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

Statement on the COT workshop on 21st Century toxicology

Introduction

1. On 11 February 2009 a COT workshop was held on '21st Century Toxicology'. The workshop focused on questions that emerged from a report published by the United States (US) National Research Council of the National Academies (NA) in 2007 called "Toxicity Testing in the 21st Century: A Vision and a Strategy"[a]. The National Academies' report sets out a 10-20 year strategy in which the goal is to develop and validate toxicological protocols that deliver better science and move away from testing in animals.

2. Toxicological investigations are carried out in animals in order to assess the safety of substances to which people may be exposed. *In vivo* studies are considered necessary to cover the many biological processes that are currently difficult or impossible to model *in vitro*. However, *in vivo* approaches have a number of drawbacks: ethical issues surrounding animal experimentation; the need to extrapolate from animal to humans in terms of physiology, biochemistry, genetics and behaviour; economic costs of long-term studies; and the potential for idiosyncratic human reactions to be missed.

3. In response to these drawbacks of animal experimentation, research is being conducted internationally to predict human *in vivo* responses through the development and validation of novel *in vitro*, lower organism and computational methods for the prediction of hazards, the determination of mechanistic information, and the integration of data. The aim is to enable predictions of human *in vivo* responses to large numbers of chemicals to be assessed under programmes such as the Registration, Evaluation, Authorisation and Restriction of Chemical substances (REACH) legislation in a high-throughput and cost-effective manner, with a reduced use of animals. Such an approach could facilitate toxicological assessments of mixtures of chemicals.

4. The workshop provided the Committee with an opportunity to hear a collection of presentations both on large-scale efforts to develop '21st century toxicology' and on the evolving or emerging techniques that are anticipated to contribute to the fulfilment of the above aspiration over the coming 10-20 years. The utility of toxicogenomics was illustrated through investigations of carcinogens and mechanisms of action. A presentation was given on how the hazards of chemicals can be assessed by using multiple computational methods within the overall framework of a tiered non-testing approach. Applications for

[a] Report available at: http://books.nap.edu/catalog.php?record_id=11970.

metabonomics/metabolomics[b] in risk assessment were outlined and the integration and validation of different 'omic approaches (transcriptomics, proteomics and metabonomics) with conventional toxicology was also addressed.

Presentation Summaries

A vision for toxicology in the 21st century

5. One of the challenges faced in regulatory and risk assessment toxicology is the large number of substances that humans are exposed to, for which inadequate toxicological data are available. Whilst such exposures are often at low levels, determining whether such exposures are a risk for public health needs to consider the toxic effects and potencies of the substances, which is where data gaps are problematic.

6. The 2007 NA report envisages a not-so-distant future in which virtually all routine toxicity testing would be conducted *in vitro* in human cells or cell lines by evaluating perturbations of cellular responses in a suite of "toxicity pathway" assays using high throughput robotic-assisted methodologies. *In vitro* biochemical- or cell-based assays have proven useful in many allied areas of science, in drug discovery and specifically in genetic toxicology. However, for the broader purposes of toxicology and the protection of public health, effective translation of *in vitro* assay results to the whole organism requires an integrated program that identifies critical biological targets and links *in vitro* results with knowledge of cellular and organ physiology and ultimately with *in vivo* effects[1].

7. The National Toxicology Program (NTP) of the US National Institute of Environmental Health Sciences published its roadmap to achieve the vision of an NTP for the 21st century in 2005 (NTP, 2004) and shares many aims with the vision outlined by the NA (2007). The vision for the NTP roadmap[2] is "to move toxicology from a predominantly observational science at the level of disease-specific models to a predominantly predictive science focused upon a broad inclusion of target-specific, mechanism-based, biological observations".

8. The NTP, the National Centre for Computational Toxicology within the US Environmental Protection Agency (EPA) and the National Institute of Health Chemical Genomics Center (NCGC), comprise the Tox21 Community. This Community is taking steps to cooperatively re-examine where and how *in vitro* biochemical- and cell-based assays, *in vivo* assays involving lower organisms, and computational modelling of biological systems can best be utilised in a high-throughput fashion[3] to provide the information needed to adequately protect human and animal health and the environment. The Tox21 community has identified 1408 substances of interest comprising pharmaceuticals, industrial chemicals, dyes, pollutants, pesticides, natural products and food-borne chemicals. Tox21 is applying the following methodologies and expertise to aid the prediction of hazard for those chemicals:

[b] Distinctions are sometimes made in the published literature between when the terms 'metabonomic' and 'metabolomic' should be used, but in other cases the terms are also used interchangeably. For clarity, only the term 'metabonomics' has been used here throughout.

