COMMITTEES ON TOXICITY, MUTAGENICITY AND CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COT, COM, COC)

JOINT STATEMENT ON THE USE OF TOXICOGENOMICS IN TOXICOLOGY

Introduction

1. The COT/COC/COM held a joint symposium on the use of genomics and proteomics in toxicology in October 2001. The following overall conclusions were subsequently agreed and published in a statement.

a). We recognise the future potential of proteomics and genomics in toxicological risk assessment.

b). 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.

c). 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 of bioinformatic and statistical analysis of data and pattern recognition and for information on the normal range of gene expression.

2. The Committees agreed to further consider toxicogenomics as part of the horizon scanning exercise initiated at the February 2004 COT meeting. It was noted that there was a considerable increase in the number of publications using toxicogenomic approaches. A number of discussion papers were subsequently prepared for the Committees which reviewed the available published literature. The data from 50 studies were considered during the review which also included available information from the HESI collaborative scientific program on toxicogenomics[1].Details of the references consulted during the review can be found in the papers cited at the end of this statement. This current review considered information on use of metabonomics in toxicology for the first time. The COT requested a further paper and presentation on the use of statistics/bioinformatics in

[1] HESI = Health and Environmental Sciences Institute (HESI) of the International Life Sciences Institute (ILSI).]

toxicogenomics. A presentation was given by Dr David Lovell (University of Surrey) to the COT at its meeting on the 7 September 2004.

3. For the purposes of this statement, the term transcriptomics refers to gene expression as measured through cDNA or oligonucleotide or cRNA microarray based approaches, proteomics refers to determination of protein levels through gel or solid phase approaches and metabonomics refers to measurement of metabolites in tissues, plasma or urine.

4. A summary of the main conclusions reached by each Committee based on the information provided is given below.

Conclusions reached by Committee on Toxicity

5. The COT reached the following conclusions after discussions at its February and September 2004 meetings.

a) There had been improvements in the design and reproducibility of studies, and the approaches to the analysis of raw data and statistical approaches to evaluation and identification of toxicologically relevant patterns for gene changes.

b) The key areas for future development were development of methods for pattern recognition, the evaluation of functional significance of changes in gene expression and distinction between adverse and adaptive changes.

c) There was a need for better toxicogenomic studies on time course for effects and dose-response assessment and the reversal of toxicological effects. At present toxicogenomic data could be considered as part of the overall toxicological data package, but could not be used in the absence of prior knowledge about the toxicity of the chemical from conventional toxicological approaches. There was a potential that toxicogenomic approaches could be designed to screen for specific mechanisms of toxicity but such approaches would require appropriate validation.

d) Regarding transcriptomic methods it was agreed that there were a considerable number of sources of variance which might affect the results of studies. The COT confirmed that for the present it was necessary to confirm key gene changes independently such as by quantitative PCR[2] analysis of mRNA. The design of experiments (e.g pooling of samples), reproducibility of replicate mRNA analyses, the approach to assessment of background changes, use of different fluorometric methods to assess gene expression changes, use of housekeeping genes, variation between laboratories regarding analysis of mRNAs in particular the use of different platforms, and validation of

[2] polymerase chain reaction

the genes incorporated into microarrays were all examples of the potential sources of variation in transcriptomic analyses.

e) There are few 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 comparatively of greater difficulty and expense to develop and the evaluation and interpretation of data is complex. Low density arrays are cheaper, easier to use and evaluate, but may miss novel mechanisms and have limited coverage of genes.

f) Regarding proteomics, it was agreed that there had been considerable important developments since the 2001 meeting particularly in respect of introduction of solid-phase separation methods. Both two dimensional gel methods and solid phase techniques were valuable and should be considered as complimentary techniques.

g) Metabonomics had not been considered in the 2001 joint COT/COC/COM meeting. There had been considerable recent advances in techniques regarding recognition of metabolite pattern changes in tissues and biological fluids. The potential for development of biomarkers was noted. The potential use of trajectomes to visualise onset and recovery for toxicity was noted.

