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MUT/04/1

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

Toxicogenomics: Update on Joint COT/COC/COM meeting held October 2001

Application of toxicogenomics to screening for mutagenicity. Initial results from ILSI/HESI trial and data from the published literature.

Introduction

1. The COT/COC/COM held a joint symposium on the use of genomics and proteomics in toxicology on 8 October 2001. A statement outlining the conclusions reached has been published on the Committee websites and a full write up of the meeting was published in *Mutagenesis*. 2003 May;18(3):311-7. A copy of the statement is enclosed as Annex 1. The key conclusions reached at the symposium are given below;
 - i) We *recognise* the future potential of proteomics and genomics in toxicological risk assessment.
 - ii) We *note* that these techniques may serve as adjuncts to conventional toxicology studies, particularly where proteins under investigation are known to be causally related to the toxicity.
 - iii) However, we *consider* that research and validation is required before these techniques can be considered for routine use in regulatory toxicological risk assessment. In particular there is a need for more research leading to development of genomic/proteomic databases, methods in bioinformatic and statistical analysis of data and pattern recognition and information on the normal range of gene expression.
2. There was relatively little data on application of toxicogenomics to screening for mutagens presented at the October 2001 joint meeting. Since then, a number of papers have been published and some preliminary information from the ILSI/HESI trial have become available. It is therefore appropriate to review this information particularly with regard to the conclusions reached in October 2001.

Outline of discussion papers for COT/COC/COM

3. An update paper was considered by the COT in February 2003 who concluded that there was no need to amend the conclusion reached in October 2001. It was acknowledged that the application of genomics,

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(this term is used in this paper instead of the term transcriptomics) proteomic and metabonomics to toxicology was rapidly developing and thus COT asked for an annual update paper to be prepared. The most recent draft review for the February 2004 COT meeting has been forwarded to COM members as background information. (This latter paper also includes the first summary of metabonomic data presented to COT.) This is a relatively bulky paper and in order to assist COM members, key outcomes of the review of published papers retrieved during 2003 are given below ;

- a) There is extensive research ongoing regarding the use of toxicogenomic approaches in toxicology. Most concern transcriptomic studies with fewer data available on proteomic and metabonomic approaches.
- b) The most useful application of toxicogenomic data is the development of hypotheses for mechanistic interpretation of toxicological data.
- c) The results of the initial screening studies (for specific target organ effects e.g nephrotoxicity, hepatotoxicity) indicate that cDNA microarrays may be of value in early screening for potential effects but there are considerable areas of further work required dealing with reproducibility both within and between studies or laboratories and the development of bioinformatic approaches to aid data evaluation.
- d) The application of Principle Component Analysis (PCA) has shown promise as a useful method for displaying complex data in a visually interpretable form. This approach uses analysis of the principle sources of variance in data and displays this information graphically, either 2-dimensionally or 3-dimensionally, e.g. PC1vPC2.
- e) There is little available research which provides a comparison of the outcome of genomics with proteomics. It was not possible to draw any definite conclusions from the one available study of the acute hepatotoxicity of bromobenzene in rats. Newer developments in proteomic methods (SELDI-TOF) may assist in such work in the future.
- f) There is little comparative data on the use of high density cDNA microarrays (e.g. with thousands of genes) and low density cDNA arrays (with small numbers of genes targeted for a limited number of toxic mechanisms). In general high density arrays are difficult and expensive to develop and it is difficult to interpret the data. Low density arrays are cheaper, easier to use and evaluate, but may miss novel mechanisms and have limited coverage.

