Committee on MUTAGENICITY

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

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STATEMENT ON THE USE OF MUTATION SPECTRA IN GENETIC TOXICOLOGY

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INTRODUCTION

1. The term 'mutation spectra' refers to the composite of the number, types and sites of all mutations observed in a given gene sequence. It is also used more broadly to refer to the number and types of mutation found, or even the main type of mutation observed (for example GC→AT transitions). It is recognised that some genotoxic carcinogens generate unique mutation spectra *in vitro* and in experimental systems *in vivo* and these can contribute to determining carcinogenic mode of action of a chemical and mechanisms of tumourigenesis (Besaratinia and Pfeifer 2006). Some of these distinctive spectra have been identified in specific tumour related genes and are considered to be diagnostic mutations. For example the COM previously advised on the significance of the high frequency of mutations in codon 61 of the *K-ras* gene in lung tumours from ozone-exposed mice. These mutations, which included AT →TA transversions, were considered to be induced specifically by ozone due to the absence of these mutations in spontaneous lung neoplasms. http://www.iacom.org.uk/statements/COM99S3.htm.

2. The COM retains a watching brief on this topic, with a particular interest in the experimental models and approaches available. Their usefulness in understanding cancer aetiology and in molecular epidemiology is recognised, for example in the measurement of mutation "hotspots" in the carcinogenic process or in interpreting specific chemical-induced spectra. This statement provides an overview of the current state-of-play of the use of mutation spectra in evaluating chemical carcinogenesis and the Committee's position and opinions on this topic.

THE CURRENT POSITION

3. A paper was presented to the COM in March 2014 which summarised a cross-section of the studies available in the recent published literature, which evaluated different test systems, chemicals and gene endpoints (MUT/2014/02). These included studies conducted in *in vitro* systems (bacterial, human, rodent and transgenic cell lines) and *in vivo* systems, primarily transgenic models (i.e. Muta™mouse, Big Blue®) and the HUPKI (human p53 knock-in) mouse model. Members were asked to consider the papers presented, to comment on the usefulness of the test systems, the validity of the approaches used to assess

individual chemicals and to provide further evaluations and opinions of the topic if appropriate.

4. Members considered the paper to be a good representation of the types of studies in the recent literature. The Committee were of the opinion that the selectable genes in the Ames test Salmonella strains and genes such as the *hprt, gpt and tk* loci in mammalian cells, whilst good systems for identifying chemically induced mutations, are not suitable for use in the identification of mutation spectra. This is because mutations in such diagnostic genes in bacteria or cells are identified following selection through growth advantage and hence may not be representative of the overall pattern of mutation. Thus, mutation spectra are most appropriately identified in phenotypically neutral genes. The *lacI or lacz* genes in transgenic rodents used for mutagenicity testing are considered to be examples of such neutral genes, mutations which are identified without *in vivo* selection. The *cII* gene is another good example of such a gene, which is used for mutational analyses principally because it is short and readily isolated and sequenced. However there are also examples of chemically-induced mutation spectra which are conserved across species *in vivo* and in *in vitro* models using selectable and non-selectable genes for example, those induced by2-amino-1-methyl-6- phenylimidazo[4,5-b]pyridine (PhIP)(Yadollahi-Farsani et al, 1996; Lynch et al 1998). The Mammalian Gene Mutation Database (MGMD) is an internet accessible database of published mutation data providing a comprehensive, standardized information resource (Lewis et al 2000). http://www.swansea.ac.uk/cget/database/web/mgmd2.htm

