineated by dose addition and response addition, referred to by some researchers as an 'additivity envelope', in which a non-interacting mixture would be expected to lie (**Figure 1**). This approach would be considered to meet this criterion. In addition, as the number of individual components in the mixtures of interest increases, it is likely that there will be a variety of chemicals with similar and dissimilar mechanisms of action and it may not be appropriate to use dose addition or response addition. In this regard, some groups are beginning to combine the two models, but as an interim, it is feasible to assume effects will lie in the additivity envelope if the mixture is non-interactive.

It should be noted, that dependent on the default non-interaction model applied, there are different demands made on the ideal single substance dose-response data (which has an impact on Criterion I). That is, for dose addition, single substance studies have to provide concentration-effect data for the same effect levels that will be assessed in the combination studies. For the application of response addition, it is necessary to have detailed resolution of the single substance dose-response relationships at effect levels below the region of interest for the mixtures.

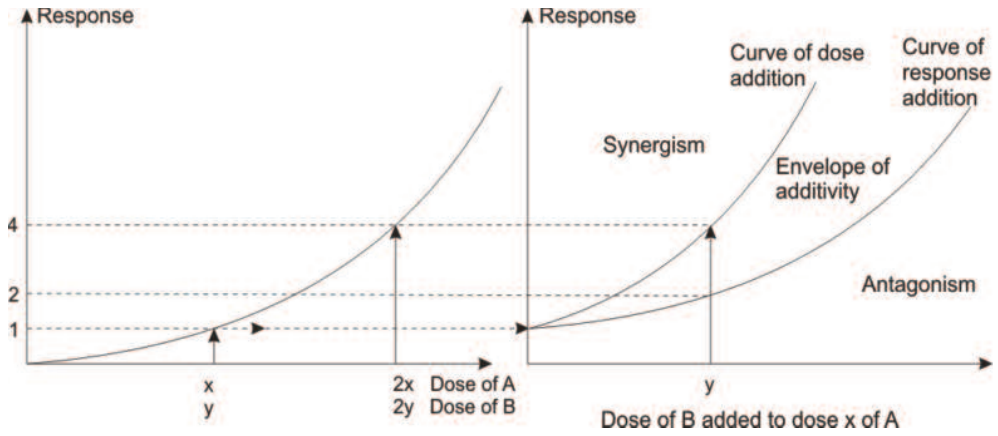

Figure 1. Schematic representation of a non-linear dose-response relationship (left hand side for two substances,A and B) **and classification possibilities for the response of a mixture of the two components** (right hand side; dose response for B added to dose x of A). Taken from Lutz *et al.* (2005). Dose x of chemical A produces a response of 1 effect unit, and dose y of chemical B has the same effect magnitude, in fact chemicals A and B have the same dose-response curves. A mixture of dose x of substance A plus dose y of substance B generated a response of effect level 4, one might postulate that A and B acted in a synergistic manner. This is interpretation is not correct when the shape of the chemicals' dose-response curves are considered. Therefore, the mixture of dose x of substance A plus dose y of substance B can be considered as dose 2x of chemical A or 2y of chemical B, and these doses generate a response of effect level 4, i.e. in agreement with dose addition. If the two chemicals acted independently of each other, the expectation would be the lower of the two curves in the right hand panel, i.e. response addition. This curve has exactly the same shape as the dose-response on the left hand panel, except that it is set off on the y-axis by response level 1 (the effect generated by dose x of A). On this basis, the mixture of dose x of substance A plus dose y of substance B would result in effect level 2 as shown by the lower dotted line on the right hand panel.

III. Combinations of mixture components should be assessed across a sufficient range of concentrations and mixture ratios to support the goals of the study

The characteristics of a mixture are clearly dependent on the components of the mixture and the concentration range of the mixture that is tested. However, there may also be considerable dependence on the ratios at which each component is present within the mixture. This is because different types of interactions can be exhibited by the same mixture of chemicals at different mixture ratios. Approaches to mixture testing routinely used include:

- full factorial design: tests a full complement of component ratios across the dose-response range of each mixture ratio.

