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DRAFT **MUT/07/18**

COMMITTEE ON MUTAGENICITY OF CHEMICALS IN FOOD CONSUMER PRODUCTS AND THE ENVIRONMENT (COM)

UPDATE REVIEW ON TOXICOGENOMICS.

Introduction

1. The COT/COC/COM held a joint symposium on the use of genomics and proteomics in October 2001. A meeting report was published in Mutagenesis.[1] published a joint statement in December 2004 on the use of Toxicogenomics in toxicology. This was based on a literature review of 50 studies which included information from the HESI/ILSI collaborative programme of research.

2. The key conclusions reached in 2001 are reported below for ease of reference.

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.

3. The COT/COC/COM published a joint statement in December 2004 on the use of Toxicogenomics in toxicology. This was based on a literature review of 50 studies which included information from the HESI/ILSI collaborative programme of research. The 2004 review was initiated during the horizon scanning exercise for 2004. The conclusions reached by COM are shown below. A copy of the full statement is given as Annex 1 to this covering paper.

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

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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]

Advice requested from COM

4. Toxicogenomics was raised during the 2006 horizon scanning exercise. The objective of the current review is to consider if the conclusions reached by COT/COC/COM need updating. The DH Toxicology unit have drafted a short overview (Annex 2) of a number of *in vitro* studies which included data on gene expression changes and in some studies data on DNA adducts and mutagenicity as well as data on transcriptomic investigations. Copies of relevant papers are appended as Annex 3. There are still a number of other papers that need to be evaluated before the committee is asked to complete the review of its 2004 conclusions. However the papers reviewed for this paper are useful in initiating the COM review.

[Members will wish to note that the COC considered a short selective overview paper at its July 2007 meeting. The draft minutes are appended at the end of this covering paper for ease of reference. The COT segment of the review is not expected till later on this year.]

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Discussion of studies included in current review paper.

5. The current studies (not included in the 2004 review) evaluated for this discussion paper only included information from transcriptomic approaches using a number of mammalian cell lines which are either p53 proficient (TK cells) or p53 deficient (LY5178Y or NH32 cells). Test compounds used were predominantly genotoxins acting by a variety of mechanisms in the absence of exogenous metabolic activation. Two genotoxic metabolites were studied (BaP9,10-epoxide and N-hydroxy-4-acetylaminobiphenyl). Most papers report information on biological replicates and technical array replicates, hybridisation procedures, approaches to visualisation and normalisation. It is evident that there is some degree of variance between studies regarding the approach to analysis involving the use of arbitrary cut off points for selected genes, the use of different approaches to clustering analysis or gene expression pathway analysis.

6. There are a number of questions which arise from the studies reviewed in the discussion paper which would be useful aids to help structure the COM evaluation. The comments given below are intended to stimulate discussion and are based on the information in Annex 2 and 3.

a. *Is it possible to describe a gene expression profile for genotoxicants and for different classes of genotoxicants?.* Amundson and colleagues (see Annex 2) infer from their study using TK6 cells and (their equivalent p53 deficient cells NH32) that DNA damaging compounds induce a strong p53 dependent responses. An approach based on statistical significance linked to hierarchical clustering was used to derive this conclusion. . Dickinson and colleagues reported on studies using cisplatin and sodium chloride but it is suggested these two compounds are so distinctly different in biological actions that differentiating on the basis of gene expression in a mammalian cell system doesn’t really advance the evaluation of a gene expression finger print for genotoxins a great deal.

b. *Are gene expression responses due to direct acting mutagens such as alkylating agents limited to a small set of genes?* There would appear to be interstudy variation where the same alkylating agent has been tested under relatively similar conditions from different laboratories. Thus for example, in the ILSI/HESI trial Islaih reports upregulation of 8 genes following a 4h exposure of LY51578Y cells to MMS following exposure to 6ug/ml and upregulation of 46 genes at 25 ug/ml. (an exposure concentration designed to result in moderate cytotoxicity and a detectable mutagen response . The statistical analysis was based on Wilcoxons signed rank test (see Annex 2). In a further study Islaih et al 2005 reported that at concentrations designed to give rise to relatively high cytotoxicity and large increase in mutation frequency, direct acting genotoxins (MMS and MMC) produced comparatively few gene expression changes in TK6 cells compared to indirect acting genotoxins (see Annex 2). In this second study Islaih et al undertook analysis