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- g) It is noted that many journals require the results of cDNA microarray experiments to be replicated by methods involving quantitative mRNA analysis (e.g. RT-PCR).
4. The current COM paper summarises the outcome of the ILSI/HESI (International Life Sciences Institute/Health and Environmental Sciences Institute) studies to investigate the use of toxicogenomics in screening for genotoxicants. Relatively little toxicoproteomic data are available at the present time from this study. A considerable amount of the relevant information was discussed at an invitational meeting held on 5/6 June 2003 at Hyatt Fair lakes, Virginia, U.S.A. and has been published in detail by ILSI/HESI on the internet. (<http://www.ilsilife.org/publications/publist.cfm?pubentityid=120&publicationid=458>) A publication from ILSI/HESI giving a summary of the current status of the project is appended as Annex 2. (A brief summary is given below.)
5. A number of selected overheads used at the meeting and the published minutes of the meeting have been included as Annex 3 to this paper. A number of additional published papers are included as Annex 4. These latter publications provide further useful insight into the potential for use of genomic approaches in genotoxicity screening.
6. It is suggested that the COT/COC/COM review the minutes from all the Committee discussions before considering if an alteration to the conclusions reached in October 2001 are required.

ILSI/HESI research: Application to genotoxicity screening (Annex 2 pages 13/14 and Annex 3).

7. In mid-1999 the membership of the ILSI Health and Environmental Sciences Institute formed a technical committee with the objective of initiating research to provide information on the potential for use of toxicogenomics. Four working groups were set up; hepatotoxicity, nephrotoxicity, genotoxicity and informatics. Initial results from each of the working groups are now available and were discussed at the workshop referred to in paragraph 4 above. Compared to the other working groups, relatively little data are available from the genotoxicity working group.
8. In brief, mutagenicity studies were undertaken using L5178Y mouse lymphoma cells (p53 deficient) and human TK6 cells (p53 proficient). Cells were exposed to chemicals for 4 hours at two dose levels and cytotoxicity and mutagenicity evaluated or mRNA extracted and isolated immediately after 4h or after a further 20 h incubation. 11 chemicals were included in the trial which were all direct acting mutagens via a number of different mechanisms (cisplatin, benz(a)pyrene diol epoxide (ILSI have confirmed this compound was used in the trials), MMS, bleomycin, taxol, mitomycin C, ENU, hydrogen peroxide, etoposide and hydroxyurea). It was reported that

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gene expression analysis was undertaken using a number of different platforms, but most laboratories migrated to using Affymetrix during the project.

9. The dose levels selected were designed to result in 20-30% cytotoxicity with a weak mutagenic response (1.5-4 fold increase in endpoint) or 70-80% cytotoxicity (in TK gene mutation assay) with a robust mutagenic response.
10. The key objectives of the ILSI/HESI research were to;
 - a) compare genomics with conventional endpoints from mutagenicity studies with regard to sensitivity and utility for genotoxicity screening.
 - b) Investigate whether it was possible to distinguish between positive data from a mutagenic effect and that from cytotoxicity and between DNA reactive and DNA non-reactive compounds.
 - c) Identify issues regarding interlaboratory variability and difficulties in data analysis.
11. Relatively few data are available at present. The secretariat understand that full papers have been submitted to EHP-Toxicogenomics and should be available soon. Most information is presented as PCA graphs.
 - a) cDNA microarray experiments could distinguish between as mutagenic response (using cisplatin) in an in-vitro micronucleus assay and a cytotoxic (false positive) response (e.g. sodium chloride).
 - b) Studies using benzo(a)pyrene diol epoxide in TK cells showed measurement of DNA adducts and TK mutant frequency were more sensitive indicators of genotoxicity than number of genes showing altered expression. (No information provided on which genes were affected.) It was evident that the use of global arrays (i.e. high density cDNA arrays containing thousands of genes or expressed sequences) was not practical for genotoxicity screening.
 - c) PCA analysis showed it was possible to differentiate between mechanisms giving rise to positive results.
 - d) The p53 status of cells used in experiments was reported not to affect the identification of DNA damage signatures.
 - e) It was reported that cDNA arrays can distinguish between DNA reactive and non-DNA reactive positive response.