- fractional factorial design: reduces the number of tests to a specified subset of mixture combinations while still maintaining a substantial proportion of the information that would be produced with a full factorial design.

- ray design: tests fixed-ratio mixtures, i.e. a constant ratio of the mixture components, across a range of concentrations.

There are no hard and fast rules as to the correct approach to take in all cases, but it is important to employ the design that will satisfy the goals of the study, and not to over-interpret the resulting data. Detailed descriptions of these different approaches have been published recently (IGHRC, US EPA etc.)

IV. Formal statistical tests should be used to determine whether the response produced by a combination is different from that predicted by the additive hypothesis.

Some researchers evaluate only whether responses differ statistically from controls and whether dose combination responses differ statistically from individual component responses. Such comparisons do not actually address the question of whether there is an interaction. As detailed in Criterion II, the appropriate non-interaction model will have been stated, and statistical tests should compare the observed mixture effect with that of the expected joint effect on the basis of the non-interaction hypothesis. Without a clearly stated non-interaction hypothesis, the results of any statistical test cannot be interpreted. Statistical methods that have been used to infer that mixture components interact include simple t-tests, linear models (including ANOVA and multiple regression) and multivariate regression. Ideally, the statistical approaches will allow confidence intervals to be placed on the observed mixture data and also on the predictions based on the mathematical models of dose addition or response addition. As the prediction is based on experimental (variable) data on the single substances, it is possible to estimate the variability associated with the predicted combined effect.

V. Interactions should be assessed at relevant levels of biological organisation.

Although the primary objective of the mutagenicity testing strategy for chemical mixtures should be to identify hazard in the tested material or mixtures, it is important to understand if the mixture poses a significantly greater hazard than the individual components. Identifying a potential interaction which might be of potential importance for public health, therefore requires not only a mechanistic rationale, *in vitro* evidence of interaction and *in vivo* evidence of interaction but also, the information must consistently point towards a synergistic interaction.

Interaction studies at the level of the whole organism or population can be difficult to interpret without information from underlying levels of biological organisation. Without knowledge of the mechanism of action of the mixture components it may not be possible to establish which non-interaction hypothesis is most appropriate. It may therefore be necessary to employ an additivity envelope approach (as detailed above in criterion II), consequently reducing the chance to detect true interactions. On the other hand, numerous interactions may be detected in studies carried out at the molecular, biochemical or cellular level, and these interactions may never manifest change in the organism. Ideally the systems used to assess combination effects should be fit for purpose, which implies use of accepted mutagenicity/genotoxicity tests.

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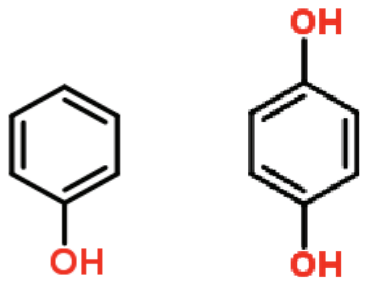
Update statement (2008) Mutagenicity of Phenol

COM/08/S2- November 2008

Introduction

Background to COM review

1 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, COM/00S1.[1] [Hydroquinone is a metabolite of phenol, see section on metabolism and figure 1 below]



Phenol Hydroquinone

Figure 1.

2 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.

3 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.

4 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 a 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 doses of around 100-160 mg/kg bw (relevant BMMN studies are reviewed in para 14 below).

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

6 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 has now been published and was identified during the 2007 COM horizon scanning exercise.[2] Members asked for a review of the paper during the COM horizon scanning exercise. In addition the HPA 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.

Introduction to current COM review

7 The COM consideration of phenol covers a period from 1994-2003. The objectives of the current review is to i) produce an updated 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.

8 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.[3] In addition relevant information from important studies on phenol were provided to the COM.