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of effects on cell cycle for the different direct (alkylating, cross linking, reactive oxygen species/generator, and topisomerase inhibitors) and was able to correlate gene expression changes with effects on cell cycle and mutagenicity. However Hu et al who also participated in the ILSI/HESI trial using MMS under similar exposure conditions (but doses of 12.5, 50 or 100 ug/ml) report 281 genes were statistically significantly changed (based on Jonckheere-Terprestra test, a dose-response trend test). Are the differences reported between these studies using MMS due to possibly different dose levels with differences in cytotoxicity, differences in analysis of data or a combination of both of these factors? Does the additional investigations of effects on cell cycle undertaken by Islaih et al 2005 provide additional supporting data to underpin the validity of this particular experiment? One general approach that has been used in evaluating the data from transcriptomic studies has been to use data from RT-PCR analyses to confirm the gene expression results for a number of selected genes. Where the data from these two approaches dose not agree, the default has been to exclude that particular experiment form the assessment (an example is the bleomycin in LY51578Y cells reported by Islaih et al 2004). Thus in the second study by Islaih by providing data on cell cycle changes and linking this to gene expression changes the authors have provided additional supporting data for that particular study. In contrast Ricicki et al reported statistically significant genes changes for 2250 genes (using a human cDNA array of 17 569 clones based on calculation of standardised Z regression/standard error coefficient) in a study using TK6 cells exposed to n-hydroxy-4-aminobiphenyl. In this study the TK6 cells were exposed to the test material for 27h and the maximum cytotoxicity was approximately 40%.

c. *Can the gene expression changes be correlated with the measures of genotoxicity such as DNA adduct and mutagenicity particularly with regard to dose-response*? Islaih et al 2004 found a five fold increase in mutagenicity in LY5178Y cells treated with MMS at the low dose level of 6 ug/ml using a 4h exposure (20 hour recovery) but without any gene expression changes. Ricicki reported evidence to show that DNA adduction, mutagenicity of N-hydroxy-4-acetylaminobiphenyl showed different dose-response relationships. The authors reported on dose-response changes for gene expression but didn't correlate gene expression with DNA adduction of mutation.

6. Some general comments on the overall evidence reviewed are given below

a. There are substantial differences in the way in which the studies are reported. This includes variation in the level of detail of experimental methodologies numbers or microarray replicates, statistical and other analytical approaches.

b. Most of the studies reviewed used high density arrays where there would be differences between platforms in the genes and oligonucleotide sequences used. The studies are very data rich. Its difficult to compare studies even when the same compound and cell system have been used

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because of substantial differences in approaches to evaluating the data and identifying relevant gene expression changes.

c. Identification of specific gene changes (gene pathways) is likely to be useful in specific chemical mode of action evaluations and for understanding time courses and dose responses.

d. Attempts to correlate mutation frequency and adduct formation with gene changes has some limited success.

e. Clustering is widely used to examine gene expression patterns induced by classes of chemicals such as genotoxins vs non-genotoxins. There is a moderate degree of success with these approaches and probably represents an effective way for the data to be applied.

Additional studies to be evaluated.

7. There are a range of *in-vivo* studies with mutagenicity, DNA adduction and gene expression changes to be further considered. These include transcriptomic and proteomic studies.

HESI Genomics Committee Symposium, Nov 7-8, 2007

8. This November 7-8, 2007 meeting will feature the HESI Genomics Committee's first public release of results from its four 2005-2007 research programs. The presentations will cover: outputs from a multi-lab pilot studying using RT-PCR and microarray to identify gene sets capable of distinguishing direct and in-direct acting genotoxins. The ILSI/HESI presentation which can be found on http://www.hesiglobal.org/search.htm?query=genotoxicity%20working%20group; suggests that identifying differentiating patterns between genotoxins and non genotoxins is plausible.

General review publication (Annex 4)

9. A copy of a recently published review which focuses on non-covalent interacting chemicals is appended. The authors provide a useful commentary on exisiting transcriptomic investigations with DNA reactive compounds within this paper.

Questions for COM

10. Is the COM able to update its views reached in 2004, on the topics identified below;

a. The HESI/ILSI trial (COM should await outcome of the November 2007 meeting).

b. Can any further conclusions be reached on the nature of patterns of transcriptomic changes for genotoxins, differentiation with non genotoxins, correlation of transcriptomic data with DNA adduct/mutagenicity data.