- Historical toxicity data (NTP and EPA).
- Experimental toxicological expertise (NTP and EPA).
- Ultra high-throughput testing (NCGC).
- Mid-high-throughput systems (EPA).
- Lower organism models (*C. elegans* at NTP and zebrafish at EPA).
- *In vitro* 3D model systems (NTP and EPA).
- Effect of human/rodent genetic background on toxic effects (NTP and NCGC).
- Testing of mixtures of chemicals (NCGC).
- Computational toxicology (NTP, NCGC and EPA).
- Validation experience (NTP, NCGC and EPA).

9. In order to identify key toxicity pathways, toxicogenomic data, human disease-gene association[4] and metabolic pathways (BioCarta[c], GeneGo[d], Invitrogen[e] and Kegg[f]) will be used. ToxCast will also develop methods for incorporating hepatic metabolism into *in vitro* assays. Limitations of the approach were:

- Not all *in vitro* assays are suitable for high-throughput screening and not all substances can be tested *in vitro.*
- Current in vitro assays do not take into consideration the route or extent of exposure *in vivo.*
- Current *in vitro* assays do not account for absorption, distribution, metabolism and excretion.
- How can genetic and environmental heterogeneity relating to differences in sensitivity be taken in to account?
- There will be a need to develop extrapolations through levels of biological organisation from genes up to whole organisms.

10. Expected needs for toxicology in the 21st century were described as the following:

- Continue to refine traditional methods and develop new methods to provide basic toxicology information for public health protection (mechanistic, exposure-response, predictivity of toxicity, life stage susceptibility and genetic susceptibility),
- Reconcile results from new “data rich” techniques (e.g., ’omics, high throughput screens) with existing testing information for conceptual validation, and
- Develop approaches to accomplish formal validation of new methods for human hazard and risk estimations.

[c] http://cgap.nci.nih.gov/Pathways/BioCarta_Pathway.
[d] http://www.genego.com/.
[e] http://escience.invitrogen.com/ipath/browse.do.
[f] http://cgap.nci.nih.gov/Pathways/Kegg_Standard_Pathways.

The US EPA's ToxCast Program for the Prioritisation and Prediction of Environmental Chemical Toxicity

11. The US EPA has estimated that there are approximately 90 000 substances in use or produced in the US with inadequate available toxicological data, which is an untenable number for widespread applications of contemporary *in vivo* strategies. In addition, it is to be expected that as substances within this large cohort would present a range of degrees of risk to people, many would not pose health concerns. This is because a lack of toxicological information does not imply a real health risk. The practicalities of such a large cohort require that prioritisations must be undertaken if hazards are to be assessed.

12. The EPA has recognised and is attempting to address these problems through its ToxCast research project, which will develop computation models for predicting and characterising hazard[5]. ToxCast will facilitate the process of characterising hazards by measuring the impact of substances on non-*in vivo* or lower-organism endpoints in a systematic high-throughput manner[6]. Phase 1 of ToxCast utilises 20 assay sources comprising 554 endpoints summarised as follows[g]:

- Biochemical assays attempting to cover known toxicity targets:
    - Radioligand binding.
    - Enzyme activity.
    - Co-activator recruitment.
- Cellular assays in human cell lines and primary cells, and in human and rat biotransformationally competent cells
    - Cytotoxicity.
    - Reporter gene.
    - Gene expression.
    - Biomarker production.
    - High-content imaging for cellular phenotype.
- Multi-cell interaction assays.
- Model lower organism developmental assays:
    - Zebrafish.
    - *C. elegans.*

13. Judson *et al*.[7] have categorised the data on substances available to the ToxCast Program *a priori* as falling into the following general categories:

- Chemical structures.
- Physicochemical properties.
- Biochemical (*in vitro* or cell-based) assay data.
- *In Vivo* toxicology assays with 1^{o} or 2^{o} data available.
- *In Vivo* toxicology assays with text reports available only.
- Expert opinion on *in vivo* toxicological modes of action.
- Regulatory listings of chemicals that are of toxicological concern.
- Phenotypes describing disease or toxicology categories.

[g] Assay details available at: http://www.epa.gov/ncct/toxcast/files/ToxCast_Assays_01aug2007.pdf.

14. The aim is that in combining the results from the ToxCast endpoints with the information available *a priori*[8], the EPA will be able to prioritise a relatively small sub-set of compounds that are likely to present the greatest hazard or risk (the EPA intends to incorporate exposure into the prioritisation scheme in the future). It is anticipated that this will be achieved through statistical and machine learning approaches used to correlate ToxCast results with the *a priori* information. Once identified, such sub-sets can then be investigated with *in vivo* toxicological assays to fill significant data gaps in order to facilitate protecting public health. This strategy aims to fill important data gaps using far fewer animals in a relatively short period of time without a greater incidence of false negative categorisations than the alternatives. An example of predictive modelling using ToxCast data has been published[9].