Overview of phenol mutagenicity

in vitro mutagenicity studies

Bacterial tests

9 The COM agreed that phenol was not mutagenic in standard bacterial mutagenicity tests.[3]

Mammalian cell gene mutation tests

10 The Committee considered the available mammalian gene cell mutation studies. Phenol induced a dose-related increase in the frequency of *Hprt* mutants in V79 cells in the absence of exogenous metabolic activation (4-fold increase at the top dose). Cell survival at the top dose was 50%.[4] A positive result had also been documented in SHE cells using the Na+/K+ and Hprt loci in the absence

of exogenous metabolic activation at the highest dose tested.5 There was no evidence of cytotoxicity reported in this study. Evidence for a positive result had been documented in mouse lymphoma L5178Y cells in the presence and absence of exogenous metabolic activation at dose levels which induced cytotoxicity.[6] A similar results had also been documented in LY5178Y cells in the presence and absence of exogenous metabolic activation.[7] Overall it was prudent to conclude a positive response in gene mutation assays in mammalian cells in the presence and absence of exogenous metabolic activation, although the mechanism for the induced effects had not been resolved.

Mammalian cell chromosomal aberration tests

11 Phenol gave a positive result for chromosomal aberrations in CHO cells in the presence and absence of exogenous metabolic activation.[8] Members noted the increase in the absence of exogenous metabolic activation was approximately 3 fold and there was no evidence for a dose response in the presence of exogenous metabolic activation. Positive results were also reported in a number of micronucleus tests in CHO cells both in presence and absence of exogenous metabolic activation[9], in V79 cells and human PBLs (both in the absence of exogenous metabolic activation).[10,11] No evidence for an aneugenic effect of phenol was reported in a test where chromosome number in metaphase spreads were scored and reported (positive results were reported for benzene in the same experiment but a known aneugenic positive control was not used).[5] Evidence for a moderate increase in both kinetochore positive and negative micronuclei was reported in PBLs indicating some evidence for both clastogenic and aneugenic activity with phenol.[11] Overall members considered no definite conclusion regarding the potential for aneugenicity could be drawn from these data.

Studies investigating DNA damage

12 A number of *in vitro* studies investigating the potential for DNA damage were available. Members noted the evidence for UDS in the absence of exogenous metabolic activation in SHE cells.[5] Members noted the evidence for ssDNA breaks in mouse lymphoma cells in the presence of exogenous metabolic activation.[12] Members considered the evidence for formation of 8-hydroxy-deoxyguanosine (8-OHdG) 3 indicated some potential for oxidative DNA damage but commented that undifferentiated HL60 cells were likely to be more predisposed towards formation of free radicals and oxidative DNA damage than differentiating HL60 cells[12] or normal human peripheral blood lymphocytes[13] Members noted the evidence for formation of DNA adducts in calf thymus DNA in the presence of horseradish peroxidase and hydrogen peroxide.[15] The data reported provided some evidence for oxidative DNA damage with phenol but the test system was likely to be predisposed to formation of free radicals and oxidative DNA damage.[15]

Conclusion: *in vitro* mutagenicity data

13 Thus phenol was mutagenic *in vitro* in mammalian cells giving rise to gene mutation and chromosomal damage in the presence and absence of exogenous metabolic activation. The mode(s) of action had not been fully elucidated although there was evidence that effects were in part due to oxidative DNA damage.

in vivo mutagenicity studies

14 The results of available studies considered in the draft EU risk assessment report reported evidence for a 2-2.5 fold induction of BMMN using oral and i.p. doses which equate to or exceed the relevant LD50 in mice. 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. Members reconsidered the three key studies supporting this conclusion. Chen and Eastmond 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.[16] Mazzarini A *et al* 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.[17] 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 48h 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.[18]

15 The COM affirmed its previous assessment of these studies. The COM agreed the overall conclusions reached in the draft EU Risk Assessment report.[3] Thus phenol should be regarded as an *in vivo* somatic cell mutagen. The COM confirmed that there was consistent evidence for a small effect at doses below the i.p. LD50.

Evidence regarding mode of action for the *in vivo* mutagenicity of phenol.

Induction of micronuclei by phenol in mouse bone marrow. Association with chemically induced hypothermia. (Spencer et al Tox Sci, 97, 120-127, 2007)[2]

16 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, 24h sampling). BT was measured prior to dosing, and 2,5,24 and 48h. Animals were killed at 24 or 48h post dose 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 24h 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 Dunett t-test. Kinetochore positive MN-PCEs were compared using Fisher exact test.