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Secretariat September 2007

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DRAFT COC MINUTES FROM 12 JULY 2007 MEETING.

ITEM 5: TOXICOGENOMICS: A SELECTIVE UPDATE OF THE LITERATURE SINCE 2004 (CC/07/12)

16. This paper was prepared for COC following a review of the literature, published from 2005 until the present, which utilised gene expression profiling methods to investigate chemically induced carcinogenesis. Members were informed that a large number of papers was retrieved, many of which appeared in isolation (i.e. the only paper of its type in the period reviewed) and, therefore, the selection of papers for review was intended to group papers together and enable draw more generalised conclusions to be made. The largest group of papers retrieved compared the effects of genotoxic carcinogens to non-genotoxic carcinogens, or carcinogens to non-carcinogens. Members were informed that, although many of the papers were apparently robust, containing a large amount of relevant data, including the identification of signature genes and fingerprints, it was difficult to compare them to arrive at overall definitive conclusions. Mechanistic investigations also provided large data sets which are likely to contribute to mode of action evaluations. Members were informed that, as methodologies appeared to vary across studies (ie microarrray, statistical and analytical methods), it was again not possible to readily compare one paper with another. Furthermore, hypothesis testing may skew data interpretation.

17. The committee commented that toxicogenomics is a difficult and emerging area. Studies tended to be heterogeneous and no consensus had emerged as yet. Members considered that the studies varied in terms of cell lines and methods used, and sample concentrations, and many of the studies relate to gene expression and not mechanistic or biological effects. It was noted that pathway analysis software packages tended to provide many solutions but these may be based on changes in a very small number of genes. Looking at pathways and networks may be more productive than studying changes in individual genes. Members considered that the results of studies should be considered to be part of the weight of evidence for a chemical and that it would be useful to include them in the evaluation of a chemical by the COC, although it was probably too early for toxicogenomic data to impact on the assessment of carcinogenicity. It would also be useful to see good overview papers as they were published, e.g. interlaboratory comparisons and comparisons of genotoxic and non-genotoxic chemicals.

18. Members agreed that the COC statement should be updated in light of the information presented, particularly as the previous statement was based on a workshop discussion. The Committee requested being kept informed of developments in toxicogenomics through mini reviews of generic issues rather than general overviews.

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References

1. Barlow T et al. Report of symposium on the use of genomics and proteomics in toxicology. Mutagenesis, 18, 311-317, 2003.

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Annex 2 to MUT/07/18

TOXICOGENOMICS: AN UPDATE ON THE LITERATURE SINCE 2004

1. This paper is an update on the paper presented and reviewed in 2004, from which a joint statement with the COC and COT was issued. This statement is appended.

2. A large number of papers have been retrieved. Those presented here have been reviewed specifically with the aim of identifying any advancements in the field which may impact upon the conclusions drawn in the last statement. The use of statistical methods and bioinformatics in toxicogenomics has not been addressed as it warrants a more detailed review which is under discussion with Dr David Lovell.

3. It is of interest that a number of international initiatives addressing the usefulness and robustness of toxicogenomics approaches in chemicla risk assessment are ongoing. Of most relevance to genetic toxicology is the ILSI/HESI initiative (see cover paper)

Results from ILSI/HESI L5178Y Trial

Islaih et al 2004

4. This study was performed as part of the ILSI/HESI programme on the use of genomics in risk assessment, this aspect particularly part of the Genotoxicity Working groups contribution to the assessment. The gene expression changes induced by two different direct acting genotoxins, MMS and bleomycin, were investigated in two different cell lines commonly used in genotoxicity testing, mouse lymphoma L5178Y and TK6 cells. The two genotoxins act by different mechanisms, alkylation and DNA strand-breaks respectively.