15. Phase 1 of the Program involves applying the ToxCast assays to 300 substances that are well-characterised *in vivo* (predominantly pesticides) and developing predictive models for *in vivo* effects. Substances assessed during Phase 1 then represent a training set, which the remaining, largely uncharacterised substances can be compared against[7]. Subsequent phases will incorporate additional compounds such as nanomaterials (as a pilot), a further 300+ data-rich chemicals (for early model validation), known human toxicants (to examine extrapolation) and then data-poor compounds for hazard prediction and prioritisation.

16. Additional databases and computational models are being generated during ToxCast to aid the risk assessment process[h]. The Virtual Liver Project is being developed to predict *in vivo* hepatic effects from *in vitro* data and to simulate mechanisms of action from *in vitro* molecular and cellular data and *ex vivo* histopathological data. A Virtual Embryo Project is also being developed for developmental toxicity. The databases are the Aggregated Computational Toxicology Resource (bringing together publicly available data on environmental chemicals), Distributed Structure-Searchable Toxicity (a repository for publishing toxicity data searchable by chemical structure) and the Toxicity Reference Database (collects data from *in vivo* studies).

Toxicogenomic tools for chemical safety assessment

17. Genomic technologies are rapidly evolving as powerful tools for discovery- and hypothesis-driven research, a fact demonstrated by the increased number of publications involving microarrays, proteomics and metabonomics. Toxicogenomics (the integration of 'omic technologies, bioinformatics and toxicology) has seen significant investment by the pharmaceutical industry for both predictive and mechanism-based toxicology in an effort to identify candidate molecules more quickly and economically. However, the judicious application of genomics may help also in the selection of safe compounds for use in other chemical sectors, such as food, agriculture and domestic products, where significant human exposure may occur.

18. Despite significant progress in its development and implementation, deciphering meaningful and useful biological information from toxicogenomic data remains

[h] Further details can be found here: http://www.epa.gov/ncct/toxcast/index.html.

challenging for toxicologists, risk assessors and risk managers. In general, toxicogenomic studies have been limited to a qualitative description of alterations in transcript, protein, and metabolite levels with little correlation to toxicity or contributions toward the elucidation of mechanisms of toxicity[10].

19. Applications for toxicogenomics in hazard/risk assessment practices include:

- Delineating mechanisms of action.
- Identification of biomarkers.
- Defining species differences.
- Candidate biomolecule comparison and selection (drug discovery).
- Interpreting or facilitating read-across, as for example under the REACH legislation.

20. Examples were presented to illustrate how genomics may be used to assist in the early selection (perhaps more precisely early elimination) of chemicals for development, for elucidation of mechanisms of toxicity, to allow read-across for members of a chemical series, and to contribute to the 3Rs by assisting in the conception and development of appropriate *in vitro* models.

21. An approach for dealing with transcriptomic data was described:

- Data processing:
    - Filter to remove genes of unknown function and those with less than 1.5-fold variation between classes.
    - Derive a probability value for each oligonucleotide describing differences in normalised expression between controls and treated.
    - Generate a 'signature' list of regulated genes.
- Data analysis and reporting options:
    - Compare differences in the effects of small numbers of compounds on small numbers of genes and/or look for trends (e.g. with dose or time)
    - Perform biological/functional classification clustering analyses to find patterns in data sets.
- Validation and interpretation:
    - Confirm interesting results with alternative analytical platforms.
    - Laser micro-dissection can facilitate testing of individual cell types.
    - Map on to and/or compare findings to biological pathway networks.
    - Consistent annotation across bioinformatic databases is needed.

22. The choice of statistical approaches will depend on the question(s) being asked. It may be desirable to simultaneously compare the effects of a variety of different compounds on the transcript levels for a specific subset of genes using a heatmap in order to determine patterns in the data visually[11]. Alternatively, temporal changes can be assessed through sequential sampling. A clustering approach may be desirable for larger data sets, whereby a heatmap can be further mathematically interrogated for trends across different genes in response to exposures, or to find similarities in the effects of different compounds (read-across).

23. An example of the application of toxicogenomics for the delineation of mechanism was also provided. Rats exposed *in utero* to dibutyl phthalate

experienced cryptorchidism and reduced testicular weight, with microarray data from fetal tissue indicating that effects occurred primarily on Leydig cells[12]. Comparison of transcriptomic, proteomic, and metabonomic data will require sampling at multiple time points as lag periods separate the responsiveness of these levels of biological integration from each other. This is a forthcoming challenge for biologists and statisticians to cooperate on.