17 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 48h observation time point. No deaths occurred at 200 mg/kg bw and below. Signs of 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 and the 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 figure1 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.

18 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 the published 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 48h 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.

19 A statistically significant increase in MN-PCE/1000 PCE was recorded at 300 mg/kg bw at 24h sampling (male 10.8 c.f. 2.1 in control and 11.3 in females cf 2.5 in controls). At 48h the mean frequency of MN-PCE/1000PCEs 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.

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

21 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. Vinblastine (VB) gave the expected positive result. The proportion of kinetochore positive MN was substantially higher in VB treated mice.

22 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 BT in mice. It was noted that in part, it was possible to speculate that BT affected spindle function thus resulting in 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 did not 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.

COM conclusions on Spencer et al 2007

23 Members agreed that the study had been well conducted but considered a dose level of 200 mg/kg bw i.p would have been valuable. The dose level of 300 mg/kg bw clearly exceeded the maximum tolerated dose level. The committee considered that the degree and duration of hypothermia reported with phenol was severe and prolonged. Members concurred with the conclusion reached by the study authors and reported in the publication '...overall, these studies suggest a role, but not necessarily a causality, for phenol-induced hypothermia in the formation of MN.'

Additional in-confidence data on thermoregulatory support study

24 Members considered the additional in confidence data on the thermoregulatory support study which had been provided by Dow Chemicals.

25 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* 2007.[2] Additional studies were undertaken to investigate the approach to thermoregulatory control induced (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 oblate phenol induce 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 thermoregulatory control conditions at five minute intervals to provide more comprehensive data on the effectiveness of thermoregulatory support. 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. 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* 2007[2]).

26 The COM accepted that thermoregulatory support was in practice very difficult to achieve. It was noted the effects resulting from dosing of phenol and also thermoregulatory support would have been stressful to the animals. Members observed that thermoregulatory support had not offset the

phenol induction of micronuclei in mice. The application of thermoregulatory support had resulted in evidence for a slight increase in micronuclei formation in control females. However overall the observed induction of micronuclei by phenol could not be discounted. Members were aware that the principal study author had written to the secretariat and had concluded that, at this time, it is tenuous to make a conclusion regarding the mutagenicity of phenol under conditions of altered thermoregulation in the mouse micronucleus test.

Additional published studies on hypo-and hyperthermic induction of micronuclei in rodents.

27 Members considered the generic paper on the role of hypo- and hyperthermia in the formation of micronuclei in rodents. [19-23] Of particular interest was the publication by Tweats DJ *et al* 2007.[23] These data support the observation that chemical induced hypothermia in mice and hyperthermia in rats and mice may be potential modes of induction 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 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 *in vivo* which need to be taken into account in the overall assessment.

COM conclusions

28 The COM agreed with the conclusions reached on phenol in its previous statement (COM/00/S1). The COM agreed the overall conclusions reached in the draft EU Risk Assessment report.[3] The following overall conclusions were agreed.

a. Phenol is mutagenic *in vitro* in mammalian cells giving rise to gene mutation and chromosomal damage in the presence and absence of exogenous metabolic activation. The mode(s) of action had not been fully elucidated although there was evidence that effects were in part due to oxidative DNA damage

b. Phenol should be regarded as an *in vivo* somatic cell mutagen. The COM confirmed that there was consistent evidence for a small effect at doses below the i.p. LD50.

c. The COM agreed that the published study by Spencer *et al* 2007 had been well conducted but considered a dose level of 200 mg/kg bw i.p would have been valuable. The dose level used in the study of 300 mg/kg bw clearly exceeded the maximum tolerated dose level. The committee considered that the degree and duration of hypothermia reported with phenol was severe and prolonged. Members concurred with the conclusion reached by the study authors and reported in the publication '...overall, these studies suggest a role, but not necessarily a causality, for phenol-induced hypothermia in the formation of MN.'

d. The COM concluded that the additional 'in confidence' data on thermoregulatory support in phenol treated animals provided inconclusive evidence regarding the role of hypothermia in phenol-induced micronuclei in mice. Thus for phenol-treated animals there was evidence of impaired capacity to modulate temperature compared to controls and a transient hypothermia. It was possible that the application of thermoregulatory control could influence the formation of MN in control and phenol-treated mice.

e. The COM concluded that all the available data on phenol suggested phenol should be regarded as a non-threshold *in vivo* systemic mutagen. There is insufficient evidence to support a threshold approach to risk assessment of systemic phenol.

