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MUT/06/19

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

HORIZON SCANNING PAPER 2006

Introduction

1. Members will wish to consider horizon scanning topics identified by the secretariat. A literature search was undertaken using PUBMED which indicated several thousand publications in 2005/6 which might be potentially relevant. Using the terms “potent mutagen”, “mutagenicity”, and “mutagenicity testing” approximately 2000 references were identified. In addition the contents lists of Environmental and Molecular Mutagenesis and Mutagenesis were scanned. The literature search was briefly scanned to highlight potential chemicals, exposures and generic areas of mutagenicity evaluation which might be of interest to members. A brief overview has been produced below. It is acknowledged that a more extensive literature search and selection of papers could have been undertaken but the objective is to provide areas of interest for discussion rather than a complete literature evaluation. The horizon scanning exercise provides an opportunity for members and advisers from Government Departments/Regulatory agencies to discuss and suggest topics for further work. The paper has been subdivided to assist members discussion, although it is recognised for example that the mutagenicity evaluation of exposure scenarios (e.g drinking water constituents) and approaches to the evaluation of mixtures are closely related subjects.

Specific chemicals/exposures

Mutagenicity of water/soil borne contaminants

2. Nisihikawa A and colleagues administered MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2-(5H)-furanone) in the drinking water to 7 week old *gpt* delta C57BL/6J transgenic mice at 0, 10, 30 or 100 ppm for 12 weeks. MX is a potent in-vitro mutagen and has been shown to be carcinogenic in rodents. There was no evidence of mutagenicity or cell proliferation in the liver or lungs. [There was no evidence of carcinogenicity in a concurrent study where 100 ppm was administered to small groups of 10 animals for 78 weeks. In contrast previous studies had shown administration of doses below 100 ppm resulted in less mortality and a high incidence of lung and liver tumours in mice.] There was no concurrent positive control in the mutagenicity study.

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Dose-dependent effects on gap junction intercellular communication in WB epithelial cells was noted. Thus MX was reported not to have *in-vivo* mutagenic effects in cancer target organs in the mouse. (Environmental and Molecular Mutagenesis, 47, 48-55, 2006)

3. The structure of a new chlorinated disinfection byproduct (DBP) was reported following laboratory based experiments of chlorination undertaken in China. (Gong H et al Environmental Sci Technol, 39, 7499-7508, 2005.) 2,2,4-trichloro-5-methoxycyclopent-4-ene-1,3-dione (TCMCD) was identified as showing very similar retention time in one laboratory to methylated MX in a GC/MS method for evaluation of DBPs from chlorinated drinking water. Dose-related mutagenic activity was identified in *Salmonella typhimurium* TA 100 in the absence of exogenous metabolic activation at between 25-100 ug/plate (approximately 3500 revertants/plate). Some preliminary re-evaluation of analyses of chlorinated drinking water indicated the presence of TCMCD in some samples of chlorinated drinking water.
4. Watanabe T and colleagues reviewed the occurrence of mutagenicity in soil and water samples (most data reported derived from studies of samples obtained in Japan). They noted the relatively recent demonstration of *in-vitro* mutagens such as nitroPAHs, phenylbenzotriazole-type (PBTA) mutagens and compounds based on dichlorobiphenyls (e.g 4-amino-3,3'-dichloro5,4'-dinitrobiphenyl). These three groups were reported to account for approximately 50% of the *in-vitro* mutagenicity of surface soils and river waters. It was noted that the identification of other mutagens in soils/waters remain unknown. The authors concluded that identification of mutagens in spoils and water sources was an important step in understanding potential risks to humans. (Environmental Sciences, 12, 325-346, 2005.)

Mutagenicity of airborne contaminants

5. The genotoxicity of water-soluble and solvent (dichloromethane) soluble fractions of two particle sizes (PM_{2.5} and PM₁₀ particulates from different regions of Mexico city were investigated using the alkaline single cell electrophoresis (Comet) assay in A549 human type II alveolar epithelial cells (which express CYP 1A1, 1B1, 2B6, 2C8-19, 2E1, 3A5 and 3A7) as modified by Rojas (J Chromatogr B Biomed Sci Appl, 722, 225-254, 1999). The chemical composition of extracts determined by ion and gas chromatography and atomic adsorption spectroscopy. These analyses showed that soluble transition metals and organic soluble (e.g PAHs, nitro-PAHs) were important determinants of genotoxicity. The authors report that the particulate composition rather than mass was more important in determining the

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extent of DNA damage and that (in contrast to other publications) the constituents of water soluble-PM extract were more likely to induce DNA damage than the organic compounds. (Gutierrez-Castillo ME et al, Environmental and Mol Mutagen, 47, 199-211, 2006).