24. Relating early toxicogenomic changes to distant effects is further complicated when only a subpopulation of the treatment group experience classical pathology, as is the case with carcinogenesis, reproductive toxicity, and teratogenesis for example. Deciphering meanings from biological information, differentiating between adaptation and toxicity and establishing links between gene-changes and physiological changes remain challenges in the field. Future evolutions of toxicogenomics could entail:

- The development of chip-on-chip approaches using microarray technology to investigate interactions between protein and DNA.
- Investigation of microRNAs, which are involved in the regulation of messenger RNA to control protein regulation.
- With DNA methylation state inheritable, this is an area of epigenetics open for further consideration.

Metabolic profiling strategies for characterisation of toxic mechanisms

25. Metabolic profiling strategies encompassing high-resolution spectroscopy of biofluids, cell extracts or tissues, in combination with multivariate statistical modelling tools, have been shown to be well-suited to generating metabolic signatures that reflect various physiological and/or pathological states. This approach provides a means of measuring dynamic biochemical responses of organisms at the systems or sub-systems level, and as they develop through time[13]. This can be undertaken at the level of the whole organism, or can be used in model *in vitro* systems (e.g. within carcinoGENOMICS project described below) to generate hypotheses relating to pathological mechanisms in either a diagnostic or prognostic capacity[14, 15], or to establish metrics for the evaluation of therapeutic interventions.

26. The use of metabonomics in toxicology has the following background:

- The interaction of pharmacological agents with cells and tissues results in perturbations of the concentrations and fluxes of endogenous metabolites involved in key intermediary cellular pathways.
- The response of cells to toxic stress generally necessitates an adjustment of their intra- and/or extracellular environment in order to maintain homeostasis.
- Metabolic adjustment to pathophysiological stimuli is expressed as a unique fingerprint of biochemical perturbations characteristic of the nature or site of a toxic insult or disease process[16], which can be detected using high resolution spectroscopic tools such as nuclear magnetic resonance (NMR) spectroscopy or mass spectroscopy (MS).

27. A generalised metabonomic strategy for toxicity studies was outlined:

- Acquisition and processing of spectra:
    - It is common to use an NMR- or MS-based analytical strategy. These techniques are complementary and can be used in isolation or, to give a better coverage of candidate biomarkers, their datasets can also be combined.
    - Phase and baseline correction.
    - Referencing for calibration.
- Data reduction if needed or desired.
- Pre-processing:
    - Removal of redundant regions.
    - Normalisation.
    - Scaling.
- Development of models through an interaction between unsupervised and supervised multivariate analyses in combination with data filtering.
- Validation of models using independent data sets or internal cross-validation.
- Biomarker identification.

28. Chemometric and bioinformatic strategies for optimising the characterisation and prediction of pathological conditions are commonly applied in order to increase the sensitivity of metabonomic analysis by reducing the influence of confounding random and systematic noise, accommodating the presence of large dynamic range in the measurement variables and/or incorporating the temporal dependence of pathologies. The choice of statistical models and visualisation method should suit the questions being asked.

29. Model and visualisation options include:

- Unsupervised multivariate methods can show differences and similarities between samples represented by their positions relative to one-another. Used for identification of inherent patterns or structure within the data and also for detection of outliers or anomalous samples.
- Supervised multivariate discriminant or regression models can be used for comparing against external variables.
- Batch processing to facilitate the following of changes over many time points.
- Orthogonal correction approaches can reduce the impact of non-classed related variation on visual representations.
- Assessment of trajectories within plot for matched samples or between classes that show trends (e.g. changes over time or differences relating to the onset, progression and recovery from toxicity).
- Clustering approaches to categorise the similarity of samples.
- Correlation networks linking ’omic data from differing levels of integration or data sets generated using different analytical platforms.
- Predictive models based on unsupervised, supervised or genetic algorithm approaches.

30. The kidney was cited as an example where metabonomics had characterised different urinary metabolite profiles for damage occurring on different regions of the organ. Puromycin, uranyl nitrate and 2-bromoethanamine, which affect the renal glomeruli, the lower regions of the proximal tubules and the renal medulla (including the loop of Henle and the collecting ducts) respectively, were shown to produce distinctive urinary metabolite profiles[16].

31. Gene-metabolite interactions can be probed using a range of chemometric tools and the metabolic signature used to direct appropriate sampling points for genomic/proteomic analysis. Using a range of multivariate analytical strategies, metabonomic data can be integrated with gene expression and proteomic data to provide a more holistic vision of biological processes at a whole systems level. An example was presented where NMR spectroscopic and two dimensional gel electrophoresis protein matrices from mouse blood plasma were combined to discriminate between control and prostate cancer xenograft animals. A correlation network then visually linked combinations of metabolites and proteins[17].

32. Tissue samples can be analysed non-destructively with NMR spectroscopy using the so called magic-angle spinning (MAS) technique. A toxicological application of MAS NMR was to 2-bromoethanamine-induced toxicity in the renal papilla where metabolite changes responsible for temporal trajectories were linked chronologically to phases of mitochondrial dysfunction and degeneration[16].