November 2008

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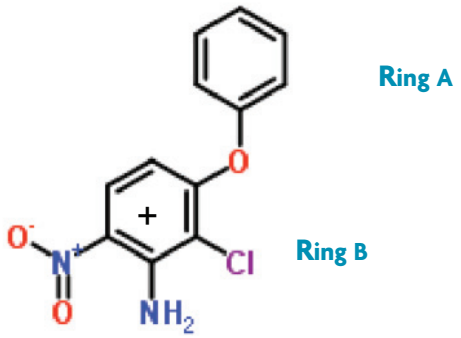
Statement on the review of Mutagenicity of Alconifen and risk assessment of its Postulated Metabolites Hydroquinone and Phenol)

COM/08/S3 - November 2008

Introduction

1. The COM has been asked for advice by the Pesticides Safety Directorate (PSD) on a pesticide active ingredient new to the U.K. which is undergoing evaluation through the independent Advisory Committee on Pesticides (ACP). The referral statement was as follows: 'ACP requested advice on the mutagenicity of Aclonifen and the genotoxicity risk assessment of the postulated metabolites hydroquinone and phenol. The referral does not include carcinogenicity data or the evaluation of mode of action for tumours in rodents observed in long-term carcinogenicity bioassays with Aclonifen'.

2. Aclonifen (2-chloro-6-nitro-3-phenoxyaniline) (figure 1.) is a selective systemic herbicide used for pre-emergence control of grass and broad leaved weeds in a range of crops.



+ = position of uniformly radiolabelled phenoxyaniline ring

Figure 1. Aclonifen

3. On 31 January 2008, at the request of the ACP Chair, an approach was made by PSD to the COM Chair for advice as to whether DNA adducts could be detected and measured in the existing stored tissues from animals dosed with Aclonifen. The COM Chair advised that it would not be advisable to undertake a retrospective analysis of stored tissues from Aclonifen treated animals for DNA adducts.

4. A teleconference was held between the data holder (Bayer CropScience), PSD and Health Protection Agency (HPA) (representing COM Secretariat) on the 13 June 2008. The comments raised by HPA during this teleconference outlined the particular need to address the metabolism of Aclonifen to hydroquinone and phenol and the assumption that these two metabolites were non-threshold *in vivo* mutagens.[1,2] Subsequent to the teleconference, the data holder submitted a revised position paper on 13 August 2008 on the relevance of phenol and hydroquinone formation following Aclonifen exposure which outlined their evaluation of the genotoxicity data on Aclonifen and metabolism of Aclonifen. ACP requested advice from COM on 30 July 2008.

5. The COM Secretariat held a teleconference with the data holder on 11 September 2008 to explain COM procedures, the referral for advice from ACP, data that COM would consider and to outline the procedures during committee with regard to a presentation from the data holder. Information on the possible areas of Aclonifen evaluation which COM Members might wish to raise questions was outlined, although it was noted that other aspects of Aclonifen might be raised.

6. The data holder submitted a presentation for the COM meeting on 13 October 2008 which was circulated to Members. In addition, on 21 October 2008 the data holder submitted a revision to the report dated 16 July 2004 on cleavage of the diphenyl ether bond in the Aclonifen molecule which had been circulated to Members.[3] The revised report was circulated to COM Members and replaced the aforementioned 2004 report.

7. The data holder attended the COM meeting of 23 October 2008 to make a short presentation and answer COM queries regarding the evaluation of the metabolism and mutagenicity of Aclonifen.