h) The Committee agreed that transcriptomics/proteomics and metabonomics each need to be considered as part of an integrated approach to toxicological risk assessment. Thus a preliminary study of the acute hepatotoxicity of paracetamol in mice using transcriptomics and metabonomics had provided new insights in the mechanism of hepatotoxicity of this chemical.[2]

i) It was too early to draw any conclusions as to whether the use of toxicogenomic approaches to toxicology could result in the use of fewer animals in testing.

j) There were no epidemiology studies retrieved during the review (i.e. up to September 2004).

Additional conclusions reached by Committee on Mutagenicity

6. The COM reached the following conclusions after discussions held at its February and May 2004 meetings.

a) No conclusions can be drawn from the preliminary results of the ILSI/HESI trial of mutagenesis in mouse lymphoma L5178Y $tk^{+/-}$ cells.[3] Further information on the detailed results from this trial and validation of the findings would be needed before conclusions can be drawn.

b) Mutagenicity may be associated with changes in expression of relatively few genes which might be potentially difficult to identify in high density arrays.[4] The COM agreed there were considerable difficulties in developing in-vitro mutagenicity screening assays using toxicogenomic approaches with regard to selection of appropriate microarray platform, confirmation of microarray results using quantitative measures of mRNA levels, identification of appropriate fold change in gene expression, and development of appropriate statistical/bioinformatics approaches for assessment of studies. However it was possible that valid approaches to screening for mutagens might be developed in the future.

c) The COM identified the need for more research on time dependent changes in gene expression using mutagens and the application of integrated toxicogenomic approaches to evaluating changes in protein and metabolic pathways in response to exposure to mutagens. No adequate proteomic/metabonomic studies of mutagens had currently been identified.

d) The COM reviewed a number of published papers which presented data using mouse lymphoma L5178Y $tk^{+/-}$ cells and agreed that no clearly defined pattern of gene expression changes which could logically be associated with mutagenesis had been identified. The COM reviewed a recent study which had used HepG2 cells and agreed that the authors had been able to distinguish between genotoxic and non-genotoxic carcinogens but only when a number of genotoxic compounds (predominantly methylating agents) were excluded.[4] Overall this latter study provided some useful information but there was a need for considerable additional research involving multiple dose levels and sampling times before conclusions could be reached.

e) The Committee considered that the limited available in-vivo studies using four hepatocarcinogens did provide some preliminary results which suggested genotoxic responses in gene expression could be identified in-vivo.[5]

f) One preliminary investigation provided evidence to suggest that transcriptomics could provide information to aid in the interpretation of conventional *in-vitro* clastogenicity assays to assist in the evaluation of mutagenic or cytotoxic responses in these tests.[6]

Additional conclusions reached by Committee on Carcinogenicity

7. The COC reached the following conclusions after discussions held at its June 2004 meeting. The COC reached a number of general conclusions on toxicogenomic studies in experimental animals regarding dose-response evaluation, investigations of reversibility, statistical handling of data and bioinformatic developments which are consistent with those reached by COT. COC members also commented on the need for “pathway mapping” for the

identification of toxicologically relevant gene changes. The COC agreed with the COM conclusion that a gene expression pattern had been reported in studies in rodents using genotoxic hepatocarcinogens.

a. A number of studies in rodents using model carcinogens had reported on toxicogenomic approaches to investigate the process leading to neoplasia from initiation to tumour formation and growth. However no conclusions could be drawn from these limited studies. It was noted from the preliminary evidence considered by the committee that it was difficult to distinguish between chemical induced changes in gene expression from those occurring as a result of the neoplastic process.