6. The mutagenicity (assessed in *Salmonella typhimurium*) and genotoxicity (assessed in the Vitotox assay, similar to SOS chromotest) of acetone extracts of PM₁₀ (particles collected on quartz filters) and semi-volatile compounds (SVOCs collected on polyurethane cartridges) derived from air samples from rural, urban and industrial sites in Flanders Belgium were assessed. Concentrations of 16 PAHs were determined in air samples. A higher level of direct mutagenic activity (i.e in absence of exogenous metabolic activation) was reported PM₁₀ extracts. The addition of exogenous metabolic activation resulted in a doubling of the mutagenic activity of the SVOC extracts but with no additional changes in the PM₁₀ extracts. The highest total mutagenic activity (i.e PM₁₀ +SVOC) was reported fro extracts derived from industrial sites, and at a rural site contaminated by pollution. A significant mutagenic activity derived from PM₁₀ was reported fro samples obtained near to the North Sea. Only a few percent (ca 3%) of the indirect mutagenic activity could be explained by the presence of selected 10 PAHs. A correlation analysis reported a significant correlation between observed and predicted total mutagenicity based on PAH concentrations particularly for indirect mutagenicity ($Q^2= 0.827$, cf $Q^2= 0.689$ for direct mutagenic activity). The authors report that there was a significant correlation between indirect mutagenic activity and B(a)P concentration ($r=0.69$ $P < 0.005$) but less than reported by other investigators. The authors concluded that biomonitoring is an important element in determining the impact of air pollutants. Du Four VA et al Mut Res, 588, 106-117, 2005).
7. The effects of residence in Los Angeles (LA a high ozone area) during the summer compared to San Francisco Bay (SF a lower ozone area) were compared in students (first year undergraduates recruited to the study between 2000-2002, total 249, of which 1216 provided buccal epithelial samples in Spring and Fall). A significant increase in MN (i.e. Fall compared to Spring results) in LA subjects (39% $P=0.001$) was reported whereas the increase in SF subjects (12.7%) was not statistically significant ($P=0.48$). In a separate acute exposure study (4h to 200 ppb ozone), an increase in mean MN and nucleoplasmic bridges was reported in peripheral blood lymphocytes and an increase in MN in buccal cells was reported in 15 subjects. The authors considered there was suggestive evidence for a cytogenetic effect of ozone in the longitudinal study and that the exposure study reported

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the first evidence of an effect of ozone on buccal cell MN formation. (Chen C et al Mutagenesis, 21, 131-137, 2006.)

8. 3-Nitrobenzanthrone (3-NBA) is a highly potent *in-vitro* mutagen. 3-NBA induced DNA adducts in all eight tissues sampled in female F344 rats. Following a single oral dose (9 mg/kg bw in triocanoine) two peaks of DNA adduct formation were identified at 6h-3d and at 10 days post dose. (This time frame also correlated with the time profile for tissue damage (which included haemorrhage, loss of intestinal villi in small intestine, intestinal fragility and oedema of adipose tissue surrounding the g.i tract)). The highest levels of DNA adducts at 6h occurred in the glandular stomach (i.e site of contact). A single peak of DNA adduct formation was reported in a previous intratracheal study in rats. The authors considered that bioavailability was affected by storage in tissues and later becomes available resulting in increased DNA adduct levels at later time points of collection. The authors considered regenerative cell synthesis decreased DNA adduct levels at around 5 days post dose. (Nagy E et al Environ Mol Mutagen, 47, 541-552, 2006.)

{It is noted that there are a number of recent publications detailing information on the metabolic activation of 3-NBA to mutagenic metabolites. Pathways appear to include both oxidation and cytosolic nitroreduction and also there is evidence to suggest self induction of metabolism. Stiborova M et al Drug Metabolism Dispos, 34, 1398-405, 2006. Arlt VM Cancer Res, 65, 2644-2652, 2005. }