5. Members agreed that in molecular epidemiology, analysis of mutations within the tumour suppressor p53 (product of the *TP53* gene) was of greatest value in evaluating chemically-induced mutations as it has been shown that mutation patterns in this gene are maintained across different species and test systems. A substantial database of *TP53* mutations in tumours has been compiled by the International Agency for Research on Cancer (IARC) and this provides significant insight into the role of this gene in the aetiology of cancers in different organs. This database has the potential to contribute to interpreting the impact of specific chemicals in the carcinogenic process (Olivier et al 2004). Analyses of *TP53* mutation patterns from over 16,000 somatic mutations within tumours indicate that 75% are missense mutations of a single amino acid substitution and these are distributed in all coding exons. Thirty percent (30%) of these are within five 'hotspot' codons (codons 175, 245, 248, 273, 282) which contain CpG sequences. These sequences are highly methylated in the human genome and it is believed that spontaneous deamination leads to the enhanced mutation frequency in these areas (Denissenko et al 1997). In cancers other than lung, CG→TA transitions within CpG sites comprise 92% of mutations. Codons 248 and 273 are observed to be most frequently mutated in the whole *TP53* database (Olivier et al 2004, Pfeifer et al 2002). It is considered that these mutations result in a decrease in transcription function of p53 which reduces activation of genes such as *p21* and *BAX*. Mutations are most often identified on the non-transcribed strand of DNA and this is attributed to the slower repair of bulky DNA adducts on this strand (Denissenko et al 1998).

Chemically-induced mutations

6. Members agreed that there is value in using mutation spectra data to aid in the identification of specific chemical induced DNA lesions. However, this

information requires careful consideration; in particular, an understanding of how the kinetics of the compound, and its tissue distribution will impact on the mutation spectra over time. Caveats for how the chemical will be activated or deactivated or how the lesion may be repaired in different tissues [or in different sites/genes] needs to be considered when interpreting /evaluating the resultant spectra. Members concluded that currently there are only a few examples of specific mutation spectra which have been identified experimentally and positively correlated with a chemical exposure and subsequent induction of tumours in humans. These are tobacco smoke, aflatoxin B1 (AFB1) and aristolochic acid (AA).

7. **Tobacco smoke** contains a wide variety of known mutagenic carcinogens including polyaromatic hydrocarbons [PAH], for example benzo(a)pyrene (BaP), tobacco specific nitrosamines such as 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK), aromatic amines, such as 2-napththylamine, and carcinogenic metals, such as chromium (Pfeifer et al 2002). Whilst there is debate surrounding which chemicals are responsible for the generation of spectra, a distinct *TP53* mutation spectrum is observed in lung tumours from smokers compared to those in non-smokers (Pfeifer et al 2002, Olivier et al 2004). It is estimated that about 30% of mutations in *TP53* from smokers are GC→TA transversions, a mutation seen in only 12% of tumours in non-smokers and 13 % in other types of cancers. There is an apparent hotspot for mutations in codon 157 of *TP53,* specific for lung cancers of smokers and this is replicated *in vitro* (Dennissenko et al 1996; Hussain et al 2001). Lung tumours from smokers, like almost all other types of human tumour, exhibit a marked predisposition towards mutations in codons 248 and 273, but uniquely in lung tumours from smokers, there comprise a large percentage of GC→TA transversions (35-45%). Taken together these data indicate that there is evidence for a distinctive mutation spectrum but it is not highly discriminating.

8. In other cancers associated with cigarette smoking, the predominance of GC→TA transversions in *TP53* is less pronounced. In larynx, oesophageal, oral and bladder cancers GC→TA transversions are present in 27%, 16%, 15% and 7% of tumours from smokers respectively (Pfeifer et al 2002). In colorectal tumours the mutational patterns did not differ significantly between smokers and non-smokers (Huang et al 2006). An understanding of the timing and dynamics of the induced mutation and its possible repair, and therefore its contribution to tumour development, could explain these inconsistencies. Mutations in *TP53* are a late event in colorectal tumours, and as such may not be detected or represented in the same way in lung tumours from smokers.

9. **Aflatoxin B1** (AFB1), a product of *Aspergillus* fungus species, is a well-established human hepatocarcinogen following the consumption of contaminated foods (IARC). The presence of AFB1 DNA adducts correlates strongly with the generation of hepatocellular carcinoma (HCC) in populations from sub-Saharan Africa and southeast Asia, where AFB1 and hepatitis B virus, a synergistic risk factor for liver cancer, are entrenched. A specific mutation predominates (90%) and this is a GC →TA transversion on codon 249 of *TP53* which results in an AGG→AGT (Arg→Ser) change (Smela et al 2001, Olivier et al 2004). This mutation is generally not present in HCC from populations who have not been exposed to AFB1. It has been suggested that this specific mutation at codon 249 provides an effective biomarker of AFB1-induced genotoxicity (Gouas et al 2009).

