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

First draft statement on COC/G07: Part c) Emerging technologies: omics and high-throughput screening

Attached is the first draft of this Guideline Statement. This is based on the overview paper, CC/2016/14, which was discussed at the November 2016 COC meeting. The conclusions in Section 5 are based on the views and opinions expressed by the Committee during discussions at the meetings in July and November 2016.

Members are invited to comment on the content of the paper and the conclusions.

Imperial College Toxicology Unit, supported by PHE
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Alternatives to the 2-year Bioassay

COC/G07: Part c) ***Emerging technologies: omics and high-throughput screening***

Abbreviations

BMD	Benchmark dose
EPA	Environmental Protection Agency
HTS	High-throughput screening
PoD	Point of departure
Tox21	Toxicology in the 21st Century
ToxCast	Toxicity Forecaster
TT21C	Toxicity Testing in the 21st Century

1. Introduction

C1. Characterisation of the carcinogenic potential of the vast number of untested chemicals present in the human environment using conventional *in vivo* bioassays is not feasible and alternative methods are required. New approaches that are being developed include omics technologies and high-throughput screening (HTS) assays. The goal of these approaches is to develop predictive methods that are rapid, cheaper than current bioassays, and/or high throughput, based on human-relevant mechanisms of carcinogenesis.

2. Omics technologies

C2. The collective term 'omics' refers to the genomic (DNA sequence analysis) and post-genomic (e.g. transcriptomics, proteomics, metabolomics, epigenomics) technologies that are used for the characterisation and quantitation of pools of biological molecules (e.g. mRNAs, proteins, metabolites), and the exploration of their roles, relationships and actions within an organism (Ward & Daston, 2014). The term 'toxicogenomics' is sometimes used to describe the application of omics technologies to the study of adverse effects of toxicants or environmental stressors (Waters, 2016). The Committee will use the term 'omics' out of preference as it avoids the suggestion of being focussed on genomic techniques.

C3. Chemicals producing similar types and levels of toxicity are expected to share similar gene, protein or metabolite expression profiles, and such patterns of toxicant-induced molecular changes ('fingerprints' or 'signatures', sometimes referred to as biomarkers) can be used to assess toxicity. Omics methods may identify changes at much earlier time points than adverse effects observed at the tissue, organ or whole-organism level, and the post-genomic technologies can be used to follow toxicant-induced changes dynamically. Omics methods produce large amounts of biological information that can be integrated and analysed using bioinformatics tools.

C4. The aim of predictive omics in carcinogenicity evaluation is to create high-resolution profiles of biological responses, to map causal events, processes and pathways that occur as a function of dose and time, reflecting carcinogenic modes of action (Waters, 2016). Omics methods are not yet suitable as high-throughput screening tests, but have shown great utility in determining mechanisms of action of chemical carcinogens, and as a prioritising and/or predictive tool for carcinogen identification. They are being developed to evaluate the effects of exposures to genotoxic and non-genotoxic carcinogens, *in vivo* and *in vitro*. Studies *in vivo* have been used mostly to identify mechanisms of carcinogenicity in rodents (e.g. Guyton et al., 2009; Fielden et al., 2011; Uehara et al., 2011) and for the classification and prioritisation of compounds for further evaluation (e.g. Ellinger-Ziegelbauer et al. 2008; Thomas et al., 2009; Watanabe et al., 2012; Yamada et al. 2012; Melis et al., 2014).

C5. Several groups have reported studies to predict the outcomes of 2-year rodent bioassays by applying omics methods to short-term (from single to 90-day