In-vitro mutagenicity of combustion products from 12 tobacco constituents

9. Chemical components* and reference tobacco blends were combusted and the mutagenicity tested *in-vitro* using *Salmonella typhimurium* TA98 and TA 100 and the *in-vitro* MN assay using V79 hamster lung fibroblasts. Cellulose fibre or powder were used as support for combustions and also served as control materials. Two methods of combustion were investigated. Tests were conducted with and without exogenous metabolic activation using Aroclor 1254 from rat liver using particulate matter derived from the combustions and cigarette smoke condensate (CSC). The investigators found significant concentration dependent increases in TA98 revertants from nitrogenous components (amino acids/proteins) in the presence of exogenous metabolic activation. In the absence of exogenous metabolic activation none of the tested TPM samples exhibited concentration dependent mutagenicity in TA98. Similar results were demonstrated in TA100 in the presence of exogenous metabolic activation for amino acids, although it was noted that a positive response was documented for

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lignin in the presence of S-9 and for proline in the absence of S-9. None of the TPM samples were mutagenic in the MN test in V79 cells in the presence of exogenous metabolic activation. In the absence of exogenous metabolic activation TPM samples derived from phenolic components of tobacco (i.e. chlorogenic acid and lignin) exhibited significant mutagenic activity using combustion method A. Similar results for MN analysis were reported when combustion method B was used. Solvent and positive (CSC) controls gave expected results. The authors report this to be the first study to characterise the toxicity of combustion products of major tobacco constituents. A number of interpretations of these data are possible including that different constituents contribute in different ways to smoke toxicity. The approach used was suggested as a possible approach to guide the selection/modification of tobacco and to identify chemicals responsible for activity.

*Combustion products from cellulose fibres, starch, glucose, pectin, praline, glycine, tyrosine, tryptophan, protein, chlorogenic acid, lignin, tobacco blends CK36, IR4F.

Some data retrieved on individual chemicals

4-Aminobiphenyl induces cancer target organ mutations in neonatal mice but not in adult mice.

10. The mechanisms underlying the susceptibility of neonatal mice to genotoxic carcinogens were investigated by analysing DNA adducts and mutations in the livers of neonatal and adult Big Blue transgenic mice following exposure to 4-aminobiphenyl. This chemical was chosen for study in view of the large amount of information known about carcinogenicity and mechanisms in neonatal and adult rodents and in humans. In a preliminary dose-response study for DNA adduct formation 4 groups of 4-7 neonatal male non transgenic B6C3F₁ mice were dosed with 0, 3, 9 or 31 mg/kg bw (i.p doses split 1/3 on PND8 and 2/3 on PND 15). Subsequent studies compared DNA adducts in neonatal and adult Big Blue mice using a dose level of 31 mg/kg (adults (aged 4 months) received the dose split into 2 equivalent doses separate by a week). Animals were sacrificed 24 h after the last dose and livers frozen (-80°C) for analysis for DNA adducts (³²P-postlabelling (using isolated nuclei by the method of Gupta Cancer Res, 45(11Pt2)5656-5662, 1985 including *n*-butanol enrichment). For mutagenicity evaluation (using *lacI* and *cII*) a dose response preliminary study was undertaken using groups of 5-6 neonatal male Big Blue B6C3F₁ mice treated with 0, 3, 9, 31 mg/kg bw and sacrificed 8 weeks after the last dose (Initial studies not reported suggested the optimum sampling time was 8 weeks after the last dose.) To compare

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neonatal and adult mutagenic response 5 female neonatal Big Blue mice and 5-6 male and female adult Big Blue mice were treated with 31 mg/kg bw and sacrificed 8 weeks after the last treatment. Livers were frozen at (-80°C) for mutation analysis which included sequencing of the *cII* gene from plaques selected at random from neonatal and adult mice treated with 4-ABP.

11. N-(Deoxyquanosin-8-yl)-4-ABP was the predominant adduct identified following all treatments. DNA adducts for adult females were significantly higher than for neonatal females ($44 \pm 4.8/10^6$ cf $25.9 \pm 2.2/10^6$) while DNA adduct levels in adult males were lower than in neonatal males ($13.5 \pm 2.0/10^6$ cf $33.8 \pm 4.1/10^6$). 4-ABP significantly increased the mutation frequency in the *cII* gene in both neonatal male and female mice but not in adult mice. Sequence analysis revealed a unique spectrum of mutations in neonatal mice characterised by a high frequency of G:C→T:A transversion while the mutation spectrum in adults was similar to control mice. The authors concluded that DNA adduction appeared to be dependent on gender but conversion to mutations differed with animal age. (It was noted that the mutation frequency in the *cII* transgene was significantly higher in female neonatal mice compared to male neonatal mice ($p < 0.01$))

Mutagenicity evaluation of mixtures

12. The COM considered an approach to evaluate mixtures of benzimidazoles and other tubulin binding compounds at its May 2006 meeting. Members questioned the premise that dose-additivity would be the default approach in cases where there were no data to support this approach. An extract from the relevant section on mixtures from the 2005 horizon scanning paper is given below.