5. Cells were treated with MMS at 6 or 25 μg/mL or Bleomycin at 1.5 or 20 μg/mL for 4 hours with or without a 20 hour recovery period. Quantification of *Tk* mutants and assessment of cytotoxicity was undertaken. RNA extracted from TK6 cells was hybridised to Affimetrix U95a human genome assay (10,000 genes) and that from L5178Y cells was hybridised to Affimetrix mouse MG-U74A array (12,000 genes). A cRNA hybridisation using biotin incorporated UTP/CTP visualised by streptavidin-phycoerythrin fluorescence. Mouse L5178Y samples from bleomycin treatment were hybridised to mouse Mu11K array (reported to be essentially the same as MG-U74A). P value was calculated using Wilcoxon's signed-rank test. Altered genes were selected based on detection and change (same direction) in two experiments with a cut-off value of 1. One array was undertaken for each time/agent/dose/ cell line from two independent experiments. Normalisation was undertaken by scaling to a common value of 1.5000 prior to using Affymetrix Microarray suite 5.0. A few select genes were confirmed by RT-PCR at 24h.

The number of genes determined to be altered by treatment with the two chemicals in the two cell lines is summarised as follows (ESTs not included):

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	L5178Y		TK	
	Up-regulated	**Down-regulated**	**Up-regulated**	**Down-regulated**
MMS				
Low 4h	8	0	0	20
24h	0	0	3	0
High 4h	46	0	6	3
24h	31	1	22	20
Bleomycin				
Low 4h	4	0	3	0
24h	0	33	5	0
High 4h	12	1	25	12
24h	0	40	133	8

6. MMS L5178Y RSG88.5%, 43.5%, MF ($x10^{-6}$) 158, 573 (0=33). Bleomycin L5178Y RSG 79%, 38%, MF 42.5, 85.0 (0=28)
MMS TK6 RSG 78.5%, 26.5%, MF 5.8, 98.1 (0=1.3), Bleomycin L5178Y RSG 73%,31.5%, MF 0.7 (cf 0=1.3), and 4.5.

7. For MMS in LY5178Y there were very few gene changes reported at the low dose at 4h. The authors focus on the upregulation of Fen1 (involved in base excision repair. There were no gene changes identified at the 24 h time point (noted there was approximately a five fold increase in MF at the low dose level). At the high dose level upregulated genes were involved in a wide range of processes including apoptosis, cell cycle regulation, stress response, transcription regulation, cell proliferation, ion transport, electron transport and protein catabolism. The only gene changes in common at the 4h and 24 h time points at the high dose were Fen1Prdx5 (protective role against oxidative stress), Atp6v1a1 and Cyb5 (hydrogen and electron transport). The three genes quantiated by PCR gave results in accordance with the microarray data.

8. In TK6 cells, at 4h, a total of 20 genes were down regulated with no upregulation reported. The genes were involved in apoptosis, mitotic checkpoints, regulation of transcription, RNA processing, cytoskeleton integrity, and protein assembly. At 20h 3 genes were upregulated including P21 involved in cell cycle arrest. At the high dose, 4h time point, P21 was one of four genes upregulated. After 24h a much wider response including upregulation DNA damage/repair genes, P21, and down regulation of genes involved in mRNA processing, protein catabolism and immune response. There were three common gene changes between 4 and 24 h (BAX (apoptosis), TNFSF9 (apoptosis) and CDKN1A (cell cycle arrest).

9. For Bleomycin in L5178Y cells the authors were surprised to note only down regulation of genes at the low dose 24 h time point compared to upregulation of two genes involved in apoptosis and one involved in cell cycle regulation at the 4 h time point. It is noted that the incvestigators were unable to confirm the gene array responses using real time PCR. There were unable to confirm the microarray results in repeat experiments. Thus the results of the bleomycin experiments in L5178Y cells are of very limited value.

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10. In TK6 cells, the low dose of bleomycin didn't result in a detectable increase in MF (hprt) but three upregulated changes were reported including p21 at the 4h time point and GADD45A and P21 at the 24h time point were documented. At the high dose many DNA damage/repair, apoptosis and cell cycle regulation genes were induced at the 4h time point. After 24h there were 123 genes upregulated and 8 downregulated. Processes involved included DNA damage/repair, apoptosis, cell cycle regulation and oxidative stress response. GADD45A, P21, TRAF1 (apoptosis), and ADA (purine metabolism) were common changes at both dose levels at 24h. RT-PCR confirmed the microarray results for selected genes.