b. It was not possible in studies in animals using model non-genotoxic carcinogens to identify common gene expression changes which might be of value in developing an approach to early detection of non-genotoxic carcinogen. The available study identified more distinct than common changes in studies in mice using two model non-genotoxic hepatocarcinogens.[7]

c. Potentially valuable information on mechanisms of carcinogenesis could be derived from experiments designed to investigate particular specific mechanisms. Some preliminary information on non-genotoxic liver carcinogenesis in mice was available.[7]

d. Comparison of gene expression changes in stomach tumours in rodents induced by a model genotoxic carcinogen had shown similarities with gene expression profiles from human stomach cancers.[8] These preliminary data could be used for hypothesis generation regarding the aetiology of stomach cancer. However caution was required in interpreting the studies considered by the committee as the range of toxicological effects in animals given relatively high doses of model carcinogens did not reflect the likely effects in humans exposed to much lower doses in the environment.

Use of statistics and bioinformatics in toxicogenomics.

8. The COT heard a presentation from Dr David Lovell on the use of statistics and bioinformatics in toxicogenomics at its 7 September 2004 meeting. The background paper for the presentation is available on the COT internet site. [9]

9. The COT reached a number of conclusions based on the main recommendations proposed by Dr Lovell;

Samples and experimental design.

a) The purpose of a study and experimental design should be clearly specified such as whether it is designed for screening compounds internally within an organization, for providing evidence

relating to a mechanism of action or is part of a formal submission to a regulatory body. Sample sizes based upon power calculations depend upon the 'probe' of interest

b) Replicates should be biological replicates representing independent experimental units such as subjects, animals or cultures. Technical replicates from repeated sampling of the same experimental unit may also be analysed. The need for biological replication should take precedence over the need for technical replication

c) Samples should not be pooled if information on individual experimental units is important and information is required on within group variability in *in-vivo* studies.

d) Sufficient information on an experiment should be provided to enable independent replication. The strongest evidence to confirm the results of toxicogenomic studies comes through data showing commonality of gene pathways affected in transcriptomic, proteomic and metabonomic experiments. The concept of phenotypic anchorage of data, i.e. linking cause and effect is crucial to the evaluation of toxicogenomic data.[10] Confirmatory evidence may also come from similar findings from the analysis of results using different platforms (cDNA and oligonucleotide arrays) and from other techniques such as reverse transcriptase (RT)-PCR. The concordance or otherwise of results from these different systems relates more to the different mechanisms underlying the systems than a formal statistical interpretation and practical issues in terms of amounts of sample may determine how much verification using other techniques will be carried out.

e) No specific guidelines should be drawn up for the reporting of expression changes between different groups. The relationship between the size of difference such as the fold-change and the significance level is complex and depends upon such factors as the statistical tests used, the sample size, whether multiple comparison methods are used and the relative level of gene expression for the probe. A useful presentation of the data is in the form of a volcano plot which illustrates the relationship between statistical significance and difference between two treatments.

f) Considerable intra- and inter-laboratory variability has been found in cross-laboratory studies of microarrays.

g) The extension of the methods from laboratory-based experimental studies to observational epidemiological studies raise a number of issues relating to the collection and preparation of samples to prevent the introduction of biases. This is critical for all biomarker / molecular epidemiology work but is particularly relevant to these sensitive multivariate analyses which are vulnerable to many

unintentional biases as a consequence of less experimental control over the material used in the study.

Data Management

h) Toxicogenomic studies generate large quantities of raw data. Management of the full set of 'raw' data collected from studies is a major challenge[11]. The approach being developed by MIAME[3] for the data management of data collected in microarray studies has proven to be a useful starting point for data management of toxicogenomics study. The development of draft international guidelines on minimum information about microarray experiments for Toxicogenomics (MIAME/Tox) which builds on the MIAME initiative is to be welcomed. Adoption of a harmonised set of guidance would greatly assist the planning and completion of Toxicogenomic studies. The development of similar standards for proteomic studies (The Proteomics Standards Initiative) is at an early stage but should provide valuable generic guidance in the future.[12]