exposures) studies *in vivo*. The majority of these studies have focussed on mRNA profiling in rat liver, but proteomics, microRNA profiling, and metabolomics methods have also been employed (e.g. Yamanaka et al., 2007; Waterman et al., 2010; Koufaris et al., 2012; Ament et al., 2013). Gene signatures have been identified to discriminate between direct- and indirect-acting genotoxic carcinogens, non-genotoxic carcinogens and non-carcinogens (reviewed by Waters et al., 2010; Auerbach, 2016). The identification of non-genotoxic carcinogens using omics biomarkers is complex due to the large variety of modes of action involved, many of which are tissue-specific. Some modes of non-genotoxic carcinogenicity, for example oxidative stress, may show very early signature gene expression changes after a single exposure and repeat-dose studies can then be useful to determine 'false positives'. Auerbach et al. (2010) reported that signatures for non-genotoxic hepatocarcinogenicity in rats were more predictive from 90-day than from shorter-term studies. From this, the authors proposed the concept of a 'shared cancer biology', whereby a common pre-cancerous biology may be identified by common gene expression markers that are to some degree independent of the specific exposure. 'Profiling to the phenotype' takes as the starting point a transcriptional profiling of tissue samples corresponding to cancer pathologies identified in 2-year bioassays, 'working backwards' to use these profiles as markers for earlier prediction based on the shared pre-cancer biology concept. Such data could be cross-referenced to archived human tissue samples to improve human relevance (Waters, 2016).

C6. Gene expression studies in cultured cells exposed to toxicants have also focussed mainly on liver, using either primary hepatocytes or cell lines. These studies have shown utility in identifying genotoxic carcinogens, for which the importance of using p53-competent cell types is emphasised. *In vitro* studies have proven less useful for discriminating non-genotoxic carcinogens, in large part due to the wide diversity of modes of action involved. Indeed, the feasibility of using *in vitro* models for predicting the development of cancer *in vivo* has been questioned, for reasons including the following: the carcinogenicity of a chemical may require the presence of and interaction between different cell and tissue types in an organism, the biotransformation of parent compounds into metabolites, and correlation of effective doses *in vitro* with corresponding concentrations in different tissues in an organism (Waters, 2016). Nevertheless, methods are considered to be useful in characterising toxicity pathways to elucidate modes of action (Doktorova et al., 2012; Luijten et al., 2016).

C7. A 'comparison approach' to the identification of non-genotoxic carcinogens using *in vitro* omics-based studies has been described, whereby a limited set of the most significantly up- and down-regulated genes is compared for overlap across different chemical exposures, to identify the best match for a chemical of interest. A test of this method to categorise chemicals by their mode of action using primary mouse hepatocytes or mouse embryonic stem cells indicated the requirement to use a combination of different *in vitro* systems, and these studies are being extended to incorporate tests over chemical concentration ranges (Schaap et al., 2015, 2016).

C8. A large catalogue of (*in vivo* and *in vitro*) datasets is now available, based on a large set of compounds, consistent study designs and standardised experimental protocols. Databases contain dynamic gene expression data over multiple doses/concentrations plus companion data (e.g. compound pharmacology, toxicology, clinical chemistry and histopathology). This information can be used for 'phenotypic anchoring' – relating specific changes in gene-expression profiles to adverse effects observed in conventional toxicity tests, to allow the identification of gene-expression changes that are causally related to the development of the toxicity phenotype (Paules, 2003). Studies should now be extended to include targeting of organs/cell populations other than liver, the abstraction from individual signature genes to higher-order levels, such as pathway enrichments and molecular interactions, and the integration of expression data obtained across multiple omics platforms (DNA, mRNA, miRNA, protein, metabolites) (Römer et al., 2014).

C9. It has been suggested that the 'parallelogram approach' could be useful to compare early key events and toxicity pathways indicated by omics studies performed using sets of chemicals with well-established apical endpoints, to evaluate the likelihood of a similar mode of action in humans. This method, initially proposed by Sobels (1977) and further developed by Sutter (1995), can be used in the assessment of risk to humans by extrapolating findings from two different *in vitro* model systems, one of which should be human (e.g. rodent *in vitro* and human in vitro) and from *in vivo* studies in the non-human species (e.g. rodent *in vivo*). The parallelogram approach has been applied to studies in hepatotoxicity, integrating phenotypic and omics data from rodent studies *in vivo* with data obtained using rodent and human hepatocytes *in vitro*. The 'concordance model' extends this approach to include data from several animal species plus several *in vitro* (human) assays, which should lead to a greater level of confidence in the biological significance of the common toxicity pathways identified (Kienhuis et al., 2016). These approaches may also be applicable to carcinogenicity evaluations.

