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

COT statement on the use of toxicogenomics data in risk assessment

Introduction

1. The term “toxicogenomics” refers to the generation of large quantities of biological information by a range of techniques, and the analysis of such information in a way that can be applied in toxicology. Genomics, proteomics and metabonomics (collectively abbreviated to ’omics) are disciplines which give insight into changes in the regulation of genes, proteins and metabolism respectively, that are induced by endogenous and exogenous chemicals. Investigations can be undertaken at the level of the whole organism, or in model *in vitro* systems. Toxicogenomics (TGX) is the integration of ’omic technologies, bioinformatics and toxicology.

2. There have been major developments in TGX in recent years, due largely to investment by the pharmaceutical industry. This has included use in predictive and mechanism-based toxicology in efforts to identify new drug molecules and to improve understanding of the effects of older substances. TGX methods are particularly being investigated as a way of improving the prediction of human responses to chemical exposures. There is an aspiration that in the future, TGX will play a role in the routine assessment of chemical toxicity.

3. The purpose of this statement is to review the aspects of chemical risk assessment to which TGX tools could contribute. This is in line with the COT’s remit to advise on important general principles and new scientific discoveries that bear on the understanding of toxic risks.

4. The statement outlines important developments in the field since the last COT statement on the topic was published in 2009, and summarises COT’s conclusions on points that require consideration when interpreting TGX studies as part of risk assessment. Potential applications for TGX in risk assessment are discussed in the context of what is now state-of-the-art, with reference to examples for illustrative purposes. Whilst TGX embraces all of the major ’omics, the statement focuses on approaches that entail analysis of gene expression (transcriptomics). This is the most mature area of TGX, and wider, integrated TGX approaches have so far not been much applied in risk assessment. However, it is likely that the Committee’s conclusions on transcriptomics could in the future apply also to other ’omic technologies.

Previous COT consideration of toxicogenomics

5. The COT, jointly with the Committees on Mutagenicity (COM) and Carcinogenicity (COC) considered TGX tools in 2002[1] and 2004[2], and TGX was discussed at an open workshop on 21st century toxicology held by the COT in 2009[3]. Also in 2009, the Committee discussed pathway analysis (a method of extracting meaningful information from large 'omic datasets, which can then, for example, aid the determination of a mode of action (MOA))[4]. In 2010, the COT began a further review of progress in the field by considering fundamental aspects of TGX: study design, analysis of results and statistical methods[5]. During 2011 the Committee considered a paper published by United States Environmental Protection Agency (U.S. EPA) that outlined a framework for the use of TGX in risk assessment[6].

Developments in "omics" since the last COT statement

6. In recent years, there has been rapid expansion in the use of high-throughput and high-content 'omic methods, in research on a large number of diseases, chemical exposures and biological species. To interpret this information fully, it has been necessary to develop a range of software tools that will provide indications, within a dataset, of potentially important perturbations in biological networks (the nature of a network will depend on the analytical approach being taken – for example, it could comprise a set of inter-related genes - but it will not necessarily represent a single linear pathway, since a compound could affect several different metabolic pathways). The aim is that outputs from analytical software should be amenable to presentation in a way that is understandable by non-specialists. In addition, outputs should be searchable so that others can assess the underlying data.

7. The availability and robustness of pathway analysis tools has improved dramatically. There are, however, a number of important caveats associated with their use. In particular, it should be recognised that they indicate the existence of networks as statistical probabilities, and not with certainty. Thus, it is important that the tools be seen as starting points for analysis, providing leads to further investigation rather than definitive results (i.e. they are hypothesis-generating). The putative impact of a derived network on biological functionality must ultimately be proven experimentally before it can be firmly accepted (Plant et al., 2009).

Applications for toxicogenomics in risk assessment

8. The Committee has identified six areas in which appropriately designed TGX studies might contribute to risk assessment:

[1] http://cot.food.gov.uk/pdfs/JointCOT-COM-COCStatement.PDF
[2] http://cot.food.gov.uk/pdfs/cotstatementtoxicogen0410.pdf
[3] http://cot.food.gov.uk/pdfs/cotstatementwkshp200903.pdf
[4] http://cot.food.gov.uk/pdfs/tox200935.pdf
[5] http://cot.food.gov.uk/pdfs/tox201018.pdf
[6] http://cot.food.gov.uk/pdfs/tox201122.pdf

- Aiding the assessment of toxicological mode of action (MOA).
- Providing information on inter-species variability and extrapolations.
- Modelling effects at doses lower than those resulting in overt toxicity.
- Interpreting or facilitating read-across between similar chemical structures.
- Aiding the development of *in vitro* models to replace animal models.
- Identification of biomarkers.