It is widely recognised that we are exposed to complex mixtures of chemicals environmentally, occupationally, therapeutically or via a combination of these. However toxicology testing strategies (including genotoxicity/mutagenicity) are largely based upon the evaluation of single chemicals. The risk assessment of chemical mixtures is characterised by models in which chemicals within a mixture are considered to fit either dose additive, response additive or interactive profiles (Jonker et al 2004). In general it is believed that genotoxic chemicals fit the dose additive model whereby they are assumed to behave similarly in terms of mode of action and combined responses can be calculated from dose responses of the constituents of the mixture. 'Bottom-up' investigative approaches, where knowledge of the toxicities of individual chemicals are modelled to give an overall assessment of risk, can be performed when the mixture is simple (few components, well defined) and/or when sufficient data is available (examples; a pesticide product). 'Top-down' approaches are used for complex mixtures (many components, ill defined) and involve testing the whole mixture and comparing results to those achieved with individual components. Examples of complex mixtures include petroleum hydrocarbons, contaminated land/waste sites and drinking water.

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For genotoxic carcinogens in the environment, the requirement is to maintain levels as low as is reasonably practicable (ALARP). With this in mind, there are two potential issues which may arise when considering the mutagenicity/genotoxicity of a mixture:

1) Is the assumption that all genotoxins will adhere to the dose additive model correct? (Expressed mathematically as : $E_{AB}(d_A, d_B) = E_A(d_A) + E_B(d_B)$: where A and B are two chemicals in the mixture and E and d represent the effects and dose of individual compounds respectively), or are there scenarios that interactions (either synergistic or antagonistic) will occur, when the presence of a first mutagen affects the potency of a second?

Said et al (1999) demonstrated the enhancement of the bacterial mutagenicity induced by N-acetoxy-acetylaminofluorene by pre-treatment with AFB1-8,9-epoxide and suggested that pre-existing adducts may distort DNA thus leading to potentiation of intercalating agent effects. It is also suggested that the ability of arsenic to modulate DNA repair *in vitro* enhances genotoxicity in CHO cells (Lee-Chen 1993, Wiencke and Yager 1992) and similar conclusions have been reached regarding the potential mechanism of cobalt induced genotoxicity (De Boeck et al 1998).

2) If a complex mixture is tested and found to be positive *in-vitro* or *in-vivo*, should the mixture simply be regarded as a mutagen or should efforts be made to tease out the active components. For example, in a study assessing the mutagenicity of settled house dust, it was demonstrated that only 25% of the mutagenicity could be accounted for by known PAHs (Maertens et al 2004). Furthermore, how should the variability of complex mixtures such as drinking water be accounted for when assessing the risk?

This concern is borne out by the analyses of available data presented in the WiGRAMP report on risk assessment of chemical mixtures. Weaknesses were revealed in the design of many studies which had attempted to assess the genotoxicity of mixtures, as it was not possible to draw conclusions on potential interactions (e.g. individual compounds not tested separately, inadequate dose responses).

Do the Committee think further assessment of the potential interactions between genotoxic chemicals is warranted?

Should specific genotoxicity testing strategies be developed to aid those involved in the risk assessment of chemical mixtures?

13. The COM evaluation of benzimidazoles was undertaken in the context of providing advice on approaches to risk assessment of combined exposures for a group of compounds that are regulated by a threshold approach. A key piece of COM advice was the need to establish whether compounds in the potential common mechanisms group acted by dose additivity (i.e. same mode of action) before considering which chemicals to include the common mechanism and attempting to rank potency.