C10. Progress is being made to integrate omics data into quantitative cancer risk assessments. Dose-response assessments are applied to derive points of departure (PoDs) for omics-derived endpoints, usually benchmark doses (BMDs; often the lowest BMD), which can be compared with PoDs from conventional/ apical endpoints. Case studies using transcriptomic biomarkers for several model compounds have been described, and the standardisation of study protocols as well as methods to derive BMD values have been discussed (reviewed by Thomas and Waters, 2016). A mode of action-based context is preferential in the application of transcriptomic dose-response in the derivation of the BMD. Dose-response studies performed over time can relate BMD value changes with adverse responses to identify transcriptional changes that are progressive or resolve. Use of the 'most-sensitive BMD' derived from omics data may lead to an over-conservative risk assessment as the most sensitive changes in gene expression/ pathway alterations may represent adaptive rather than toxicity endpoints. At present, this issue is addressed by phenotypic anchoring to traditional apical endpoints, with the intention that there will eventually be sufficient well-validated data that apical endpoints *in vivo* will no longer be required. The parallelogram

approach and concordance model can be integrated to select pathways of human biological significance (Kienhuis et al., 2016).

C11. Thomas et al. (2013) outlined a framework for applying transcriptomic data to (non-cancer and cancer) risk assessment. The proposed weight of evidence analysis incorporates estimation of genotoxic potential and an extrapolation factor based on the PoD estimated from the lowest BMD determined from transcriptomic dose-response studies in eight specified tissues at a single time point between five days and thirteen weeks in rats and mice. The assumption is that basing the PoD on the most sensitive pathway is generally protective until key adverse effect pathways are identified. This approach might be applicable to obtaining margins of exposure when cancer data are not available, but advice on relative risk is required. Thomas and Waters (2016) commented that although there may be issues of concern in using such an approach, pragmatically, a PoD based on such information may be preferable to no PoD, which is currently the case for the vast majority of chemicals.

3. High-throughput screening

C12. Individual omics-based assays can provide information about multiple changes (e.g. expression levels of large numbers of genes) in response to a chemical exposure, but they currently have limited applicability for use in high-throughput screening (HTS). Conversely, HTS methods, which evaluate only one or a small number of genes or processes per assay, are adapted to screen large numbers of chemicals over a wide range of assay conditions. They have the advantage of providing rapid, high-throughput, standardised testing of chemicals. A number of these methods were initially developed in the pharmaceutical industry for the rapid screening of libraries of candidate drugs or small molecules for specific types of biological activity or disease processes (Pereira and Williams, 2007) and are now being applied robotically to study chemical perturbations of biological pathways in relation to toxicity.

C13. HTS assays comprise two general categories. Biochemical (cell-free) assays are usually homogenous reactions that measure effects on specific molecular targets and can be easily miniaturised. Cell-based assays can determine perturbations at different points in cellular pathways and are often run in multiwell formats (from Waters, 2016).

C14. HTS approaches are being developed with the aim to predict carcinogenicity *in vivo.* A wide range of doses can be tested in each individual assay allowing the description of dose-response curves at low (human-relevant) doses, which can be useful for comparison with low-dose omics and *in vivo* study data. HTS is of particular value for hazard identification and prioritisation for further testing, and can be run in parallel with structure-activity relationships (SARs) to predict potential targets prior to screening. A major challenge is how to incorporate the toxicokinetic and toxicodynamic parameters of *in vivo* studies, and some authors have questioned whether *in vitro* methods can actually be useful in risk assessment to support regulatory decision-making (see paragraph C6).

C15. The landmark report, 'Toxicity Testing in the 21st Century: A Vision and a Strategy' proposed a paradigm shift in toxicity testing from high-dose studies *in vivo* to an approach based on *in vitro* assays using human-relevant cells or tissues using a mode of action approach based on the evaluation of dynamic pathways underlying biological response (National Research Council, 2007; Bhattacharya et al., 2011). This concept has been generally labelled 'TT21C'. The aim stated is to test whether chemical compounds have the potential to disrupt processes in the human body that may lead to negative health effects. The two central aspects of the TT21C approach are the evaluation of innate cellular pathways that may be perturbed by chemicals and the determination of chemical concentration ranges in which these perturbations are likely to lead to adverse health effects. The TT21C approach is being evaluated in proof-of-concept studies using well-studied prototype compounds whose toxicity has already been examined with *in vivo* and *in vitro* assays.