Examples of how TGX data might be useful in risk assessment

9. The following sections give examples of publications that have applied TGX in a way that could be useful in risk assessment. Examples were identified for the first four of the applications listed above. Where possible, publications were selected that reported better-designed studies showing a greater potential to inform risk assessment.

Applications of TGX in studies of MOA

10. In an MOA study, Naciff et al. (2005) examined the effect of a range of doses of three different oestrogen receptor agonists (17 alpha-ethynyl estradiol (EE; 0.001-10 µg/kg bw/d), genistein (0.001-100 µg/kg bw/d) and bisphenol A (BPA; 0.002-400 µg/kg bw/d)) given subcutaneously to pregnant Sprague Dawley rats over gestation days 11-20. These doses did not elicit morphological changes in development of the testis or epididymis in the fetus. One dam in the group given the top dose of EE experienced vaginal bleeding and an early parturition. Male and female fetuses of dams exposed to the highest EE and BPA doses had prominent nipples/areolas. EE, genestein and BPA exposure led to dose-related changes in the expression in pooled tissue (testicular and epididymal tissues from five or more litter mates) of 59, 23 and 15 genes respectively.

11. Naciff et al. (2005) concluded that the dose-response characteristics for the affected genes in male rat reproductive tissues were monotonic for each compound. The authors noted that robust quantifiable gene responses were not elicited by the lowest doses. The Committee considered this study to be of good quality, because of the phenotypic anchoring and focus on dose-response relationships. The authors suggested that gene expression changes could aid the identification of MOA and support the assessment of dose-response characteristics for toxicological endpoints. The Committee noted that for those expectations to be realised, other substances with an MOA operating through the same pathways would need to be studied. The evidence of a monotonic response in a study incorporating low doses was considered to have particular value for risk assessment.

12. A paper by Currie et al. (2005) also exemplified the use of pathway analysis and phenotypic anchoring of gene changes to stages of biological change in the investigation of MOA. The data analysed came from a study published previously by the same group (Moggs et al., 2004), which had investigated the effects of administering a single subcutaneous 17β-estradiol dose (400 µg/kg bw) or vehicle to

immature female CD-1 mice over time using the rodent uterotrophic assay. Groups of animals were sacrificed at intervals up to 72 hours following exposure.

13. Vaginal opening was recorded, the uterus was removed, weighed and examined histologically, and transcriptomic analysis was performed on pooled uterine samples. Moggs et al., (2004) noted that uterine growth and maturation following exposure to 17β-estradiol was preceded and accompanied by multiple stages of molecular changes. The pattern began with the induction of genes involved in transcription regulation and signal transduction. That was followed, sequentially, by the regulation of genes involved in protein biosynthesis, cell proliferation, and epithelial cell differentiation. Currie et al. (2005) took into account biological networks and gene ontology to interpret the gene expression data in terms of affected biological processes and pathways, and phenotypic changes.

Application of in vitro *TGX in investigating inter-species differences*

14. Goetz and Dix (2009) investigated which perturbations of biological pathways were similar in rats and humans following exposure to three triazole fungicides. Myclobutanil, propiconazole and triadimefon each induced varying degrees of hepatic toxicity, disruption of hormone homeostasis and adverse reproductive outcomes in rodents. Microarrays containing 31,000 rat or 54,000 human gene probes were used to assess changes in the livers of rats dosed orally and in primary cultures of rat and human hepatocytes in order to explore extrapolation across species and between *in vivo* and *in vitro* systems. Pathways that were commonly affected by each triazole in the rat and human models were observed to be related to lipid metabolism, sterol homeostasis, steroid homeostasis and xenobiotic metabolism.

15. It was considered by Goetz and Dix (2009) that the perturbations represented a plausible MOA for effects of triazoles on reproductive outcomes and serum testosterone. Noting the degree of difference between the sizes of the rat and human arrays, the Committee considered that incomplete concordance in expression profiles would not necessarily reflect species differences, and could be a technical artefact. Moreover, even if real, observed inter-species differences might be unrelated to toxicity. The Committee considered that many of the probes would be redundant or of unknown function. Inter-species differences in gene transcription do not necessarily imply a different toxicity profile, and there had been no consideration of the nature of effects that would be expected in human cells.

