

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)**UPDATE REVIEW ON TOXICOGENOMICS**

(Previous COM consideration 2004 (statement published), October 2007, October 2008).

Introduction.

COM reviews 2004 and 2007

1. The COT/COC/COM held a joint symposium on use of genomics and proteomics in October 2001. A joint statement based on review of available published literature and information from the HESI/ILSI collaborative programme of research was published in December 2004.
2. The conclusions reached by COM in 2004 are reproduced at the end of this draft discussion paper. The COM discussed a partial update review in October 2007 (MUT/07/18). A copy of this is appended as Annex 1 for information. The key messages from the October 2007 COM review are summarised below.
 - i) The studies reviewed focused on *in-vitro* studies using genotoxicants (predominantly in the absence of exogenous metabolic activation) in P53 proficient (TK cells) and deficient cells (LY5178Y).
 - ii) A review of bioinformatics/statistics would await the COT consideration of this topic.
 - iii) There was a need to also consider some additional studies on proteomics and the additional data to be considered by ILSI/HESI in November 2007.
 - iv) It was difficult to compare the available studies which had used diverse approaches derive conclusions with regard to gene expression responses resulting from *in-vitro* exposure to alkylating agents.
 - v) There was some evidence to suggest that transcriptomic approaches could distinguish between cytotoxicity and genotoxicity *in-vitro*.
 - vi) COM members considered that further evaluation of pathways and networks of gene expression responses rather than the response of individual genes. It might be eventually possible to focus analyses on specific gene pathways which

would assist in extrapolating data from one toxicogenomic approach to another (e.g. from transcriptomics to proteomics).

- vii) The COM noted the magnitude of gene expression changes was not necessarily the key part of any evaluation and it was possible small changes in activity of genes were important. Members agreed the most appropriate approach to evaluation of data would be unsupervised analysis of data with no threshold. Members noted the value of assessments based combined information from different toxicogenomic approaches.
- viii) Some preliminary data from the ILSI/HESI trial suggested DNA adduct data was more sensitive than transcriptomics for detection of DNA reactive genotoxins. The COM agreed to review more data from the ILSI/HESI project before reaching any definite conclusions.

COM review October 2008

3. The COM considered a number of recent publications of studies at the October 2008 meeting. The key messages from the October 2008 review are summarised below;
 - i) Approaches to data evaluation of microarray data supported by investigation of altered biological functions provided more relevant conclusions compared to analyses based on hierarchical clustering approaches. The data reviewed supported the previous COM conclusion that the use of arbitrary cut off values for changes in gene expression for inclusion in analyses limited the assessment of data from microarray studies.
 - ii) Microarray studies in *S.cerevisiae* indicated that up to 20-30% of the genome might respond to exposure to alkylating agents. In-vitro studies using mammalian cells indicated significant inter-study variation with regard to the identification of mode of action of genotoxins using microarray approaches. In addition there were evidence for significant inter-laboratory variation regarding data from microarray studies. [This aspect is being specifically investigated by ILSI/HESI]
 - iii) The available proteomic studies of genotoxins had not provided any useful data regarding DNA damage responses to genotoxins.

- iv) The use of a combined approach to testing specific hypotheses regarding mode of action of genotoxins involving cDNA microarray data supported by specific RT-PCR measurements for key genes and Western blotting for key proteins associated with genotoxic responses provided a more complete approach to the assessment of mode of action of genotoxins compared to use of microarray data alone.

COM discussion; based on topics raised by Thybaud et al

- 4. Use of Toxicogenomics to differentiate classes of compounds according to their genotoxic mechanism of action.
- 5. Members agreed that there was evidence for significant inter-laboratory variation in the conduct and an analysis of toxicogenomic studies applied to genotoxicity, particularly with regard to gene expression changes which could be used to differentiate between genotoxins and non-genotoxins, although all groups separately report that they can distinguish between different modes of genotoxic activity. Possible explanations include technical reproducibility of transcriptomics, experimental variation (e.g. different culture conditions and sampling times), and analysis variation (e.g. the use of hierarchical clustering analysis and arbitrary cut off for analysis maybe highly relevant to the gene expression results highlighted by investigators)..
- 6. Use of Toxicogenomics to better understand the mechanism of action;
- 7. Members agreed that the use of high density microarrays could be particularly valuable identifying hypotheses for mode of action which have not been previously considered. The suggestion of apolipoprotein E as a marker for formaldehyde systemic toxicity is one possible example. (Im et al J Proteome Res, 5, 1354-1366, 2006). An important distinction was between cytotoxic and genotoxic mechanisms. Members agreed that there were insufficient information to draw definite conclusions for the available test systems where data had been provided.
- 8. Limitations of Toxicogenomic approaches
- 9. Members commented that data previously available from the ILSI-HESI trial had indicated that conventional approaches to genotoxicity may be more sensitive to the detection of low exposures to genotoxins than current toxicogenomic approaches. Members noted that it was necessary to evaluate post-

transcriptional changes (i.e. protein levels and function) in order to elucidate the functional responses to exposure to genotoxins. Members agreed that RT-PCR data for gene expression changes considered relevant to interpretation of microarray studies was important for validation of microarray studies.

Update on HESI/ILSI trial

10. Members will wish to note the comments from Dr Aubrecht (Annex 1 to this discussion paper). Does and COM member wish to respond regarding the request for support/input to the ILSI project?
11. The secretariat suggest that the drafting of the COM statement on the subject at least awaits review of the 1st manuscript identified by Dr Aubrecht.

Update from published papers (Annex 2)

12. Additional studies of investigations (DNA microarray and proteomic approaches) of MNNG and BPDE in human Amniotic FL cells were retrieved.