COM consideration of areas for discussion

8. The COM considered the submitted data, which included an extract from the detailed record of ACP consideration of Aclonifen at ACP meeting 329, extracts from draft EU assessment report on metabolism and genotoxicity of Aclonifen, which presented information on structure, use as a pesticide, ADME studies, toxicology, mutagenicity, carcinogenicity and reproduction, data from mutagenicity test reports on Aclonifen, copy of the report on the investigation of the potential for DNA-binding of Aclonifen and the revised position paper from the data holder on the cleavage of the diphenyl ether bond of Aclonifen.[3-13]

9. The Chair asked COM to consider the questions to ask the data holder and proposed Members should first consider the metabolism of Aclonifen followed by the mutagenicity data on Aclonifen. The discussion of mutagenicity data focussed on determining whether it was possible that the potential genotoxic effects of hydroquinone and phenol formed from Aclonifen could be assessed in these studies.

The areas for discussion related to:

10. Evaluation of data for the metabolism of Aclonifen and evidence for the systemic formation of hydroquinone and phenol from absorbed Aclonifen. It was noted that there were published papers in the peer reviewed scientific literature which provided examples of diphenyl ether breakage in a variety of species.[14-17]

11. Evaluation of the comparisons made by the data holder between mutagenicity data on hydroquinone and phenol with Aclonifen.

12. Evaluation of the mutagenicity testing strategy used by the data holder and specifically the reasons for undertaking an *in vitro* rather than an *in vivo* rat liver UDS study.

13. The COM noted the evaluation of carcinogenicity data was not included in the referral to COM, but agreed the data holder should be asked if there were data on tissue exposure from the carcinogenicity studies which might assist in evaluation of the mutagenicity data.

Data holder presentation

14. The data holder was asked to make a short presentation to the COM and to answer Members' queries.

15. The data holder presented an overview of Aclonifen rat metabolism studies, genotoxic potential of Aclonifen and their conclusions on the genotoxicity of Aclonifen.

16. Aclonifen had been 14C-labelled on the phenoxyaniline ring (B) but no radiolabelled studies had been undertaken with the phenyl ring (A) (figure 1.). The data holder noted that Aclonifen was rapidly absorbed via the oral route of administration and extensively metabolised with the majority of administered material (>90%) eliminated in the first 24 hrs via urine for both single dose and repeat dose studies (at 30 mg/kg bw). Approximately 40-48% of the absorbed dose was eliminated via the bile following an oral dose of 30 mg/kg bw. Tissue levels of radioactivity were very low. Aclonifen was metabolised by hydroxylation, methylation, reduction of the nitro group, N-acetylation, cleavage of the diphenyl ether bond and phase II conjugations. Potential diphenyl ether breakage had been inferred from the formation of glucuronide and sulphate metabolites from ring B. The data holder noted there were uncertainties in determining the total potential diphenyl ether bond breakage but overall this was estimated to be 9.2% in males and 7.3% in females. The data holder noted there was no evidence for cleavage metabolites in the repeat dose metabolism study and proposed that it was necessary for Aclonifen to be hydroxylated, and glucuronidated and sulphated before diphenyl ether breakage to form the conjugated forms of hydroquinone and phenol. This would provide an explanation for the negative findings in genotoxicity tests with Aclonifen.

17. With regard to the available mutagenicity studies on Aclonifen, negative results had been obtained in Ames tests, an *in vitro* chromosome aberration study in human lymphocytes, an *in vitro* gene mutation study in V79 cells (HPRT locus), and an *in vitro* rat liver UDS assay. Negative results had also been obtained in a mouse micronucleus test using the oral route of administration and no evidence for DNA binding in liver and urinary bladder had been reported in mice dosed orally with ^{14}C-labelled Aclonifen (labelled in ring B). The data holder considered the higher concentrations used and evidence for reduced toxicity in the presence of exogenous metabolic activation in *in vitro* mutagenicity studies in mammalian cells suggested that Aclonifen was being metabolised. The data holder noted that hydroquinone and phenol had given positive results in comparable studies for clastogenicity and gene mutation in V79 cells. In particular, Aclonifen was negative in an *in vitro* rat liver UDS study where metabolism would have been expected. In addition, phenol and hydroquinone were positive in *in vitro* UDS tests in Syrian Hamster Embryo (SHE) cells at dose levels almost 100-fold lower than tested with Aclonifen. The data holder noted the negative *in vivo* oral mouse bone marrow micronucleus test (high dose level 7260 mg/kg bw) with Aclonifen and compared this with evidence for positive results in studies with hydroquinone (80 mg/kg bw) and phenol (265 mg/kg bw). The data holder concluded that Aclonifen was not genotoxic and that, if hydroquinone and phenol