Application of TGX in modelling effects at doses lower than those resulting in overt toxicity

16. Benchmark dose (BMD) modelling was applied by Bercu et al. (2010) to changes in the expression of genes in the liver induced by two non-genotoxic hepatocarcinogens. In order to undertake BMD modelling, the authors used the BMDExpress software published by Yang et al. (2007). Fenofibrate (30 or 1000 mg/kg bw) was administered orally twice per day by gavage for two days to groups

of three female rats (strain not specified). Methapyrilene (10 or 100 mg/kg bw) was administered orally by gavage once per day over a period of 1, 3 and 7 days to male Sprague-Dawley rats (Waring et al., 2004). Approximately 9000 genes were probed and assigned to groups representing pathways and processes which the authors noted were likely to encompass key events for carcinogenicity. $BMDL_{10}$ values[7] were calculated for each gene probe considered to be significantly perturbed by one-way analysis of variance. Where multiple probes were found to correspond to a single gene, the $BMDL_{10}$ values were averaged.

17. Sets of biologically-related genes were grouped together. The authors then estimated the "lower 95th percentile of all $BMDL_{10}$ values"[8] (sic.) for each group of genes. The lowest of those group "lower 95th percentiles" was proposed by Bercu et al. (2010) to offer a potential reference point for risk assessment. An objective was to see if modelling of genomic changes at low doses in short-term studies could provide reference points (referred to as points of departure) for use in risk assessment as a surrogate for a reference point from a chronic study.

18. The calculated $BMDL_{10}$ values for gene expression changes were compared to no observed adverse effect levels (NOAELs), lowest observed adverse effect levels (LOAELs) and $BMDL_{10}$ values for precursor events and for tumorigenesis. Fenofibrate exposure led to gene expression changes in seven groups of biologically-related genes, for which the "lower 95th percentiles" of the distribution of the $BMDL_{10}$ values were between 2.4 and 3.6 mg/kg bw. These values were lower than the reported $BMDL_{10}$ values for peroxisomal β-oxidation activity of 5 mg/kg bw per day in a 4-day study and of 40 mg/kg bw per day for hepatocellular carcinoma. Following 7 days of methapyrilene dosing, the "lower 95th percentiles" of the distribution of the $BMDL_{10}$ values for biologically-related genes were between 7 and 53 mg/mg bw, whereas for cell proliferation in a 13-week study, the NOAEL was 4 mg/kg bw per day and the LOAEL was 10 mg/kg bw per day. The $BMDL_{10}$ for methapyrilene-induced hepatocellular carcinoma was 4 mg/kg bw per day.

19. This type of surrogate approach will be valid only if the critical tissue(s) are sampled. Another methodological challenge is the timing of sampling in order to capture high responses in transient effects, since in general, the identity of critical effects will not be known *a priori*. The reported investigation was an attempt to address questions relating to both the sensitivity and the timescale of gene expression changes, but did not reach definitive conclusions on either. Gene changes were reported at doses below those that had caused cancers in laboratory animals. It was unclear, however, whether the gene changes were relevant to the MOA for carcinogenicity. It would have been more useful to have investigated MOA separately from the question of whether gene changes could be used for determining a reference point for non-genotoxic carcinogenesis.

[7] The BMD_{10} is the dose that is estimated, using statistical models, to a produce a 10% change in an outcome measure. The $BMDL_{10}$ is the lower 95th confidence limit of a BMD_{10}.

[8] Members noted that the meaning of "lower 95th percentile" was unclear and assumed it could be either a 2.5th or 5th percentile.

Application of TGX in read-across between similar chemical structures

20. Gu et al. (2007) used cardiac gene expression changes to investigate the S and R enantiomers of each of two structurally-similar inhibitors (called 1 and 9) of acetyl-CoA carboxylase II (ACC2), which were candidates for use in treatment of type 2 diabetes. Inhibition of ACC2 was seen with the S enantiomers but not the R enantiomers, whereas cardiotoxicity had been seen with substance 1, but not substance 9. ACC2 is expressed in the mitochondrial outer membrane of oxidative tissues. Groups of rats were dosed twice daily for 3 days with 100 mg/kg of one of the four enantiomers, and gene expression profiles were measured in the heart. Both enantiomers of substance 1, but not of substance 9, had a heart tissue transcript profile similar to that of the positive control cardiotoxic agent, doxorubicin.