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14. One recent study on potential interactions between mutagenic chemicals has been published. Lutz W et al (Toxicological Sciences, 86, 318-323, 2005) undertook mutagenicity studies for the production of micronuclei in LY5178Y cells using three binary mixtures of the methylating agents methylmethanesulphonate (MMS) and N-methyl-N-nitrosourea (MNU) and the topoisomerase inhibitor genestin. (2×10^5 cells using $\leq 1\%$ DMSO as a vehicle, with 4h incubation, 20 h expression time during which 5ug/ml cytochalasin B was added). The induction of MN was assessed for both response and dose additivity. The authors report the use of the concept of “envelope of additivity” to quantitatively define responses between response and dose additivity. Evidence of synergism or antagonism would occur for responses reported to be outside the “envelope of additivity.” For MMS and MNU the effect was compatible with dose addition. For MMS and GEN the response was above response addition but below dose addition. For MNU and GEN the response was below response addition indicative of antagonism. The observed difference between MNU and MMS when tested with GEN would not have been predicted on the basis of a simplistic interpretation of methylation.
15. The approach used by these authors does highlight the potential difficulties in interpreting studies where there has been no evaluation of dose and response additivity.
16. What further work should COM be undertaking with regard to mixtures. Initial literature searches suggest there is a relatively small number of studies using both top down (e.g. diesel exhaust Ostby L Arch Tox, 71, 314-319, 1997, Oh SM Tox Letts, 161, 226-235, 2006) and bottom up approaches (e.g chlorination byproducts MakiPaakkanen J in Environ Mol Mutagen, 43, 217-225, 2004) which could be reviewed. (Data have not been screened for adequacy at the present time.) Any generic guidance which could be developed might be of value in a number of areas such as;
 - i) developing advice on possible strategies for assessing the mutagenicity of chemical mixtures in order to help define potential marker compounds or mixture specific approaches to regulation.
 - ii) developing advice on possible strategies to assess the adequacy of regulatory standards for mixtures which are based on single marker compound. (It is noted that COC/COM have agreed that mutagenic potency and DNA binding at the site of contact can act as a surrogate for site of contact carcinogenic potency for PAHs.)

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17. The COM raised the possibility of interactions between mutagenic chemicals not being additive during discussions on benzimidazoles. One possible approach might be to review the literature for evidence of clear synergistic interactions and to consider whether the available data suggests the need for generic guidance on possible approaches to testing or advice on mechanisms of interaction with respect to mutagenicity which might need to be considered more widely.

Mutation “fingerprints” in assessing cancer aetiology.

18. The COM previously considered whether mutational fingerprints resulting from exposure to genotoxic carcinogens can be established from mutational hotspot analysis of tumours a number of years ago (e.g in 1999 when some preliminary data relating to exposure to ozone was considered and 2001 when some data on p53 mutational spectra in lung tumours of tobacco smokers was considered). The general advice provided has been that with the exception of a number of exposure scenarios such as tobacco smoking, mutational fingerprints for exposure to genotoxic carcinogens cannot be elucidated since mutations arising during carcinogenesis cannot be differentiated from those originating from exposure to genotoxic carcinogens.
19. Besaratinia A and Pfeifer G have published a review on this subject. In brief it is proposed that in-vitro studies of specific genes (e.g hprt) and use of rodent transgenic *in-vivo* mutation assays (e.g *lacI* and *cII* in Big Blue TM mice) are cited as potential approaches to the identification of mutational fingerprints which can be used to derive hypotheses for investigation in molecular epidemiology studies (e.g p53 and *ras*). Examples of specific PAHs present in tobacco smoke and aflatoxin B1 are cited with regard to in-vitro studies of mutational fingerprints using normal bronchial tissue and Hep2G cells respectively. The data from these studies are compatible with the data from molecular epidemiology studies of cancers from individuals exposed to these chemicals. In assessing data from such studies the cascade hypothesis suggested by Loeb LA (Proc Natl Acad Sci USA, 100, 776-781, 2003) is cited. Essentially Loeb argued that specific exposure-associated mutation spectrum recovered from a cancer related gene in a tumour is of relevance to cancer etiology only if i) the mutations occur early on in the carcinogenic process and ii) the gene in which mutations occur is essential and required for tumourigenesis and retained throughout the tumour selection process.
20. Do members agree with the approach suggested by Besaratinia and Pfeifer regarding approaches to the use of mutational fingerprints for

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carcinogen identification. Can the approach be used for any tumours given that appropriate hypotheses can be identified from available *in-vitro* and *in-vivo* approaches in cell lines and rodent studies respectively?

21. In a recently published investigation, different p53 mutation patterns were reported for colorectal tumours from smokers compared to non smokers. In brief, 153 tumour tissues samples (63 smokers, 90 non smokers) were available and were examined for p53 mutation and p53 protein expression by direct sequencing and immunohistochemistry. P53 mutations were identified in 77 of the 153 colorectal tumours. (22 smokers, and 31 non smokers). There were no statistically significant differences for transition or transversion mutations (it was noted that G:C→A:T transition was relatively more common in non smokers than smokers. A statistically significant increase in the number of smokers with deletion mutation was reported (7 compared to 1). Immunohistochemistry showed immunoreactivity correlated with p53 mutated tumours. The authors considered the higher occurrence of deletion mutation as evidence for a mutational pattern associated with smoking. Would members agree with this conclusion?