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12. Overall, the limited data available at present suggest that genomic analysis of L5178Y mouse lymphoma cells and TK6 cells may aid in the evaluation of positive results in mutagenicity assays but appear to be less sensitive at predicting in-vitro mutagens. The information suggests cDNA microarrays are not a practical approach to screening for genotoxicity. It is noted that no information on the functions of genes affected by exposure to genotoxicants was reported in the available information from ILSI/HESI.
13. Information from a number of recent published papers is given below which provide some initial information on gene expression changes in cells exposed to genotoxicants. **Relevant papers are appended at Annex 4.**

In-vitro studies

Identification of transcriptome profiles for Bleomycin and hydrogen peroxide in L5178Y mouse lymphoma cells. (Seidel SD et al Environmental and Molecular Mutagenesis, vol 42, 19-25, 2003)

14. The research presented in this paper (see Annex 4) was undertaken as part of the ILSI/HESI trial.
15. Bleomycin and hydrogen peroxide were chosen as examples of chemicals which produced oxidative DNA damage via different mechanisms with bleomycin producing damage at specific loci whilst hydrogen peroxide induced generalised damage. Dose response data for mutagenicity and cytotoxicity in L5178Y cells using the microwell method were produced in two independent experiments. Cells treated with bleomycin at 2.5 ug/ml and 20 ug/ml were chosen for cDNA microarray studies. For hydrogen peroxide dose levels chosen were 5 ug/ml and 10 ug/ml. The lower dose of hydrogen peroxide produced more mutagenicity/cytotoxicity than anticipated (using the ILSI/HESI criteria set out in para 9 above). The microarray screening used the 1.2K Clontech cDNA array and visualisation of hybridised cDNAs through incorporation of ³²P- into cDNAs. (It is noted that many research groups have now preferred to use fluorescence probes as used in the Affymetrix system). The authors noted variable background labelling with ³²P in this study which affected gene expression analysis of the mRNA replicates.
16. The analysis of gene expression patterns was based on combining data from both dose levels and experiments. There was considerable variation between analyses of the same mRNA in the same experiment and between the two experiments for both bleomycin and hydrogen peroxide. The authors considered that the higher specificity of the cDNA probes used in this experiment may have accounted for the inter experimental variance. The authors report on 10 specific gene expression changes noted with bleomycin and 6 gene expression

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changes with hydrogen peroxide. It is not evident how these results are obtained, whether this involved a hierarchical clustering analysis or PCA. It is stated that these genes showed a dose-dependent change in at least one experiment. None of the genes identified was involved in DNA repair or oxidative stress response. The general pattern of response was down-regulation at 4hours and up-regulation at 24 hours. A few of the gene changes were considered to be associated with apoptosis and/or cell-cell communication (thymus cell antigen1, syndecan 1, integrin $\alpha 7$, granzyme A, cathepsin D and mPin). There were three common gene changes identified between bleomycin and hydrogen peroxide at the 24 hour time point (granzyme A, integrin $\alpha 7$ and 45ka calcium binding protein precursor (Cab45). It is noted that none of these changes in gene expression were confirmed by PCR analyses. The authors considered that the lack of response of DNA damage genes might be due to the deficient p53 status of the cells. The increased expression of apoptosis related genes at the 24 h time point might reflect the induction of apoptosis pathways not involving p53.

17. Although the authors conclude that a unique and common patterns of gene expression were identified in this study, the apparent variance in the results seen confound the interpretation of data. Thus the lack of response of DNA repair/damage genes or oxidative damage requires explanation. In addition there was no independent confirmation by PCR analyses. It is unclear what conclusions, if any, should be drawn from this study.

cDNA microarray gene expression profiling of three in-vitro genotoxins in L5178Y cells. Lee M et al (Environmental and Mol Mutagenesis, vol 42, 91-97, 2003.)

18. cDNA microarray studies were undertaken in L5178Y mouse TK^{+/-} lymphoma cells treated with one of three compounds previously shown to give a positive mutagenic response in the assay. The dose level chosen for cDNA microarray experiments was designed to result in 60% cytotoxicity. A 2h sampling time point was used. Data on mutagenicity was not presented. Compounds studied which were known to be positive in mouse lymphoma cells but exhibit positive (hydroxy urea) or negative (p-anisidine) or unknown (paclitaxel) carcinogenic potential in rodents;

Hydroxyurea. Inhibitor of DNA replication, in-vitro and in-vivo mutagen and transpecies carcinogen.

p-anisidine, Positive in Ames test and in-vitro chromosomal aberration assay but negative in in-vivo MN test and in carcinogenicity bioassay in mice.