33. As an aid to drug discovery, the COMET consortium[18] has constructed a large database of metabolic and pathological data for 147 compounds administered to rats and mice during single dose 1-week or 28-day repeat dose studies. Sample classification using a SIMCA-based analysis of biofluid NMR data led to percentages of correct classifications of toxins of the liver, heart muscle and kidney of 86-100% and of control Han Wistar and Sprague Dawley rats of 86-96% respectively.

A Tiered Approach for the Use of Non-Testing Methods in the Regulatory Assessment of Chemicals

34. To promote the availability of reliable computer-based estimation methods for the regulatory assessment of chemicals, including chemicals used as food additives, the European Commission's Joint Research Centre (JRC) has been developing a range of user-friendly and publicly accessible software tools[i]. Computational methods can provide predictive information for use within various steps of the risk assessment process. These include hazard identification (property estimation and mechanistic information), hazard characterisation, estimation of a threshold of toxicological concern and exposure assessment (environmental distribution).

35. Toxtree predicts various kinds of toxic effect by applying decision tree approaches. The set of decision trees currently includes the Cramer classification scheme, the Verhaar scheme, the BfR rulebases for irritation and corrosion, the Benigni-Bossa scheme for mutagenicity and carcinogenicity, and the START rulebase for persistence and biodegradability. A recent addition is the ToxMic plug-in,

[i] Further details can be found at: http://ecb.jrc.ec.europa.eu/qsar/.

which considers structural alerts for the *in vivo* micronucleus assay in rodents[19]. New rulebases can easily be developed and incorporated. In the area of food safety, the Cramer classification scheme can be used to apply the Threshold of Toxicological Concern (TTC) concept and thus to predict the level of concern based on systemic toxicity.

36. Toxmatch generates quantitative measures of chemical similarity. These can be used to compare datasets and to calculate pairwise similarity between compounds. Consequently, Toxmatch can be used to compare model training and test sets, to facilitate the formation of chemical categories, and to support the application of read-across between chemical analogues. DART (Decision Analysis by Ranking Techniques) was developed to make ranking methods available to scientific researchers. DART is designed to support the ranking of chemicals according to their environmental and toxicological concern, and is based on the most recent ranking theories. Different kinds of order ranking methods, roughly classified as total and partial-order ranking methods, are implemented.

37. Finally, the JRC is developing a web-based inventory of quantitative and qualitative structure-activity relationship ((Q)SAR) models (the JRC QSAR Model Database), which will help to identify relevant (Q)SARs for chemicals undergoing regulatory review. The JRC QSAR Model Database provides publicly-accessible information on QSAR models and will enable any developer or proponent of a (Q)SAR model to submit this information by means of a QSAR Model Reporting Format (QMRF).

38. Following the REACH guidance on the assessment of chemicals, it is recommended that these and other tools should be used in a stepwise (tiered) approach. A flexible, integrated testing strategy (ITS) was outlined for REACH, which would be designed around the compound in question and utilise the available information. The ITS would be endpoint-specific and draw on exposure information and the results from *in vitro* tests, (Q)SAR models, read-across assessments and existing toxicological information. The ITS would then be used to support a risk assessment, an assessment of potential for persistence and bioaccumulation, and decide on issues of classification and labelling. Finally, decisions would be made on whether the substance was safe to use in conjunction with available risk management options and on whether additional targeted testing was required.

39. In order for a (Q)SAR result to be adequate for a given regulatory purpose, the following conditions must be fulfilled:

- The estimate should be generated by a valid (reliable) model.
- The model should be applicable to the chemical of interest with the necessary level of reliability.
- The model endpoint should be relevant for the regulatory purpose.

40. The above conditions can be met by the use of the QMRF and the (Q)SAR prediction reporting format (QPRF). The QMRF is a robust summary of a (Q)SAR model, which reports key information on the model according to the OECD validation principles; no judgement or “validity statement” should be included. A QPRF is a

description and assessment of the prediction made by a given model for a given chemical. A (Q)SAR should be associated with the following information[j]:

- A defined endpoint.
- A transparent algorithm.
- A defined applicability domain[k].
- Appropriate measures of goodness-of-fit, robustness and predictivity.
- Mechanistic interpretation, if possible.

41. A generalised non-testing strategy would comprise these six steps[l]:

(a) Collection of existing information:
- Chemical composition (components, purity/impurity profile).
- Structure generation and verification.
- Key chemical features.
- Experimental data: physicochemical properties, (eco)toxicity, fate.
- Estimated data: pre-generated QSAR or read-across estimates.

(b) A preliminary assessment of abiotic/biotic reactivity and fate:
- Identify reactive potential.
- Identify possible transformation products or metabolites.