Approaches to mutagenicity testing

In-vitro testing

Mouse Lymphoma Assay

22. A further report on the development of the Mouse Lymphoma Assay (Thymidine Kinase gene mutation) updating the work of the International Workshop on Genotoxicity Testing (IWG) (Aberdeen 2003). Acceptance criteria for positive and negative controls were reached. Initially the WG had considered that a single reference chemical could be used in studies undertaken in participating laboratories. Two approaches were considered acceptable for positive control response. In the first the laboratory should use a dose of a chemical that yields an absolute increase in total MF (i.e. an IMF) that is an increase of at least 300×10^6 over the spontaneous background (at least 40% of the IMF should relate to small colonies). In the second approach a dose of the chemical should result in an IMF of small colonies of at least 150×10^6 above the background for small colonies. The upper limit of cytotoxicity should be the same in the positive control and also for experimental studies (RTG > 10%). A single dose of positive control is acceptable.

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23. With regard to data evaluation the WG agreed that there was no one statistical approach which could be recommended. With regard to small increases in MF, the group agreed that data sets where there was little variance between duplicate cultures or data points fitting tightly to a linear or quadratic curve were more likely to be judged positive (by statistical analysis) than experiments showing a wider variability. The WG reports that the variability in response is not adequately defined in the MLA until approximately 50 experiments are included in the analysis. Thus evaluation cant be based on variability alone. The WG agreed a biologically relevant approach to MLA data by requiring the IMF to exceed a global MF. The concept of a Global Evaluation Factor was derived. Using the multilaboratory data available the WG derived a GEF (in excess of background) of 90 for agar and 120 for the microwell methods. A test chemical is positive if both the IMF for any treatment exceeds the GEF and a positive trend test is obtained. It is noted that experiments where one of these criteria were satisfied should be evaluated on a case-by-case basis and it is advisable to retest (particularly in the 10-30% TRG cytotoxicity range). Compounds that didn't reach the GEF levels but reproducibly gave a positive trend might be weakly mutagenic in the assay. Further chemical characterisation or changes to exogenous metabolic activation should be considered. (Moore M et al Environ Mol Mutagen, 47, 1-5, 2006.)

In-vitro MN assay using flow cytometry (FCM)

24. A FCM method is reported following *in-vitro* exposure of L5178Y cells to a range of genotoxicants and non genotoxicants where discrimination between apoptotic/necrotic cells and true MN formation was reported. A sequential staining methods was used to label apoptotic/necrotic cells and MN. Ethidium monoazide (EMA) was added to cultures following heat shock to monitor the development of apoptotic cells. A linear response with time was reported. EMA bound to chromatin from dead and/or dying cells following photoactivation. The cell membrane was then digested with RNase plus a pan nucleic acid dye (SYTOX Green). FCM was performed using 20,000 cells per sample. Concurrent microscopy (1000 cells x2 cultures) was also undertaken. Additional investigations using a range of genotoxicants (hydroxyurea, MMS, B(a)P, etoposide, cyclophosphamide and vinblastine were undertaken (using 4 h treatment of cells plus 20 h exposure (S-9 used for B(a)P and cyclophosphamide). Non genotoxicants used were sucrose, tributyltin methoxide and dexamethasone. Concentrations used were up to 5 mg/ml or level inducing cytotoxicity. The FCM data corresponded to the microscopy data. The authors noted that it was possible to dissociate tightly

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bound metaphase chromosomes if the specimens were handled too vigorously and these could result in apparent MN formation. The authors reported positive results for all genotoxicants studied and also that an enhanced response was detected with vinblastine if a 24 h exposure was used. Negative results were reported for all the non genotoxicants including under conditions where considerable necrosis was reported with tributyltin methoxide and dexamethasone. The authors suggest that high throughput in-vitro MN assay was possible using the sequential staining approach. (Avlasevich S et al Environmental Mol Mutagen, 47, 56-66, 2006.)