21. The changes induced by substance 1 were in the expression levels of genes involved in mitochondrial oxidative phosphorylation. The authors concluded that the observed cardiotoxicity was most likely to be independent of ACC2 inhibition and probably related to an alkyne moiety that was present in substance 1 but not substance 9. Whilst the approach taken by Gu et al. (2007) related to lead candidate molecule selection within drug discovery, it is of relevance to risk assessment. For example, a question might arise as to whether a substance that was poorly characterised toxicologically (perhaps a contaminant) could induce a particular effect that had been observed in only a sub-set of structurally-similar substances.

Considerations when interpreting TGX studies in risk assessment

22. There are major limitations to the value for risk assessment that can be derived retrospectively from TGX studies which were originally designed for other purposes. Dose selection may not suit the question at hand, analytical and statistical methods may be sub-optimal by contemporary standards, and the original data may be unavailable for reanalysis. Ideally, experiments should be designed to address specific questions in risk assessment, and use a range of doses to support dose-response modelling. Where they are available, it may be more informative to reanalyse original TGX data, rather than to base interpretation on historical analyses. There could be benefits in developing rules for data-generation to make datasets more amenable to reanalysis should this be needed to address future questions.

23. It is not possible to reach firm conclusions on MOA from TGX results in isolation. However, TGX offers a way of generating hypotheses about potentially relevant pathways, which could be investigated further in follow-up work. Where TGX changes were consistent with an established MOA, it would be important not to ignore other changes that did not relate to that particular MOA, and which might point to other MOAs. Unless a single gene is known to be critical, investigations should look at clusters of genes for relevant pathways.

24. It is important to be able to distinguish critical effects from other effects. As chemicals frequently induce multiple toxicological and adaptive responses, it will not always be straightforward to distinguish which TGX changes indicate MOAs for critical effects. TGX changes seen at high doses or later time points could reflect secondary effects. The observation of a TGX effect (e.g. a change in gene

expression) does not necessarily imply that an adverse effect has occurred at the dose in question or would follow in time or at higher exposure.

25. Appropriately designed TGX studies might aid qualitative assessments of whether a particular MOA could or could not occur in different species. However, apparent homology between species could be misleading because even minor differences in a single gene can alter function substantially. Ultimately, determining human relevance from toxicogenomic results may not be possible without recourse to more focused investigations.

26. Knowledge of the internal dose at a particular target site is important, but not necessarily straightforward to generate. An internal dose could be extrapolated to an external dose for use in risk assessment by, for example, use of physiologically-based pharmacokinetic modelling.

27. For TGX changes to be useful in risk assessment, it is essential that they be clearly linked to specific pathways relevant to toxicological effects. In some cases reported TGX findings may relate not to a toxic MOA but to non-critical physiological pathways (e.g. of metabolism).

28. Transcriptional changes could not provide a basis for establishing a health-based guidance value unless there were clear evidence that the relevant gene was critical to the MOA.

29. TGX outcome measures need to be sufficiently reproducible within and between laboratories. Challenges to establishing reproducibility could relate to differences in dose levels, the timing of measurements and methods of statistical analysis, and to unstandardised analytical technology.

30. In any use of TGX as part of risk assessment, the requirements for sensitivity and specificity will depend on the question at hand. An example of an application requiring relatively high sensitivity would be the assessment of whether a particular MOA was relevant to humans. Another would be the use of a TGX result as a biomarker to assess whether a substance could induce an effect for which there was a structural alert. Specificity would be a priority in the assessment and comparison of dose-response relationships.

Case study: application of TGX to the risk assessment of dibutyl phthalate

31. In 2009 the U.S. EPA published a document entitled An Approach to Using Toxicogenomic Data in U.S. EPA Human Health Risk Assessments: A Dibutyl Phthalate Case Study (US. EPA, 2009a; US. EPA, 2009b). The Committee considered this a useful reference document and noted that it illustrated well the problems that can be encountered when using existing TGX data to address new questions in risk assessment. The systematic approach to the evaluation and use of toxicogenomic data in health risk assessment that was outlined by the U.S. EPA was directed at their risk assessors and the wider scientific community.