11. Overall gene responses in TK6 cells were more comprehensive than L5178Y cells for these two compounds probably reflecting the active p53-dependent genes in TK6 cells. There was no clarly discernable pattern differences between bleomycin and MMS. An overall pattern indicative of a genotoxin was reported by the investigators involving pathways of cell cycle arrest, activation of DNA repair, and apoptosis. The authors contrast their findings to other investigators in the ILSI/HESI trial and it would be relevant to further consider this aspect.

Akerman et al 2004

12. This study, part of the ILSI/HESI project, aimed to compare the effects of Benzo(a)pyrene;s ultimate mutagenic metabolite Benzo(a)pyrene diol epoxide (BPDE) on adduct levels, and mutation frequencies with changes in gene expression profiles. Briefly, TK6 cells (number of cells exposed not stated) were cultured in the presence of BPDE (0, 0.01, 0.1 and 1.0ug/mL) for 4 hours, biological measurements were undertaken at the end of exposure and after 24 hours. Mutant frequencies for *TK* and *HPRT* loci were determined and ^{32}P-post labelling was used to quantify adduct levels. Gene expression data was generated using Human 350 low density microarray cDNA probes (Phase-1 Molecular Toxicology). Visualisation of cDNA used the Cy3-dCTP/Cy5-dCTP system with an additional split control hybridisation to account for bias in lower detection of Cy5. Microarrays were normalised using Lowess methodology, all statistical analyses and the level of significance ($P<0.001$) were determined for 8 technical replicates for each gene using students unpaired two-tailed t-test. RT-PCR was undertaken for 10 selected genes. ANOVA was used to test for effect of BPDE concentrations on measured parameter and Dunnett's was used to compare each dose level to controls. Genes expressed greater than 1.5-fold or repressed less than 0.67-fold and significantly different by $P<0.001$ compared to control. Two independent experiments were undertaken. The population growth rate, cloning efficiency and cell viability (measured by flow cytometry 24h time point only) were all reduced at 1.0 μM at 4 and 24 h.

13. Mutation frequencies were significantly increased for HPRT and fast growing TK clones at 1uM only, and evidence of a dose response at 0.1μM. dG-^{2}N-BPDE adducts were significantly increased in a dose dependent manner at both 4 and 24 hours, although consistently lower after 24hours compared to 4 hours. 800 and 9600 adducts/cell were detected at 0.1 and 1uM respectively.

14. At 4 hours only 2 genes were consistently upregulated and only at 1μM (GADD45, glutathione peroxidase). Fifteen genes were down regulated at 1μM, many

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associated with cell-cycle and apoptosis (e.g Cyclin A, c-Myc, topoisomerase-II, SRC-homology 2 domain protein) and 1 at 0.1µM.

15. At 24 hours 16 genes were upregulated and 10 down regulated at 1µM. A dose response increase in expression of GADD45, SSAT and TK and decrease for c-Myc, ODC, Cyclin A and SAMdC was reported (confirmed by RT-PCR). P53 and RAD23 were not up-regulated, confirmed by RT-PCR. The authors reported a good correlation using linear regression between the two experiments for 4h (r^2=0.91) and 24 h (r^2=0.89)

16. Most notable, was that gene expression changes were detected only at concentrations which caused cytotoxicity but not at lower doses at which adducts were detected. The authors considered the pattern of gene expression changes were consistent with DNA damage and cell stress. This may be associated with the cell type, or the timings of sampling in relation to treatment. Discussed were the alterations in genes associated with c-Myb, and those apoptosis and cell cycle arrest/progression(e.g c-myc, cyclin A, p55CDC) and p53/GADD45. However it is concluded that the lack of response may be due to the test system and that apoptosis in invoked in TK6 cells rather than DNA repair following exposure to BPDE. The rationale for using a low density array as opposed to a high density array was not provided.

Amundson et al 2005

17. TK6 cells and their p53 null derivative NH32 cells, were exposed to a large variety of DNA and non-DNA damaging stresses with the aim of understanding p53 responses to these stresses. Osmotic shock, heat shock and 12-o-tetradecanoylphorbol 13-acetate treatment were used as non-DNA damaging stressors. Genotoxic agents included ionizing radiation, H_2O_2, sodium arsenite (the later two as oxidative stressors), MMS $CdCl_2$ and cisplatin. Cells were treated for 4hrs (at single concentration,) a time-point considered to be appropriate for the measurement of early gene responses. No rationale for dose selection was given. Cytotoxicity and mutagenicity measurements were not reported.