Possible use of Expanded Simple Tandem Repeats (ESTRs) for detection of germ line mutagens

25. ESTRs were reported to be useful biomarkers of response to germ line mutagens. The authors quoted references to published data on ionising radiation and a number of in-vivo mutagens (e.g ENU, isopropyl methanesulfonate, etoposide). ESTRs are long (>1kbp) non-coding tandemly repeat arrays of short sequence elements (4-10 bp). It is noted that ESTRs are distributed throughout the whole genome (in mice). The response of these loci to mutagens includes loss or gain of whole repeat units generating new mutant length alleles. In the experimental study, C3H/10T1/2 cells were grown in the presence of low concentrations (3nM etoposide, 0.64 mM ENU, 0.5 nM okadaic acid or 1 uM B(a)P) in acetone (0.045 v/v in culture medium) for a period of 6 days and grown for 4 weeks. DNA was isolated and approximately 4.8kbp for the *Ms6-hm* locus was amplified by PCR and then subject to gel electrophoresis for identification of alleles. Mutant alleles were identified by mass changes compared to reference alleles. A positive response was documented for etoposide, ENU and B(a)P but not okadaic acid. (Polyzos A et al Mutation Res, 598, 73-84, 2006.)

In-vivo testing

Skin MN assay

26. Results from skin micronucleus tests using 10 PAHs in hairless mice were reported. In brief, the chemicals were applied once daily for three days (at dose levels up to that producing mild/moderate irritation after the last dose or at the maximum soluble level (acetone olive oil 4:1)). Following treatment to the dorsal skin (3cm x 4cm), the treated area was excised and pressed between layers of filter paper, cut into 0.5 cm pieces rinsed with PBS, 0.02% EDTA/PBS, and treated overnight with

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0.25% trypsin in PBS at 4°C. The dermis was separated and the basal cell fraction isolated by agitation, filtration through a nylon mesh and centrifugation. The cell pellet was suspended in CS-MEM treated with hypotonic KCl (15 min 37°C) and immersed in methanol/glacial acetic acid 3:1. The cells were washed, air dried on glass slides and stained with acridine orange. A total of 2000 basal cells/animal were scored by fluorescence microscopy on coded slides. MMC was used as a positive control. The solvent control frequency 0.8‰ (8/1000) was similar to other studies using this method.

27. The authors considered that DMBA, 3-MC, B(a)P and DB(ah)A produced positive results which demonstrated a similar order of potency in the skin MN assay compared to skin carcinogenicity studies in mice. A positive but less potent response was also reported for DB(ac)A (considered to be an initiating agent but not a complete carcinogen). The non carcinogen (in rodents) B(e)P, Pyr, and ANT were negative in this assay. The authors concluded that the skin MN assay is predictive of *in-vivo* site of contact mutagenicity. The data are also potentially relevant to the discussions COM/COC have previously had with regard to PAH carcinogenic potency. However it is noted that the authors appear to have used a relatively simple qualitative approach to evaluating mutagenic potency and its not possible to evaluate the conclusions reached. The authors did not use or propose any published method for determining *in-vivo* mutagenic potency (e.g NOEL for mutagenicity based on dose applied/day or total dose or an ED10 or 25 for mutagenicity. (Nishikawa T et al Mut Res, 588, 58-63, 2005)

Statistical analysis including the positive control

28. Hauschke D et al reported on a proposed method for evaluating *in-vivo* dose response effects for mutagenicity by including the fractional increase in mutagenicity induced by the positive control (compared to background) as a factor in determining the minimal biologically effective increase. (Biometrical Journal 47, 1, 82-87, 2005.)

***In-silico* approaches**

29. A tiered approach to mutagenicity evaluation was proposed following analysis of mutagenicity data (*Salmonella typhimurium* data), structure (presence or absence of epoxide) and prediction of *Salmonella* response using three *in-silico* approaches either singly or combined. The approaches used were selected because of their different modes of analysis. DEREK is a rule based system, MCase is a database/substructure based system, and AWorks is a discriminant

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system based mainly on physicochemical descriptors. Databases used for the analyses were the CGX data base (www.lhasalimited.org/cgx) (703 chemicals assessed for Salmonella mutagenicity) and the Japanese ECJ database (206 chemicals assessed for mutagenicity). A separate analysis was undertaken for influence of Mw on mutagenicity. Using a criteria of two *in-silico* approaches giving concordant results then for the CGX database a sensitivity of 87.8% (positive correct) and a specificity of 85.6% (negative correct) and a concordance of 86.7% (proportion of positive and negative correct) and applicability (86.7%). For the EJC database the sensitivity, specificity, concordance and applicability were 73.1%, 86.5%, 93.9% and 95.1% respectively. There were improvements in sensitivity, specificity, and concordance of Salmonella prediction if the requirement was concordance from all three *in-silico* approaches but as consequent reduction in applicability (proportion of chemicals for which relevant data were available).