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Paclitaxel (inhibits tubulin disassembly), negative in Ames test, positive in in-vitro cytogenetics and in in-vivo MN assay. Carcinogenicity unknown.

18. cDNA was prepared from total RNA by reverse transcriptase. Flourescent labelling of control cDNA with Cy-3 and treated cDNA with Cy-5 was used to visualise the outcome of hybridisation experiments. The mouse twin chip 7.4 K microarray was used. Quadruplicate RNA samples were used for each chemical. Analysis was undertaken using a combination of reported old-change and estimated false discovery rate.
19. The authors reported 7 up-regulated genes and 12 down-regulated genes which common to all three chemicals and were also identified in each of the quaduplicate hybridisation experiments. This observation maybe of relevance to in-vitro testing in mouse lymphoma cells but not with regard to prediction of in-vivo mutagenicity. The reason for any correlation with outcome of mutagenicity testing in mouse lymphoma cells in unclear. None of the gene identified related to DNA damage or repair. Up-regulated genes included Inter-alpha trypsin inhibitor, heavy chain 2 and two binding proteins (zinc finger protein 385 and binding protein 3. Down-regulated genes included Bmil (encoding zinc finger protein 52), Mypt1 (myosin phosphates target subunit 1) and Zfp52 (zinc finger protein 52). It was noted that the function of approximately half of the selected genes was unknown. It is not possible to establish a link between the common genes identified in this study and genotoxicity.
20. The authors also looked for differences in gene expression changes between the chemicals which might assist in differentiating mechanisms of genotoxicity and also give insight into the mechanisms of carcinogenicity. However, with regard to carcinogenicity, since the mouse lymphoma assay was designed to evaluate potential for mutagenicity in-vitro and was never intended to provide information on the mechanism of carcinogenicity, there is no rationale for using gene array data from L5178Y cells to aid in evaluating mechanisms of carcinogenicity. [Members will wish to note that a paper on genomic changes during the formation of tumours is being prepared for the COC].
21. The authors undertook pair-wise comparisons of gene expression patterns and claimed that the data supported a difference between hydroxyurea and paclitaxel and hence the approach might be able to distinguish between different mechanisms of genotoxic activity. However they didn't provide evidence that gene expression changes with these chemicals were consistent with those anticipated for genotoxic chemicals and thus no conclusions can be drawn.

In-vivo study

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Characteristic expression profiles induced by genotoxic carcinogens in rat liver. Ellinger-Ziegelbauer H et al (Toxicological Sciences Advance access published on internet 4 November 2003)

22. The authors evaluated gene expression profiles in the liver of rats treated with four genotoxic liver carcinogens for periods of up to 14 days. Chemicals used were dimethylnitrosamine, 2-nitrofluorene, aflatoxin B1 and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK). Dose levels were not reported, but were selected to give rise to histological evidence of liver damage and were set at levels which would ultimately lead to carcinogenesis over extended dosing periods. Samples of liver were taken at days, 1,3,7,14. Tissue was snap frozen (at -80°C) for storage prior to RNA extraction. Other tissue samples were processed for conventional histology (H&E). Serum samples were taken for analysis of AST,ALP, GGT, cholesterol, and total/direct bilirubin.
23. For the 2-nitrofluorene and dimethylnitrosamine, pooled RNA from 5 animals/time point were processed in triplicate for cRNA hybridisation using Affymetrix u34A rat chips (8740 probe sets, 7000 rat genes, 1740 expressed sequence tags). For the NNK and Aflatoxin B1 studies liver samples from three individual rats were processed for cRNA hybridisation. Biotinylated cRNA was used in the microarray studies and visualisation was by staining with streptavidin-phycoerythrin. Gene responses were selected for one dimensional cluster analysis by a number of different analysis methods (including fold change, changes in gene expression for genes present in treated and absent in control (and vice-versa), or applying a statistical test on all genes including low expression genes). A number of gene expression changes were confirmed using quantitative analysis of RNA (RT-PCR).
24. The authors reported histological changes (as predicted from the literature) for the chemicals under study.