18. RNA was extracted and cDNA synthesised (using ^{32}P incorporation to aid in determination of hybridisation). Hybridisation was undertaken using a high density array (source not reported). Visualisation was undertaken using Cy3/Cy5 system. Normalisation was undertaken using 88 house keeping genes (set to 1.0). Gene selection and clustering analysis was undertaken using four procedures; pair wise statistic, distance based methods, Wilcoxon-Kruskal Wallis and class correlation methods. Genes that had a statistical weight greater than expected for 10,000 randomised interactions were considered discriminating. Final discrimination sets were based on statistical significance in at least 3 out of the 4 methods used. The microarray contained 7668 identified genes, and from these a set of 1451 genes were selected which were considered to be good quality target genes from preliminary experiments. Multidimensional scaling (MDS) plots were used to group responsive genes.

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19. A strong general mechanism of action was indicated and thus further subsets of genes (346) were defined to distinguish between the four stressor groups; non-DNA damaging, ionizing radiation (IR), non-IR DNA damaging and oxidative stressors. A set of 117 genes discriminated between DNA and non-DNA reactive mechanisms. Signatures were defined for each treatment, a notable signature identified following treatment with CdCl2 (heavy metal). The $CdCl_2$ experiment was undertaken separately as a test of the discrimination pattern system. The closest pattern was to that of arsenite.

20. Analysis of the changes in the p53 wild type vs p53 null cell types: from the 1451 gene set, only 13 were identified that discriminated p53 status. However, the MDS plot showed that genotoxic treatments elicited a strong p53 dependent response, which was not shown by non-genotoxic treatments. The genes used to discriminate p53 status were CDKN1A, CCNG1, BA13, BTG1, ST14, PHLDA3, PLXNB2, MDM2, LIF, BTG2, PPMID, CTSD, TRIM22, XPC, DDB2, TNFSF9.

21. Where data for analysis of p53 status was repeated suing genotoxicants a 16 gene signature was identified that discriminated p53 status broadly across all genotoxicants. (including 13 genes reported above and BTG1, BTG2 and ST14)

22. The authors suggest this work identifies a gene set for future studies and that this approach can be used to predict mechanism based toxicity. Further analyses were undertaken using Expression Array Systematic Explorer (EASE). For genotoxicants molecular functions most represented in the data set were DNA binding, regulation of nuclei acid metabolism, transcription factor activity, nuclei acid binding, mitotic cell cycle.

23. This study provides some evidence that discriminant analysis of high density microarray data can identifiy genotoxic compounds. No data to correlate the findings with mutagenicity or the time course of gene responses were provided. The authors considered that p53 genotype was not a major factor determining gene response for non genotoxicants.

<u>Dickinson et al 2004</u>

24. This investigation focused upon comparing gene expression changes with the genotoxic endpoints. L5178Y cells were treated with cisplatin or sodium chloride and micronuclei, DNA-protein cross links and platinum-DNA adducts were measured concurrently.

25. RNA was hybridized to Affimetrix Genechip and expression levels were computed from Affimetrix probe level data using Iziaarry's Robust Multi array Average (RMA). Differences in log 2 base expression were compared to controls using ANOVA. Expected fraction of false positives 1/20 using false discovery rate (FDR).

26. Increases in MN were observed following treatment with both cisplatin and NaCl, for cisplatin there was a sharp decrease at 100µM (compared to 10 and 30µM).

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Selection of 50% reduction in growth was used to provide equitoxic concentrations for the assessment of gene expression profiles.

27. Gene expression data indicated 176 genes with statistically significant changes for at least one treatment. These data were used to generate principle component analysis plots (PCA). There was little overlap of gene expression changes between the two compounds examined, with 14 in common. In cisplatin treated cultures, 71 genes yielded clear dose responses– although most only of modest <3-fold increases. These included GADD45 family, cFos, CDK-4 binding protein, these increases were linearand directly reflected the amount of Pt adducts. MN frequency did not correlate with adducts numbers.

28. NaCl caused statistically significant changes in 55 genes. In contrast to cisplatin, most were down regulated, were only modest repression and many remained unidentified.

29. In conclusion, there were clear differences between DNA reactive and non-DNA reactive compounds when examined in this test system.