32. COT Members observed that in comparison with most substances that might be evaluated, an exceptionally large number of TGX datasets were available for dibutyl phthalate (DBP). Even so, it was difficult to draw conclusions of relevance to

risk assessment. The Committee noted that that if conclusions for risk assessment could not be drawn from a TGX evidence base the size of that for DBP, it would be even more difficult to make use of historical studies for substances which had been less investigated.

33. Across the DBP publications that had been examined by the EPA, many different statistical approaches had been applied to address problems associated with multiple testing, and there was no consensus on the best method. In some studies, Bonferroni correction had been used, but this was simplistic since prior expectations were likely to differ from one finding to another. It was observed that some of the most striking TGX changes could stem from the way in which data were analysed, and that better standardisation of statistical methods was desirable. The Committee considered that the EPA's proposed framework for using TGX in risk assessment, was useful, but emphasised that it would best be applied to studies designed to answer specific questions of relevance to risk assessment.

34. Within the EPA assessment of the TGX studies of DBP, reference was made to alterations in the expression of the 3 beta-hydroxysteroid hydrogenase (3β-HSD) gene at a lowest dose of 0.1 mg/kg b.w. per day. The Committee had previously[9] noted that the Tolerable Daily Intake (TDI) for DBP established by the European Food Safety Authority was based on a LOAEL of 1.5 mg/kg bw per day. For risk assessment to be based on transcriptional changes, the Committee considered that there should be clear understanding that the specific gene was critical to the MOA. In the case of DBP, a dose-response relationship was not shown for 3β-HSD, and therefore the Committee concluded that this change should not be used as the basis for a TDI.

Conclusions

35. TGX data could contribute to risk assessment in a number of ways. As part of hazard identification, TGX could aid the assessment of toxicological MOA, read-across between structurally-similar substances and the development of *in vitro* predictive models. In hazard characterisation, TGX could inform assessment of inter-species differences, allow modelling of effects at sub-pathological doses, and might in the future assist the establishment of heath-based guidance values. TGX could also contribute to exposure assessment and risk characterisation through the identification of biomarkers, although the Committee identified no examples of such applications to date. Ideally, experiments should be designed to address specific questions in a risk assessment and use a range of doses to support dose-response modelling.

36. Toxicogenomic studies are often hypothesis-generating. Where TGX changes are consistent with an established MOA, it will be important not to ignore other TGX changes. However, care will be needed in their interpretation. The observation of a TGX effect following a particular exposure may not mean that an adverse effect was occurring at that time. However, it is also possible that it is part of a pathophysiological response to unobserved toxicity. For TGX changes to be

[9] http://cot.food.gov.uk/pdfs/tox201036.pdf

useful in risk assessment, it is essential that they be clearly linked to specific pathways relevant to toxicological effects. TGX measurements used as part of risk assessment need to be sufficiently reproducible within and between laboratories, and their sensitivity and specificity must be adequate for the purpose to which they are applied.

37. Where relevant TGX studies were available, they could be used in risk assessment as part of a weight of evidence approach alongside the results of studies using other experimental approaches. However, transcriptional changes could not provide a basis for establishing a health-based guidance value unless there were clear evidence that the relevant gene was critical to the MOA.

38. Whilst TGX embraces all of the major 'omics, these COT conclusions are based on studies using transcriptomics, since this is the technology that has been most widely applied to date. In the future, these conclusions concerning transcriptomics may be applicable to other 'omic techniques and integrated approaches.

39. TGX is a rapidly emerging field and further developments should be reviewed by the COT in the future.

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Abbreviations

3β-HSD	3 Beta-hydroxysteroid hydrogenase
BMD	Benchmark dose
BMD_{10}	The dose producing a 10% change in an outcome measure
$BMDL_{10}$	The lower 95th confidence limit of a BMD_{10}
BPA	Bisphenol A
DBP	Dibutyl phthalate
EE	17 Alpha-ethynyl estradiol
LOAEL	Lowest observed adverse effect level
MOA	Mode of action
NOAEL	No observed adverse effect level
TGX	Toxicogenomics
U.S. EPA	United States Environmental Protection Agency
TDI	Tolerable Daily Intake