(c) Use of classification schemes and consideration of structural alerts:
- Models and rulebases for mode-of-action classification, hazard identification, hazard classification and potency prediction.
- QSAR Model Databases.

(d) A preliminary assessment of reactivity, fate and toxicity.

(e) Chemical grouping and read-across[20]:
- Chemical read-across within analogue and category approaches.
- Biological read-across between endpoints or species.
- Chemical grouping by a top-down or bottom-up approach.

(f) Use of existing published (Q)SARS:
- Note step 3 above; need to identify and use relevant, reliable and well documented (Q)SARs.
- The JRC QSAR Model Database is a searchable inventory of peer-reviewed information on (Q)SAR models.

43. To optimise the use of non-testing data, a conceptual framework is provided in the REACH guidance documentation[m]. An increasing number of models are being

[j] Principles adopted by 37th Joint Meeting of Chemicals Committee and Working Party on Chemicals, Pesticides & Biotechnology (2004); ECB preliminary Guidance Document (2005); OECD Guidance Document (2007); OECD Guidance summarised in REACH guidance (IR and CSA).

[k] The applicability domain of a (Q)SAR model describes the chemical structures (e.g. aromatic amines) that formed the reference library used to build the (Q)SAR and thus the types of structures for which its predictions will be most reliable. In general there is a trade-off between (Q)SAR models with high reliability but with a narrower domain and those with a broader domain, sacrificing on reliability.

[l] Freely-accessible software and databases capable of providing information for this strategy were noted: AIM, AMBIT2, ChemSpider, CRAFT, Danish QSAR database, DART, Episuite, ESIS, JRC QSAR Model Database, OECD Toolbox, OpenTox framework, PubChem, START, Toxmatch and Toxtree.

[m] http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r6_en.pdf?vers=20_08_08.

implemented in a range of software tools and there is a need to facilitate the use of multiple tools by developing automated workflows. Going forward, there is also a need to incorporate mechanistic knowledge in the models (e.g. based on chemical reactivity and 'omic data), and for further guidance on how to assess the adequacy of non-testing and alternative test data by weight-of-evidence approaches.

'*Omics-based in vitro/in vivo approaches for the purpose of cancer risk assessment in humans*

44. We have come to learn that certain chemicals possess particular features for inducing carcinogenesis. This insight has provoked the development of a battery of *in vitro/in vivo* tests assessing carcinogenic properties of chemicals for the purpose of regulating chemical safety. The accuracy and specificity of these tests has, however, been challenged[21, 22]; as has the suitability of the rodent bioassay for the prediction of carcinogenic risk posed by compounds at low doses[23].

45. Estimations of how many rodent bioassays will be needed under REACH are from 121-2600. Cautiously assuming a 20% prevalence of human carcinogens within such REACH compounds, a bioassay sensitivity of 100%, and a bioassay specificity of 75%, rodent bioassays of 121 chemicals would result in 48 positive outcomes, only half of which were correctly positive. There is increasing demand in the domain of chemical safety assessment for tests that produce better and more reliable data, at higher speed and lower costs, and preferably, by taking fewer animal lives. The emergence of 'omics technologies over recent years has stirred hopes that such may become feasible.

46. It was noted that within the EU REACH Regulation rules[24] it was written that:

> "The Commission, Member States, industry and other stakeholders should continue to contribute to the promotion of alternative test methods on an international and national level, including computer supported methodologies, *in vitro* methodologies, such as appropriate, <u>those based on toxicogenomics</u>, and other relevant methodologies".

47. Examples were given of where toxicogenomics has been previously applied to the classification of genotoxic and non-genotoxic carcinogens both *in-vivo*[25, 26, 27] and *in-vitro*[28, 29, 30, 31, 32, 33, 34]. In reference to carcinogenicity and 'omics studies, the REACH Implementation Plan 3.3 reported the following[35]:

> "...other studies on mechanisms/modes of action, e.g. '<u>OMICs studies</u> (toxicogenomics, proteomics, metabonomics and metabolomics): carcinogenesis is associated with multiple changes in gene expression, transcriptional regulation, protein synthesis and other metabolic changes. Specific changes diagnostic of carcinogenic potential have yet to be validated, but these rapidly advancing fields of study may one day permit assessment of a broad array of molecular changes that might be useful in the identification of potential carcinogens."