Statistics

i) No single method can currently be considered the most appropriate way to analyse toxicogenomic data. No attempt should, therefore, be made at present to define specific statistical methods or software that should be used for the analysis of toxicogenomic studies. Toxicogenomics studies may have different objectives and, therefore, the methods used need to be appropriate for the specific aim of a study. Many of the methods currently available within the standard statistical packages and the specialized software associated with genomics are 'mature' methods. Considerable research is in progress in developing new and modified versions of these methods to analyze the multivariate datasets being generated. It is unlikely that this will result in a 'single' agreed method in the short-term.

j) It is critical, though, that the statistical methods used are reported comprehensively. The methods used should be clearly specified and any assumptions or options used in the analysis should be explicitly identified.

k) Ideally, the data collected should be in a form that it is possible to repeat the analysis using both the specific software initially used and using alternative statistical methods using other software and packages. There is likely to be a need for 'in house' expertise to analyse data using standard significance testing, Principal Component

[3] Minumum Information About a Microarray Experiment

Analysis, cluster analysis and classification packages to get an overview of conclusions being drawn and to try out sensitivity analyses.

Overall discussion and conclusions

10. The Committees agreed that there had been rapid and extensive developments regarding the application of toxicogenomic methods to toxicological hazard characterisation. It was possible that toxicogenomic approaches could potentially be applied in a limited number of situations but could not be applied routinely to toxicological risk assessment. Thus the available evidence supported the view that these techniques could be applied, particularly when used in combination, for the initial screening for a limited number of toxicological mechanisms where appropriate validation was available. The Committee recognised the application of proteomic and metabonomic studies to the examination of chemically-induced responses in whole animal and the possible development of biomarkers of toxicological effect. but could not currently be used for routine screening for target organ toxicity. The available information provided preliminary and limited evidence that toxicogenomic techniques could also provide additional information to aid in the assessment of dose-response relationships but it was important to interpret such data in collaboration with information from existing conventional toxicological methods such as microscopical examination of fixed, sectioned and stained tissues.

11. The main factors which limited the current application of toxicogenomic methods predominantly relate to the limited database of studies available (with regard to limited duration of dosing predominantly to short-term periods, few species and toxicological mechanisms examined, and limited information on interlaboratory reproducibility of results) and to the need for improved methods of data evaluation and interpretation (particularly with regard to the need for interpretation of data in respect of gene expression changes in toxicologically relevant pathways).

12. Members agreed the conclusions and recommendations proposed by Dr Lovell. It was recognised that the statistical methods were undergoing rapid development. It was, however, considered that there was a need for some generic guidance on the most suitable methods for statistical evaluation of different types of toxicogenomic data.

13. The Committees agreed that the overall conclusions reached in 2001 were satisfactory subject to a number of amendments. The following overall conclusions were agreed.

a) We recognise the rapid development in toxicogenomic methods (transcriptomics, proteomics and metabonomics) in toxicological hazard identification and characterisation since 2001.

b) We confirm that these techniques may serve as adjuncts to conventional toxicology studies. There is a need to provide appropriate data from studies on gene expression, protein levels and metabolite changes in order to provide sufficient information on toxicologically relevant pathways.

c) However we consider that further research and validation is required before these techniques can be considered for routine regulatory toxicological risk assessment. At present toxicogenomic approaches can provide valuable supportive data on mechanisms of target organ toxicity which can aid in the risk assessment process.

d) There is a need for further refinement and optimisation of methods used, approaches to data interpretation and evaluation using statistical and bioinformatics methods and development of appropriate publicly accessible databases.

e) We note the need for generic guidance on the most suitable methods for statistical evaluation of different types of toxicogenomic data.

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Discussion papers

TOX/04/2 & Annexes; MUT/04/1, 2 & 11 and CC/04/5 & 6 for his topic can be found on the COT, COM & COC websites.

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