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Studies using TK6 cells

Islaih et al 2005

30. In this study, TK6 cells were used to examine the gene expression changes following genotoxic stress induced by diverse modes of action; MMS and MMC (mutagens), H_20_2, bleomycin (reactive oxygen species) and etoposide doxorubicin (topoisomerase inhibitors) with the aim to investigate the differing DNA repair and other cellular responses.

31. TK6 cells were treated with the mutagens (single concentration) for 4hr and then allowed to recover for 20hr. Cytotoxicity assays and mutant frequency studies were conducted and dose levels were selected so that each compound induced a comparable effect (ca 65% cytotoxicity). Cell cycle analysis was also undertaken, using flow cytometry after 8 and 24hrs. RNA was hybridised to two separate AffimetrixGeneChip HuGeneFL microarrays (5600 human genes supplemented with additional genes). Affimetrix Microarray Suite Software was used to analysis data – criteria 1.5 fold change consistently between duplicate hybridisations. Confirmation of some genes by RT-PCR was undertaken. RSG after 48h was between 20-36%. An increase in MF was not documented for bleomycin or H_2O_2. One sample of RNA was used for each genotoxicant with duplicate arrays run (i.e no replicate cultures used).

32. It was demonstrated that, at 8 hrs, all treated cultures had a significant increase in the number of cells in S-phase, but at 24hrs etoposide was the only compound to sustain the S-phase arrest. Data for MMS, MMX, doxorubicin showed G2/M arrest at 24h. Bleomycin and H_2O_2 showed an increase in percentage of cell population in G_0/G_1 phase at 24hr. An inverse relationship between mutation frequency and the number of altered genes was noted when number of transcripts altered was plotted agains MF. H_2O_2 altered the expression of the greatest number of genes (150), followed by etoposide (128), bleomycin (84), doxorubicin (69), MMC (~40) and MMS (~20). Induction of target genes that are involved in the p53, TNF, ERK or JNK pathways were highlighted although magnitudes of changes were not presented.

33. Quantitative RT-PCR confirmed the pattern of gene expression for p53-activated transcripts p21 and GADD45a. A 2-5 fold increase in p21 and GADD45a was reported at 4 and 24 h for MMS amd MMC. For Bleomycin and H_2O_2 , p21 and GADD45a were increased 25 fold at 4h but this decreased quickly thereafter.

34. Pathway analysis was also conducted using Pathway assist software which looks for known direct interactions between genes. It was noted that 46% of altered genes had one or more direct interactions; such as p53, cyclin/cdk, TNFα, TGFβ pathways and chemokine receptors. Each compound activated most or all of these pathways suggesting similar provoked responses in the tests system. The authors considered the gene expression changes induced by genotoxicants were not random.

35. A direct relationship was noted between p53 activation, G2 arrest and mutagenic potency. MMS and MMC increased transcript levels of p21 and GADD45α and caused 50% of cells to arrest in G2 at 24hrs. In contrast the transient increases

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induced by H_2O_2 and bleomycin were reflected by a return to more normal cell cycles at 24hrs. There appears to be some consistency between the pattern of gene expression changes and the point of cell cycle arrest.

36. Therefore, this paper provides some evidence that high density arrays could distinguish between gene responses in TK6 cells exposed to direct and indirect acting genotoxicants. The study was limited by not investigating the effect of biological replicates in the test system or effect of dose-response.

Rickicki et al 2006

37. Herein gene expression profiles were compared to N-(deoxyguanosin-8-yl)4-aminobiphenyl adducts and mutation frequency (HRPT and TK loci) in TK6 cells after treatment with N-hydroxy-4-aminobiphenyl. Cells were treated for 2, 6 or 27 hrs with 0.5, 1.0 and 10μM. Human cDNA microarrays were constructed with 17 569 clones from Resgene Human Unigene Set and labelled cDNA's hybridised in triplicate. Mean log2 ratios of fluorescence were clustered using J-Express Software unsupervised algorithm. Normalised data were analysed using statistical and visualisation approaches. A 2-fold increase was used as an arbitrary cut-off for inclusion in the hierarchical clustering.

38. A total of 2250 genes exhibited statistically significant changes ($P<0.05$); 2245 up regulated, 5 down regulated. A total of 521 were found to change under the conditions emplotyed using the 2-fold cut-off criteria and these were used in the clustering. Upregulated genes highlighted included those identified as being a stress response, inflammatory response, tumour suppressor and oncogenes and those associated with cell growth and cell death. Some showed clear dose responses, whereas others showed saturation of the response at a low dose.