48. An example was given where toxicogenomics had been applied to look for biomarkers of non-genotoxic hepatic carcinogens[36]. In this study, groups of 3 male Sprague-Dawley rats were exposed to the maximum tolerated dose for five days to 1 of 100 training compounds (25 non-genotoxic hepatocarcinogens and 75 non-hepatocarcinogens) or 47 validation compounds (21 hepatocarcinogens and 26 non-carcinogens) before the gene expression of ~5500 hepatic genes was analysed. The classification algorithm was designed to select the shortest list of genes that best-classified 3, 5 and 7-day hepatic gene expression profiles of the training set, which was 37 genes, and it resulted in an estimated sensitivity of 56% and a specificity of 94%. Validation of the 37-gene signature on 47 test chemicals indicated an assay sensitivity and specificity of 86% and 81%, respectively[36]. Chemicals with similar modes of action clustered together into four putative modes of action of hepatic tumorigenicity.

49. The carcinoGENOMICS project aims to develop in vitro methods for assessing the genotoxic/carcinogenic potential of compounds, as an alternative to current rodent bioassays[37]. The aim is to develop high-throughput genomics-based tests for assessing genotoxic and carcinogenic properties of chemical compounds *in-vitro*. The project has the following characteristics:

- Metabolome and transcriptome profiling.
- Major target organs: the liver, the lung, and the kidney.
- *In vitro* systems (rat/human).
- Inter-individual variability.
- Exploring stem cell technology.
- Well-defined set of model compounds.
- Phenotypic markers for genotoxic and carcinogenic events.
- Biostatistics for identifying predictive pathways.
- *In silico* model of chemical carcinogenesis.
- High throughput technology.

50. The 2004 Children's Environment and Health Action Plan for Europe expressed increasing concern about environmental health effects in children. It was noted that "in the EU, about one third of the total burden of disease from birth to 18 years can be attributed to unsafe and unhealthy environments in the home and the broader community, resulting in significant social and economic costs". There has been an apparent increase in the age-specific incidence rates of lymphoid leukaemia in European children and adolescents between the 1970s and 1990s[38]. The EU FP6 project called NewGeneris is an integrated project that seeks to analyse children's perinatal exposures to carcinogenic agents as well as long term effects in later childhood that may relate to perinatal exposures, by developing and applying 'omics-based biomarkers for cancer risk[39]. Previous examples of transcriptomics analysis in carcinogen-exposed humans were noted[40, 41, 42].

European Union 6th and 7th Framework Programmes Contributing to the Vision

51. Being able to identify early those chemicals that would ultimately lead to unacceptable toxicity in chronic bioassays would be welcome as that would offer

substantial savings in terms of monetary cost and time before patents expire, and would have the potential to reduce animal usage and suffering.

52. The EC FP6 programme called InnoMed PredTox is utilising 'omic technologies in order to meet the above drug discovery objectives[43]. Transcriptomics, proteomics and metabonomics are being applied in short-term bioassays with a number of failed drug candidates possessing known chronic toxicity towards the liver or kidney. The consortium will then look to predict future chronic toxicity from the 'omic profiles following short-term exposure.

53. In general, the application of 'omic technologies was able to obtain indications of effects in short-term studies. However, in only a few cases were 'omic technologies able to detect changes before the onset of pathology, and thus they were usually not more sensitive as compared to this "gold standard".

54. Metabonomics was used to characterise known toxicities and to define novel biomarkers of effects in specific models of organ toxicity. 'Omic technologies were also valuable to delineate mode of action for certain specific chemicals. The application of 'omics technologies to derive biomarkers for known toxicities could be successfully applied to characterise responses in a 90-day toxicity study with the renal toxicant and carcinogen ochratoxin A in the rat, resulting in the detection of several mechanistically linked markers. In addition, metabonomics was able to identify subtle changes in conventional biomarkers not detected by clinical chemistry.

55. The use of 'omics technologies is also being applied in a current FP7 programme called Predict-IV[n], where the focus is on improving the prediction of drug toxicity to accelerate the drug development process and to reduce failure rates late in development. This is hoped to be achieved through combining *in vitro* testing, cell biology, mechanistic toxicology, computational modelling, toxicogenomics, metabonomics and prediction of pharmacokinetics. Margins-of-safety will be deduced, and the data generated by the proposed approach may also identify early biomarkers of human toxicity for pharmaceuticals. The results obtained in Predict-IV may enable pharmaceutical companies to create a tailored testing strategy for early assessment of drug safety.

56. In summary, the application of omics may provide valuable new information in toxicity studies. However, to apply 'omics, specific study designs and a very detailed validation of the methodology may be needed to serve as a basis for valid conclusions. In addition, the use of changes indicated by 'omics data in risk assessments will need to be discussed since the adverse nature of changes seen is often unknown.

Committee Discussion

Large-scale efforts to drive forward a vision of better toxicology using fewer animals

[n] Further details can be found here: http://www.predict-iv.toxi.uni-wuerzburg.de/en/.