10. The characteristic GC→TA transversion observed in *TP53* from liver of HCC patients who have been exposed to AFB1 can be reproduced experimentally *in vivo.*

In liver from Big Blue rats and neonatal mice exposed to AFB1, GC →TA transversions predominate (Dyacicao et al 1996, Chen et al 2010). In an *in vitro* FASAY assay (Functional Analysis of Separated Alleles in Yeast - considered to distinguish between silent *TP53* mutations and those which inactivate the resultant protein) in human fibroblasts, mutations induced by AFB1 treatment were distinguished primarily by GC→AT transitions, followed by GC →TA transversions and AT→GCtransitions. However, these occur at codon 245 and not codon 249 (Paget et al 2008). Studies *in vitro* with HepG2 cells revealed that although adduction at codon 249 of *TP53* did occur following exposure to AFB1 in the presence of an exogenous activating system, this was not the major site of covalent binding. Strong adduction was observed at a number of sites, including codons 226, 243, 244, 245 and 248 of exon 7 (Denissenko et al, 1998). However some studies have shown a relatively poor correlation between reactivity of AFB1 for a site and extent of mutation and that the hotspot mutation cannot be recreated in animals (Smela et al, 2001).

11. Aristolochic acid[1] (AA) is a nitrophenanthrene carboxylic acid, a component of plants of the genus *Aristolochia* many of which have historical use in herbal medicine. Its inadvertent use as a slimming aid was ultimately associated with a specific nephropathy and urothelial cancer (Arlt et al 2002, Chen et al 2012, Poon et al 2013). The same characteristic changes were found in specific areas of the Balkans (Croatia, Bosnia, Serbia, Bulgaria and Romania) and this was eventually shown to be due to AA present in bread flour from these regions following contamination of the wheat fields with *Aristolochia clematitis* plants (Slade et al 2009).

12. AA adducts (aristolactam[2] (AL)-d-adducts) have been identified in the renal cortex of >50% patients with urothelial cancer associated with AA exposure and a specific mutation spectrum in *TP53* has been identified in these tumours (Hollstein et al 2013). Amongst such tumours, 53.1% harbour AT→TA transversions in *TP53*, which is consistent with the 64.5% of this mutation found in tumours from those in the Balkan nephropathy areas but differs markedly from the occurrence in urothelial tumours not associated with AA (<5%) (Chen et al 2012). In addition there was an 84% concordance in patients with this mutation and the dA-AL-I adduct.

13. This pattern of mutations has been consistently documented in urothelial cancers associated with AA exposure (Slade et al 2009; Poon et al 2013). The same mutation spectrum has been demonstrated in a variety of experimental models; treatment with AA results in the development of AT → TA transversions at codon 61 of *c-Ha-ras* in resultant rat and mouse tumours (Schmeiser et al 1991;), in HUPKI mice (Arlt et al 2011), in *cII* gene in Muta mouse (Kohara et al 2002) and in BigBlue transgenic rats (Chen et al 2006; McDaniel et al 2012), which corroborates the relevance of animal models for the investigation of AA diagnostic mutations. AA represents an unusual example where the mutation spectra are consistent across species and time.

[1] Aristolochic acid consists of two main forms, I and II, differing by the presence of an aromatic methoxy group in the latter.

[2] Aristolactam I and II are metabolites of aristolochic acid I and II, respectively, produced by nitro-reduction. The intermediate nitrenium ion in their formation is believed to be the ultimate genotoxic form.