were formed during the metabolism of Aclonifen, then the results of the oral micronucleus test in mice should have been positive. The data holder drew the attention of COM to the detailed supporting slides in the presentation.

COM questions for data holder

18. A summary of the response given by the data holder on the areas for discussion is given below.

19. The data holder commented there were no specific data available on the formation of hydroquinone and phenol from Aclonifen. The data holder had considered the aspect of there being no evidence for diphenyl ether breakage of Aclonifen in the repeat dose study and suggested the breakage metabolites in the single dose studies being artefacts of mass spectrometry in these studies as one possibility for this observation. It was unlikely that there were individual animal data for the diphenyl ether breakage metabolism of Aclonifen as samples had been pooled prior to analysis and thus no assessment of the potential extent of inter-animal variation in metabolism could be made. With regard to the potential metabolism of Aclonifen to hydroquinone and phenol in exogenous metabolic fractions used in mutagenicity tests, the data holder considered the higher doses used and evidence for reduced toxicity in the presence of S-9 (compared to tests in the absence of S-9) in *in vitro* mutagenicity studies in mammalian cells with Aclonifen provided some reassurance that exogenous metabolism had occurred although there were no specific data on metabolites formed. Members considered that alternatively it was possible that protein binding occurred in the presence of exogenous metabolising fractions reducing the dose available to cells.

20. The data holder commented that the comparisons of mutagenicity data on Aclonifen and that available on hydroquinone and phenol were based on the best available data and acknowledged that there were uncertainties, for example comparing different cell lines, and historic data from different laboratories. The COM considered there were likely to be quite substantial differences in metabolic competency between SHE cells (used for tests with hydroquinone and phenol) and primary rat liver cells (used for the test with Aclonifen). In addition, differences in solubility of the test materials in vehicles used would also affect any comparison of the mutagenicity data. With regards to mutagenicity testing strategy, the data holder noted the rationale used for undertaking an *in vitro* rather than an *in vivo* rat liver UDS study was based on decisions on testing strategy reached at the time of testing rather than the specific question of *in vivo* metabolism of Aclonifen to hydroquinone and phenol.

21. The data holder considered there were no relevant data from the carcinogenicity studies with Aclonifen on tissue concentrations in carcinogen target tissues (brain female rat), urinary bladder (mouse) which might assist in the understanding of potential genotoxicity of Aclonifen.

22. The data holder considered the data on polyploidy in the chromosome aberration study with Aclonifen to be within historical control levels for the laboratory.

23. The data holder withdrew from the meeting so that the COM could derive its conclusions.

COM discussions

24. The COM noted peer-reviewed scientific literature which provided examples of diphenyl ether breakage in rats, mice and one bacterial strain (*Sphingomonas wittichii*) and considered it was therefore feasible that metabolism of systemic Aclonifen could result in the formation of free (unconjugated) hydroquinone and phenol, although there were no specific data on this aspect. The COM considered if exogenous metabolic activation systems such as Arochlor-1254 could metabolise Aclonifen to hydroquinone and phenol and agreed there were no specific data available.

25. The COM discussed the revised metabolism pathway for Aclonifen submitted by the data holder and agreed the proposal was feasible but not supported by appropriate data. Members were informed by the data holder that formation of phenol and hydroquinone prior to conjugation was equally unsupported as a second hypothesis in terms of available data. Members noted the proposal from the data holder that, if hydroquinone and phenol were formed from Aclonifen, then some positive results should have been recorded in the mutagenicity studies on Aclonifen.