30. The tiered approach included exclusion if Mw was above 3000 and inclusion as positive mutagen when an epoxide was present as the first two steps. The third step included the *in-silico* assessment using positive predictions from two out of the three *in-silico* approaches outlined above. The authors note the need for mutagenicity data on a large number of existing chemicals and propose that the approach presented could eventually replace mutagenicity (presumably *Salmonella typhimurium*) testing as part of the approach to evaluating mutagenicity. The authors note the approach could be adapted depending on use (e.g inclusion of all three *in-silico* approaches for some categories of chemicals where appropriate data are available.) Hayashi M et al Mut Res, 588, 129-135, 2005
31. It is noted that a comparative review of MCASE, DEREK and TOPKAT (which has similarities to AWorks) reported that if non structurally alerting in-vitro mutagens (identified from the Physicians Desk Reference) were evaluated using these approaches then the sensitivity (ie. Identification of Ames positives) fell to between 18-27%) whilst specificity and concordance remained above 74%. This later assessment is based on a highly selective small test set excluding a range of compounds such as nucleoside analogues, flouroquinolones but provides some information to suggest that the strategy suggested by Hayashi et al would work well for structurally alerting compounds but could potentially miss might miss non structural alerting Ames positive compounds. (Snyder RD and Smith MD Drug Disc Today, 10, 1119-1124, 2005.

COM discussion: Possible areas for further work

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32. A number of topics raised during the 2005 horizon scanning exercise have been taken forward as discussion papers for the COM. This included a review of the utility of the rat liver UDS assay compared to rat liver data derived using the Comet assay (draft working paper to be considered at the October 2006 meeting, a review of transgenerational mutagenicity involving changes in DNA methylation (under discussion at the October 2006 meeting), and a review of the background variance in genotoxicity indices (chromosome aberrations and micronuclei) in peripheral blood lymphocytes (draft working paper to be considered at the October 2006 meeting). Other areas raised in the 2005 paper which have not currently be taken forward included the mutagenicity evaluation of mixtures and potency indicators for mutagens. Development of COM thinking on this latter subject could be highly valuable with regard to the Margin of Exposure approaches which are to be discussed by the COC in November 2006.
33. A number of papers identified for the 2006 Horizon scanning paper further develop the evaluation of mixtures both with respect to genotoxicity of components of water and air pollution and tobacco smoke and also with regard to the possible approaches to evaluation of the mutagenicity of mixtures. What further review work would the COM suggest? Should any work be restricted to specific exposure scenarios?
34. The COM completed its review of the background variance regarding CA and MN formation in PBLs, which was a follow up work recommended as apart of the COM review on pesticide applicators. Some preliminary discussions with colleagues at the Health Protection Agency (HPA) have identified that a possible review of air pollution biomonitoring studies using the criteria developed by the COM could aid in identifying key studies that are considered adequate. It is estimated that the amount of published literature for such a review would be approximately the same order or possibly less than the review of pesticide applicators (from Bonassi S et al. Environ Mol Mutagen, 45, 258-270, 2005). It would be valuable if the available studies were screened and information on exposures from adequately conducted studies were available. This could potentially depending on availability of information, compliment any work undertaken on mixture assessment.
35. The generic areas identified in this current paper where further work could potentially be undertaken include the evaluation of mutational fingerprints as an approach to molecular epidemiology studies and the

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developments in approaches to mutagenicity testing which might be of value when the COM reviews its guidance.

36. A paper on the recent publication by Jenkins et al (Mutagenesis, 20, (6), 389-398, 2005) concerning the development of biologically relevant thresholds for methylating agents has been drafted for the October 2006 meeting. The new data does not appear to alter the generic approach used by COM for risk assessment of in-vivo mutagens and genotoxic carcinogens. However members may wish to consider further work in this area.

Potential Areas for further work (Draft suggested list, not prioritised)

37. COM members will wish to note the following suggested areas for further work and to add or delete from this suggested list as appropriate.
38. Evaluation of chemical mixtures for mutagenicity including specific evaluation of exposure scenarios and evaluation of approaches to risk assessment.
39. Biomonitoring review of air pollution.
40. Evaluation of generic areas of testing relevant to future consideration of testing strategy.
41. Use of mutational fingerprints in molecular epidemiology.
42. Further consideration of approach to risk assessment of in-vivo mutagens
43. Possible consideration of potency ranking and approaches to margin of exposure.

**Secretariat
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