HUPKI mouse model

14. Members considered the human p53 knock-in (HUPKI) mouse to be a relevant and useful model for investigating chemically induced mutations. The mice carry a human wild-type *TP53* DNA sequence (from exon 4-9) which replaces both copies of the murine *TP53* sequence and which is expressed at physiological levels and functions as normal p53 (Luo et al 2001). The spontaneous tumour responses are similar to those of mice with murine p53 (Kucab et al 2010). AFB1 exposure resulted in enhanced formation of HCC in treated HUPKI mice compared to wild type mice. However the typical codon 249 *TP53* mutations observed in humans were not observed in either wild type mice or in HUPKI mice (Tong et al 2006).

15. Embryonic HUPKI cells can be cultured, providing *in vitro* systems in which chemically-induced mutation spectra can be examined (Olivier et al 2014). The embryo fibroblasts readily undergo immortalisation in culture, generating cells in which *TP53* is dysfunctional, enabling selection from their growth characteristics (Luo et al 2001). Mutation spectra from treated cultures are compared with mutations from spontaneously immortalised cultures to determine the specificity of any mutations. In HUPKI cells, the AA-induced mutation spectrum comprised primarily AT→TA transversions (57%) and this compares with 78% AT →TA transversions in human urothelial cancer from the Balkans (Hollstein et al 2013). BaP generated predominantly GC→TA transversions (49%), GC →CG transversions (22%) and GC →AT (19%) transitions in comparison to 30% GC→TA transversions, 12% GC→CG transversions and 26% GC→AT transitions in lung cancers from smokers (Kucab et al 2013).

'Next generation sequencing'[3]

16. Members agreed that 'next generation' sequencing would soon start to provide better insight into the evaluation and interpretation of chemically-induced mutation spectra. 'Next generation sequencing' methodology reads DNA templates randomly, enabling a picture of the entire genome to be generated. The more detailed pattern of mutations will provide greater resolution of mutation spectra and improve mechanistic insights (Alexandrov and Stratton 2014). Whole-genome sequencing will identify genetic variants, including single nucleotide polymorphisms, small insertions and deletions, and structural and genomic variants (>1000 bp) across the entire DNA sequence and not just in specific genes such as *TP53* from single gene sequencing studies. This methodology is combined with advanced bioinformatics methods and database searching to enable detailed analyses of cancer genomes.

17. Mathematical models are being developed which facilitate the process of extracting mutational signatures from the complex data sets generated from the whole genome sequencing of tumour DNA. It is possible to identify a variety of mutational patterns (in 96-element signatures) and to quantify the contribution of each signature for each tumour (Helleday et al 2014).

18. 'Next generation sequencing' is being used to identify the entire mutation spectra of cancers in an attempt to examine the mutational changes which lead to

[3] 'Next generation sequencing' is the term currently used to describe high-throughput, whole genome sequencing

cancer more accurately. It has been used to investigate gene expression signatures in a wide variety of cancers, including a review of over 7,000 cancers from 30 different sites when more than 20 distinct mutational spectra were observed (Alexandrov et al 2013). It is envisaged that correlating these changes with mutational signatures from known chemical exposures in defined systems will advance the understanding of cancer aetiology.

19. In conclusion, Members agreed:

- Despite an extensive literature describing studies which examine chemically induced mutation spectra in a wide variety of *in vitro* and *in vivo* systems, there are still very few examples where a specific mutation spectrum clearly establishes a specific genotoxic mode of action for a chemically-induced human tumour. Of those highlighted, there is still some uncertainty surrounding the robustness of the spectrum for AFB1 and liver cancer, and although the relationship between smoking, BaP exposure and the induced spectrum is characterised, the causal link to BaP remains unconfirmed. Aristolochic acid appears to provide the best example for a diagnostic mutation for a human cancer induced by a chemical.

- Mutation signatures using current, single gene, approaches may, on a case-by-case basis, provide useful mechanistic insight into genotoxic modes of action and contribute to weight-of-evidence genotoxicity assessments.

- The embryonic stem cells cultured from the HUPKI mouse do not always reflect human *TP53* mutation response, but as an *in vitro* model they do appear to offer some advantages over other models.

- Major advances in understanding the processes of mutagenesis and carcinogenesis are anticipated when data from studies using ‘next generation sequencing’ become available. We await data to evaluate whether this is the case.

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