MNNG (Li et al Mutation Res, 644, 1-10, 2008)

13. A high density cRNA microarray experiment was undertaken using exposure of human amniotic FL cells to MNNG (0.2, 1.0 and 10.0 μ M) for 2.5h. The approach to analysis included use of biological pathway analysis and RT-PCR verification for a relatively large number of genes (95). It is noted that the majority of gene expression changes reported using cRNA microarrays were less than two-fold. Addition RT-PCR analysis indicated confirmation of direction of effects for 33/42 probes for which RT-PCR results were significant. The authors reported at 1.0 μ M MNNG a significant down regulation of CDK6 was reported (-1.8 in microarray and -1.5 in RT-PCR). CDK6 might contribute to G1/S cell cycle arrest. At 0.2 μ M MNNG a significant down regulation of ZNF302 was reported (-1.5 microarray, -3.7 RT-PCR) indicating possible effects on DNA binding and transcription factors. The authors note changes in Zinc Finger proteins from a previous proteomic study of MNNG in FL cells and considered further investigations were needed to confirm the effects of MNNG on Zn-finger proteins.
14. A previous paper from this research group on proteomic approaches to MNNG effects in FL cells has been seen by the COM (Jin, J et al Environmental and Molecular Mutagenesis, 43, 93-99, 2004, Annex 2). This study used exposure of FL cells to 0.25 μ M MNNG for 2.5h with 2-DGell separation and MALDI-TOF identification of 18 out 32 spots with significant expression changes. It is noted that there was evidence for up regulation of ZN14 and

down regulation of ZN224, and up regulation of CDK6. Overall these results are not consistent with the results of the current microarray experiment and need further investigation before any conclusions can be drawn.

BPDE (Shen W et al J Proteomics, 6, 4737-4748, 2007 and Genomics, e-pub 2009)

15. Proteomic and transcriptomic studies of FL cells exposed to BPDE have been published by one research group. The first publication reported proteomic investigations (2d-gel separation, MALDI-TOF identification) of FL cells exposed to 0.005 μM , 0.05 μM , or 0.5 μM BPDE. A dose level of 0.5 μM slightly reduced cell viability at 22h post exposure. Sixty-three spots were reported to be differentially exposed following exposure. There was only very limited dose response for 10 proteins. A wide number of functions were altered across all dose levels; regulation of transcription, cell cycle, cell proliferation, transport, signal transduction, metabolism. A number of changes reported (e.g. Eukaryotic Elongation Factor 1 increased at 0.5 μM) were considered to be compatible with cell cycle arrest at G2/M checkpoint. Overall though, no specific BPDE response was identified.
16. In the subsequent publication of transcriptomic investigations (high density human U33 microarrays) using the same treatments of FL cells with BPDE as were used in the proteomic study, and hierarchical clustering with gene ontology identification of genes, a wide range of functions were reported to be altered including, protein metabolism, transport, cytoskeleton and DNA repair. At 0.5 μM there was comparability between data for cell viability and evidence for gene expression changes indicative of cell cycle block at the G2/M checkpoint. Overall though there seems to be little or no comparability between the proteomic studies and transcriptomic investigations regarding specific gene expression changes and protein changes in FL cells exposed to BPDE.

Investigation of B(a)P and BPDE in human colorectal carcinoma cells (HCT 116). Hockley S et al, Carcinogenesis, 29, 202-210, 2008

17. This publication was retrieved just in time to be briefly summarised in this cover discussion paper. The publication is also appended in Annex 2.
18. Cells (both p53 WT and p53-null HCT cells) were exposed to BaP (2.5 and 5.0 μM) for 6, 24, or 48h or BPDE (0.5 μM and 1.0 μM for 2, 6, or 24h (triplicate). cDNA hybridisation was undertaken using Cancer Research UK Human 22K microarray. Data were analysed after normalisation using a significant expression of 1.4 ($P < 0.05$) as the cut off for analysis. RT-PCR analysis (CYP1A1 and CYP1b1)

were undertaken and Western Blot analysis for protein levels for CDKN1A and GAPDH were also undertaken.

19. The authors report that gene expression changes reported in both cell types with BaP and BPDE were subtle. Of interest with regard to comparing transcriptomic and proteomic approaches is the finding that there was evidence for accumulation of p53 in HCT-p53-WT cells, but no evidence for p53 accumulation from microarray studies. CDKN1A was upregulated in microarray studies using BaP. A dose level of BPDE of 1 µM resulted in significant reduction in cell viability in HCT-P53-WT cells. Principle Component Analysis indicated P53 status had a greater influence (compared to time of exposure) after 24 h. P53 dependent genes upregulated in p53 WT cells but not P53 null cells included CDKN1A and DDB2, and genes associated with apoptosis (FAS, TNFR10B). Further analysis of biological functions using Expression Analysis Systematic Explorer reported evidence for up regulation of apoptotic processes in p53 WT cells exposed to BPDE consistent with cell viability data.
20. The authors also reported that DNA adduct formation after BaP but not BPDE was p53 dependent, suggesting loss of p53 affected metabolic activation of BaP.

COM Discussion

21. Members are asked to consider the suggestion from Dr Aubrecht for advice to the HESI/ILSI trials.
22. The published studies reported in this draft discussion paper contain both transcriptomic and proteomic data for MNNG and BPDE in FL cells. It is notable that although there is some comparability between the two different toxicogenomic approaches for both MNNG and BPDE regarding evidence for overall biological functions affected, there is very little comparability at the individual gene level. This observation appears to be consistent with the observation from the additional study by Hockley and colleagues regarding p53 response in HCT-WT cells.
23. Members are asked to comment on these data which will contribute to the overall update COM statement on toxicogenomics.
24. It is hoped to bring the HESI/ILSI data to the June 2009 COM meeting.

Secretariat January 2009