57. Some reservations were expressed with regard to the practical applications that might be developed in the short-term from methodologies discussed at the workshop. It was noted than during the past 20 years, the European Centre for the Validation of Alternative Methods has only been able to establish very few validated *in vitro* studies for use in risk assessment, in part because there was not a focus on mechanisms of toxicity. It was felt that in the short and medium term, holistic models would still be required in toxicology in order to detect multi-compartmental effects (e.g. liver-hormone axes or the effects of distal biotransformation on the hazard posed to target organs by toxicants).

58. In order to aid the interpretation of toxicological results obtained *in vitro* and *in silico*, such results will need to be incorporated into physiologically-based toxicokinetic (PBTK) models and the pathway analysis strategies (PAS) that underpin systems biology. It was noted that *in vivo* research in fields outside toxicology may provide good examples of practical applications. It was considered possible to design appropriate *in vitro* tests based on mechanistic understanding, and relate the results to the *in vivo* situation using PBTK models.

59. It is not possible to draw conclusions on human relevance without understanding of the mechanism of toxicity. It was felt that whilst the Tox21 Community was generating a large body of data derived from multiple assays and chemicals, there was great uncertainty about its practical exploitation. However, it was also noted that it was early days for the initiative, use of mechanistic data in contemporary toxicology could be better, and that the Committee should monitor what happens over the next several years through its horizon scanning programme.

Computational non-testing methods for hazard identification and characterisation

60. In relation to the *in silico* models being developed by the JRC, a number of questions were raised: With differing endpoints having different models, what endpoints should be selected for poorly-characterised compounds? Within the context of REACH, an ITS would be formulated on a case-by-case basis and would, for example, consider physicochemical properties, structural alerts and possible applications of read-across. In general terms, the structural class of a compound should be linked to models possessing a complementary domain of applicability, and the results of external validation exercises can be of use in refining the selection process[44].

61. In relation to ‘read-across’ there was a question over how close similarity would need to be in order for a compound to be acceptable for a class of compounds in the absence of further tiers of mechanistic testing? Currently it was recognised that expert judgement was required in some such situations; going forward, a standardised approach would be welcomed. The Committee wondered if it may be useful to consider in terms of classification the conceptual grouping of chemicals by particular modes of action rather than ‘all hepatotoxins’ for example.

Challenges to an increasing implementation of non-animal models in risk assessment

62. When considering strategies for moving toxicology away from primarily observations in mammals' *in-vivo* towards a predictive science based on *in vitro* experimentation and computational modelling, Members considered it essential that an understanding of mechanisms of toxicity be incorporated, and as early as possible. Where such considerations are not made, it was noted that this can pose serious limitations. For example, when testing compounds in cell-based or biochemical assays, the Committee consider it important to ensure that the test material used *in vitro* would reflect the chemical structure that would be exposed to the targets or tissues *in vivo*.

63. In this regard, one of the key questions identified by the NRC that needed to be addressed when implementing the Vision was recognised as "How can adequate testing for metabolites in the high-throughput assays be ensured?" Similarly, the Pathways/Assays Focus Group of the Tox21 Community has set out to "develop methods for incorporating hepatic metabolism into *in vitro* assays" and plans to introduce metabolic competency to assays through years 4-7 of their plan.

64. Where immortalised or cancer cell lines are used, cells can be phenotypically quite different from their source tissue. The NA Vision noted that the molecular evolution of cell lines over time and across laboratories could pose problems. In relation to the time scale for the Vision, the NA also recognised that at the current time, it was not known to what extent the use of primarily human cells, cell lines, and cellular components *in vitro* could replace or improve on *in vivo* systems as predictors of effects in humans. The NA noted that in the regulatory context, results obtained *in vitro* were often used to support or complement *in vivo* results, and that correspondingly, an increased reliance on non-*in vivo* results would require extensive validation.

Overall Conclusions

65. The substantial investment by members of the Tox21 Community to try to push forward understanding of toxicity was recognised, and the idea of such a systematic approach was welcomed. Advances in the use of *in vitro* and *in silico* approaches, particularly in North America, were welcomed in view of the toxicological data gaps that are common to compounds the COT has considered.

66. After validation, the mechanistic data from such methods could be incorporated into conventional toxicological paradigms. On this point, Members felt that toxicological research needed to be increasingly mechanistically-driven and focused, as opposed to being based on batteries of non-*in vivo* approaches. In the short term, such 21st century approaches to toxicology may have use for 'read-across' amongst toxicologically similar chemicals under the EC REACH initiative and during the development of novel substances to predict/rule out undesirable effects in a high-throughput manner.

67. One area of emerging interest that the workshop was unable to cover was small interfering RNAs, which have many undefined roles likely to include some of

importance to toxicology. The workshop was also not aimed at addressing the toxicological assessment of mixtures. It was also noted that approaches such as PAS and PBTK modelling would be needed as part of an overall process for linking *in vitro* and *in silico* results logically when extrapolating to the *in vivo* situation.

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