C16. To date, the major initiatives applying the TT21C approach have been based in the US, in projects such as Tox21 and ToxCast. There are also various European projects moving to a toxicity pathway approach linked in with a reduction, replacement and refinement in the use of animals in toxicity testing. The AXLR8 consortium includes details of other EU funded research investigating these (http://axlr8.eu/, accessed 10/10/16).

C17. Tox21 (Toxicology in the 21st Century, https://www.epa.gov/chemical-research/toxicology-testing-21st-century-tox21, accessed 17/10/16)) is a collaboration in the US between partners at the National Institutes of Health (NIH), Environmental Protection Agency (EPA) and Food and Drug Administration (FDA) that began in 2008 in response to TT21C. The stated goals are to identify environmental chemicals that lead to biological responses and determine their mechanisms of action on biological systems, prioritize specific compounds for more extensive toxicological evaluation, develop models that predict chemicals' negative health effects in humans, and annotate all human biochemical pathways and design assays (tests) that can measure these pathways' responses to chemicals. Tox21 utilises quantitative HTS *in vitro* assays and computational toxicology approaches to cover a range of cell responses and signalling pathways to rank and prioritise chemicals. The HTS assays target multiple genes, proteins, pathways and cancer-related processes. To date, over 10,000 chemicals have been screened in approximately 50 assays.

C18. The EPA ToxCast (Toxicity Forecaster) project is related to, but separate from, Tox21. The results from ToxCast form a contribution to Tox21. ToxCast uses a similar approach to Tox21, but includes a much wider range of assays and endpoints. To date, more than 1800 chemicals, including industrial and consumer products and food additives have been screened in the ToxCast program for over 700 endpoints. All of the resulting information is publicly available on a database, together with tools for visualising and analysing the data (https://www.epa.gov/chemical-research/toxicity-forecasting, accessed 29/09/16).

C19. In ToxCast Phase I, a set of around 300 chemicals with pre-existing toxicity data were run through >600 HTS assays. ToxCast HTS data relating to perturbation of carcinogenesis-related pathways were then used to develop a model for classifying carcinogens (mostly non-genotoxic) based on 2-year data in the EPA Toxicity Reference Database (ToxRefDB), comprising largely pesticides. This dataset was applied to an external test set of 33 pesticides. The model showed some (limited) capability to discriminate between possible/probable and negative/unlikely carcinogens, but several known carcinogens were identified as false negatives (Kleinstreuer et al., 2013). Further, independent analyses using this data set have found that assay design and coverage are not yet adequate and need development to improve the accuracy of prediction of rodent carcinogenicity and of the relevance of predictions to humans (Benigni, 2013; Cox et al., 2016). Problems faced in developing and improving the ToxCast assays are discussed in the review article by Benigni (2014), who concluded that the next phase should focus on including exogenous metabolic activation in the HTS assay systems and developing a set of well-characterised, standard carcinogens.

4. COC conclusions

C20. Use of the 2-year rodent bioassay to evaluate the carcinogenicity of the vast numbers of untested chemicals that are currently marketed is not feasible and alternative methods are required for this purpose. Newer approaches are being developed, such as omics technologies and high-throughput screening (HTS) assays. The goal of these approaches is to develop predictive methods that are rapid, cheaper than current bioassays, and/or high throughput, based on human-relevant mechanisms of carcinogenesis.

C21. Omics technologies may be useful as a part of new strategies based on human-relevant modes of action. To date, most studies have used transcriptomic methods, but newer approaches such as metabolomics show promise for the future. Omics approaches may be used to extrapolate between animal *in vivo* and *in vitro* experiments and human *in vitro* experiments to predict likely outcomes for humans *in vivo*. This requires the development of biomarkers, and while a lot of information has been generated in this area, a better understanding of the key markers is required before this can progress.

C22. High-throughput screening (HTS) technologies using biochemical or cell-based assays that allow rapid screening of large numbers of chemicals over a wide range of concentrations may be useful for hazard identification and prioritisation, but are currently not useful for risk assessment.

C23. These emerging technologies are not yet sufficiently developed or validated to be used in the formal assessment of carcinogenic risk to humans from chemicals in the environment. However, the Committee is aware of these developments and will keep progress in this area under review.

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