26. The COM considered that the comparisons made between mutagenicity of Aclonifen and hydroquinone and phenol were useful but had reservations regarding whether definite conclusions could be reached. Thus it was possible that, when Aclonifen was orally administered to mice, hydroquinone and phenol were formed but failed to induce a detectable increase in micronucleus frequency in the polychromatic erythrocytes of the bone marrow.

COM conclusions

27. The COM agreed that further data on Aclonifen metabolism was required. This could involve more *in vivo* tests with specific analysis for the formation of hydroquinone and phenol. Alternatively, it might be possible to undertake comparative *in vitro* studies using rodent and human tissues (with specific measurement of hydroquinone and phenol formation). It was considered this could provide evidence that exposure to Aclonifen was unlikely to be associated with significantly increased genotoxic risk, although this would not preclude the possible need for additional mutagenicity tests dependent on the outcome of the metabolism studies.

28. The COM noted the approach to risk assessment had not been considered during the presentation, but that the data holder had included a proposed Margin of Exposure approach in the submission dated 13 August 2008. This would need to be considered further when appropriate metabolism data were available.

November 2008

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Ms S Kennedy	Administrative Secretary – Health Protection Agency

Declaration of COM members' interests during the period of this report

MEMBER	Personal Interest		Non Personal Interest	
	COMPANY	INTEREST	COMPANY	INTEREST
Professor P B Farmer (Chair)	Banco Santander Bradford & Bingley Foreign & Colonial Friends Provident Health Effects Institute Torotrak ILSI HESI	Shareholder Shareholder Shareholder Shareholder Research Committee Member Shareholder Committee Member	American Chemistry Council CEFIC	Research support and conference attendance expenses. Research Support
Dr C Allen	NONE	NONE	NONE	NONE
Dr B Burlinson	Huntingdon Life Sciences	Salary Employee Share Option Holder	NONE	NONE
Dr G Clare	Covance Allied Domecq AstraZeneca Diageo HBOS Marks & Spencer	Salary Shareholder Shareholder Shareholder Shareholder Shareholder	NONE	NONE
Dr J Clements	Covance	Salary Share Option Shareholder	NONE	NONE
Dr B M Elliott	Syngenta AstraZeneca	Salary Share Option Holder Shareholder	NONE	NONE
Dr D Gatehouse	Covance Friends Provident GlaxoSmithKline	Salary Consultant Shareholder Pension Share Option Holder	NONE	NONE

MEMBER	Personal Interest		Non Personal Interest	
	COMPANY	INTEREST	COMPANY	INTEREST
Mrs R Glazebrook	BT Group Lloyds TSB National Grid	Shareholder Shareholder Shareholder	NONE	NONE
Professor N J Gooderham	Banco Santander CENES Silence Therapeutics Hargreaves Lansdown Proctor & Gamble	Shareholder Shareholder Shareholder Shareholder Consultant	FSA GlaxoSmithKline FEMA (USA)	Research contract CASE studentship Research support
Dr D P Lovell	National Grid Transco Pfizer	Shareholder Shareholder Share Options Pension	AstraZeneca National Grid Transco	Spouse Shareholder Spouse Shareholder
Dr I Mitchell	Kelvin Associates IM Enterprises Chilfrome Enterprises GlaxoSmithKline Allergy Therapeutics BG Cadbury Schweppes GEC GSK ICH Mitchell & Butler Pfizer Real Good Food Renishaw Royal Dutch Shell RTZ Unilever Vedanta BP Centrica Green King Scottish & Southern	Associate Consultant Director/Creditor Director Pensioner Option and Shareholder Consultant Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder PEP Holder PEP Holder PEP Holder PEP Holder	NONE	NONE

MEMBER	Personal Interest		Non Personal Interest	
	COMPANY	INTEREST	COMPANY	INTEREST
Dr E M Parry	Invesco Fleming Legal & General Quintiles	PEP Holder PEP Holder PEP Holder Consultancy	NONE	NONE
Professor D H Phillips	Aviva Banco Santander BG Group Bradford & Bingley Centrica National Grid ECETOC Servier Butler Jeffries (solicitors)	Shareholder Shareholder Shareholder Shareholder Shareholder Shareholder Honorarium Honorarium Honorarium	NONE	NONE