2-nitrofluorene, modest hypertrophy at day 7, 14
DMN, necrosis and inflammation day 7 and 14, and mitosis day 7.
NNK, apoptosis day 14
AFB1 necrosis day 1, weak inflammation up to day14.
25. Several genes known to be induced by p53 were up-regulated by all four carcinogens which included apoptosis inducer BAX, cyclin dependent kinase inhibitor p21, cell growth regulator gene 11, B cell translocator gene 2, cyclin G1, ubiquitin E3 ligase MDM2. In addition the DNA repair enzyme O6 methylguanine-DNA methyltransferase was up-regulated by all four carcinogens. There were additional gene expression changes regarding genes involved with detoxication, mitochondrial damage and apoptosis inhibition. Regarding the latter tissue inhibitor of metalloprotease 1, growth differentiating factor 15 and growth potentiating factor were up-regulated by all four

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carcinogens and were considered to represent survival signalling. Other changes are discussed in detail in the appended paper (Annex 4). Overall though, the changes seen in this *in-vivo* study were consistent with the predicted effects of genotoxic carcinogens in rat liver and reflected the observed histology changes. RT-PCR analyses undertaken for a few selected genes confirmed the microarray experimental findings.

COM Discussion

26. Members are asked to discuss the results of the ILSI/HESI trial as far as is possible given the limited data available. The Working Group appears to be supporting the view that routine use of cDNA microarrays for screening genotoxin is impractical and that conventional approaches are more sensitive. It is proposed that cDNA microarray studies might aid in interpreting the mechanisms of positive finding in mammalian cell mutation studies. However it would appear that substantially more data are required before any conclusion could be reached on this aspect.
27. The results of the most recently published studies using L5178Y mouse lymphoma cells are difficult to interpret. The pattern of gene expression changes appear to be difficult to link directly to genotoxicity. It is noted that the absence of functioning p53 may partly explain why up-regulation of DNA damage and DNA repair genes weren't found. However an early study undertaken in HeLa cells using bezno(a)pyrene (appended as Annex 5, Yu Z et al Environmental and Molecular Mutagenesis vol 36, 210-205, 2000) also reported a lack of effects on DNA damage and repair genes. It is possible that the gene expression in HeLa cells may also be abnormal possibly including reduced p53 expression. A different interpretation which has been proposed is that p53 is normally maximally expressed in cells and that proteomic methods might be more appropriate.
28. A further observation from the published *in-vitro* studies was the apparent lack of reproducibility between arrays using the same RNA samples. This is a key area for further research in the use of microarray technologies and has been noted by the ILSI/HESI working groups.
29. In contrast to the *in-vitro* studies, the limited *in-vivo* study of four carcinogens yielded gene expression results which were in accordance with predictions based on the known genotoxic mechanism of the compounds under study. Substantial further research is needed to investigate whether a pattern for genotoxic carcinogens can be determined. This aspect is to be considered by COC. (COM members will be copied the COC paper in due course).

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30. These data suggest that microarray studies using cell lines may be unsuited to delineating gene expression patterns for genotoxicants and not likely to be of value for screening for genotoxicity.
31. The proposal outlined in the COT paper (Forwarded to COM members for information) is that the COT/COC/COM should review the available information in the series of papers prepared for consideration at the first meetings in 2004 and consider whether the conclusions reached at the October 2001 joint meeting should be amended.

Question for COM

32. Members are asked to consider if a revision to the existing statement is warranted and to agree that any further work should be undertaken in conjunction with COT and COC and should cover all aspects of toxicogenomics including genotoxicity screening.

Secretariat. January 2004.