39. Although this study appears to have closely examined adducts mutations and gene expression changes and time-courses and dose-responses were explored, no attempts were made to correlate one with the other and only generalised comments were made.

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Studies using L5178Y TK^{+}/$^{-}$ cells

Hu et al 2004

40. This study aimed to compare direct and indirect acting genotoxicants using L5178Y cells. The chemicals examined were: direct acting, DNA x-linking agents MMC and cisplatin; an alkylaying agent MMS; and indirect acting agents hydroxyurea (ribonucleotide reductase inhibitor), taxol (microtubule inhibitor) and etoposide (topo II inhibitor). Three independent studies for each chemical were conducted in which cells were exposed to chemicals for 4 hours and harvested 4 or 20 hours post dosing. Cytotoxicity and micronuclei (MN) were also assessed as direct comparitors.

41. For gene expression profiling, Affimetrix microarray was used with a total of 9977 probe sets. Data analysis utilised ANOVA for general treatment effects on log-transformed signal values and nonparametric test for trends (Jonckheere-Terpestra). Analyses assessed: 1) treatment effects for genes regulated by all 6 chemicals 2) genes differentially expressed between direct and indirect acting chemicals. Hierarchical clustering analysis was also used with Euclidean distance measure of fold chance for genes $p<0.001$ to discriminate between indirect and direct genotoxins at both timepoints.

42. At 4 hours MMS and HU altered the expression of 281 and 148 genes respectively. The other chemicals only altered small numbers of genes (20-38). At 24 hours, all chemicals induced large numbers of changes (151-643). At 4 hours 38 genes were affected by all chemicals, most of which were involved with processes expected to be involved in DNA damage such as cell cycle, transcription regulation and chromosome organization. At 24 hours, there were 1046 genes with $p<0.0001$; 39 were singled out as particularly significant and included those involved in signal transduction, immune/stress response, transcription regulation and protein synthesis.

43. Discrimination of direct-acting vs indirect-acting chemicals was shown by hierarchical clustering. At 4 hours, it was demonstrated that 43 genes were differentially expressed in cells treated with direct compared to indirect genotoxins. Analysis of the biological pathways showed that there is suppression of cellular survival pathways by direct acting chemicals and moderate activation of apoptosis pathways by indirect acting chemicals. At 24 hours, a set of 58 genes discriminated the two classes of chemicals. It is considered that fingerprints can be generated to classify chemicals of unknown mechanism of action.

Kim et al 2005

44. L5178Y Tk $^{+/-}$ cells were treated with 1,2-dibromomethane, glycidol (genotoxic carcinogens), 8-hydroxyquinolone, emodin (genotoxic non-carcinogens), methylcarbamate, o-nitrotoluene (non-genotox carcinogens), d-mannitol and 1,2-dichlorobenzene Inon-genotoxic non-carcinogens). Dose levels used were those that induced maximum mutation frequencies in the absence of excessive toxicity for the positive chemicals and 90% viability or 5000µg/mL for the others. TwinChip Mouse 7.4K microarray used (7,400 genes); gene expression ratios were normalized using LOWESS regression. Data analyses included q-value computing, SAM and

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hierarchical clustering (Eisen Lab Web). Genes were considered differentially expressed when log ratios in 4 independent hybrisdisations were more than 1 or less than -1 (ie 2-fold difference in expression, q values <5%).

45. One gene consistently unregulated by genotoxins was *Trp63* (although not identified using real time PCR), which is required for p53 dependent apoptosis. In total there were 10 genes consistently altered by the genotoxins and 7 by the carcinogens.

46. Clustering categories included signal transduction, cell cycle control, cell growth and death, response to stress, transcription and apoptosis. >2 fold changes only considered. Differences in expression profiles for non-genotoxins vs genotoxins were noted for cell cycle control, response to stress and immune response related genes. No gene clusters were found in any category that distinguished carcinogenic from noncarcinogenic chemicals. Genes altered by nongenotoxic noncarcinogens were excluded.

46. It was concluded that although gene expression analyses are potentially useful in the evaluation of genotoxicity, the diversity of mechanisms of carcinogenesis means there are inherent limitations in the application of the technique.

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