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NEW PERSPECTIVES ON SAFETY



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I. WORKSHOP OBJECTIVES

Safety assessment requires an understanding of the intrinsic potential for chemicals and drugs to cause adverse effects (hazard identification) and the exposure conditions that translate this potential into human health risks (risk assessment). Currently hazard identification for many toxicity endpoints relies heavily on the use of animals as models. Animals allow an integrated evaluation of the potential to cause adverse effects relevant to humans, but do not provide the possibility of understanding fully how all the biological processes function individually and in concert. This workshop was held to consider how recent developments in science and technology might be harnessed and exploited to provide new and imaginative approaches to characterising potential health hazards without the use of animal models. This means completely new approaches to provision of the key toxicological information that is required:

- the intrinsic hazards associated with the material
- the organs or physiological processes affected
- the relationship between route of exposure, dose of material and magnitude of effect
- persistence or reversibility of the effect

The Workshop is part of an EPAA strategy to identify new developments that will enable hazard identification and risk assessment without the need for repeated systemic exposure of animals to test materials. The goal is to be able to predict, without recourse to animal models i.e. through Replacement, the likelihood that adverse health effects of any kind might result from long-term exposure of people to substances.

The question the workshop addressed was framed as '*Against the current landscape of expertise in biology and chemistry, and drawing upon recent developments in technology, what opportunities now exist to design alternative approaches to toxicity testing – the goals being to improve our ability to characterise the potential of chemicals and drugs to cause adverse health effects while providing animal welfare benefits*'.

To this end, and to bring new thinking to the subject, the Workshop was populated not only with experienced toxicologists, but also by eminent investigators from other disciplines who would not normally engage with toxicology issues.

The workshop objectives were as follows:

- To identify truly novel approaches for the characterization of the potential hazards of chemicals and drugs.
- To develop a view of which areas of science and technology should be exploited to create new approaches to safety assessment, and of which activities may inform and shape the forward research agenda.
- To invest in alternatives research a greater legitimacy among the scientific community.

To engage a wider segment of the research community in consideration of the ways in which new developments in science and technology can be exploited for improved safety assessment.

II. BRIEFING SESSION FOR the SCIENTISTS (28/04/08)

A briefing session led by the science team of EPAA was held to place the workshop objectives in an appropriate context.

A review of a selection of papers (review and references in appendix 2) was provided before the workshop as background to give a flavour of new science approaches which have potential. It was pointed out that today regulatory safety testing remains largely the observation of responses present at tissue and organ level in dosed animals. This is because the outcomes are typically relevant to humans, standard procedures exist to extrapolate safe exposure levels for humans and because we normally have an incomplete understanding of all the possible toxic processes, mechanisms and interactions involved, including toxicokinetics and possible key interactions between different cell/tissue types. This final point is key as animal studies provide the reassurance we have not "missed something", an important concern in simpler test systems.

New technologies are becoming increasingly available to address these concerns and to move the science towards greater emphasis on prediction rather than simple observation. Without intending to be in any way complete, the background papers highlighted areas of possibility: *in vitro* mechanistic studies, toxicogenomics, toxicogenetics, databases relating chemical structure to toxic activity, advancing power of informatics and computing, and systems biology – looking at the issues bottom-up rather than top down as in animal studies.

Investment to date within the framework of the 3Rs (Reduction, Refinement and Replacement) has been substantial, and has paid dividends in delivering animal welfare benefits. It must be recognised, however, that the areas of toxicity testing where most progress has been made in the design and use of non-animal methods are those that are intended to model discrete effects induced by chemicals over a relatively short period of time (such as for instance skin corrosion and eye irritation).

Defining the potential for longer-term

systemic adverse health effects is a much greater challenge, as at its core is the art of identifying the unexpected. It is much more difficult to design non-animal methods that would allow identification of potential toxicants where the site and mechanism of action are not known and, importantly, when there is the possibility that interaction between different cell/tissue types might play a key role in the toxic process. New chemicals or drugs could potentially have the ability to provoke adverse health effects by targeting one or more of many dozens of tissues, many hundreds of cell types, or many thousands of molecular events. In this regard it was acknowledged that there may be important benefits to be realised from studies conducted using well defined, lower organisms (e.g., zebra-fish, invertebrates).

The Participants discussed the differences between fundamental toxicology and regulatory toxicology. Fundamental toxicology was described as the science of understanding the molecular basis for adverse effects induced by xenobiotics; regulatory toxicology as the definition of the type and extent of adverse effects that might be caused by a new chemical or drug, particularly adverse effects in humans which might occur at relevant doses. With modern techniques and the availability of omics, it is possible to access vastly increased amounts of biological information; the key step is to turn all these data into useful knowledge, which ultimately can be used to make sound judgements. We can for instance search for gene expression patterns to assess secondary effects or use classical genetics to characterise specific genes. We have to keep in mind, however, that the level of mRNA expressed, or the protein produced, is not necessarily a signal of its importance for toxicology. Weak effects may still be critical and there is a danger in restricting the problem to known effects, thereby failing to identify the unexpected. Additionally, it is important to consider the possibility that an interaction between different cell types/tissues might play a key role in the genesis of a toxic effect and this issue bears directly on questions relating to which cell type(s)/tissue(s) should be evaluated. The

regulatory burden can be excessive if the challenge is to prove that there is no effect.

These points were further exemplified with 3 case studies highlighting the practical challenges facing toxicologists today. These examples were selected to give a flavour of the variety of different types of toxicity that a new chemical or drug can cause, and not because they are considered of any greater priority/importance than the many other potential types of chemical/drug-induced toxicity.

Steatosis (disturbing the process by which fat is processed by the liver) was described first as an example of the dilemma of which organ to study, given the vast array of potential target organs and possible toxicological mechanisms present in each. It highlighted the difficulty of knowing what you might be looking for and where. The impact of steatosis is variable in terms of effect on the organism and eventual cell death.

Phospholipidosis (abnormal accumulation of phospholipids in cells) was described as an example of toxicity related to the chemical structure of a molecule. It has the effect of overloading the detoxification mechanisms and impairing cell function depending on exposure duration, dose and gender. The effects are usually reversible but can lead to secondary damage in vital organs which is difficult to predict. Activity maybe predictable only in narrow structural classes of chemicals and predicting impact on humans is particularly difficult.

The final example focussed on central nervous system effects and the difficulty of predicting convulsions or seizures. The response to a chemical depends on its ability to penetrate the central nervous system, the effective concentration in the brain, and the specific mechanisms of action. They may also be caused by secondary effects of systemic toxification. The key difficulties in predicting human seizures and convulsions are the wide individual variation in susceptibility and the lack of capacity for animal models to identify the variety of sensory warning signs that can precede the clinical manifestation in humans.

The discussion centred on 4 points:

- The need to recognise that within toxicology as practiced today, many effects are known to be highly dependent upon the extent, duration, timing and route of exposure, and on the species, strain, sex and age of animals used. Moreover, in some cases changes may be considered adaptive rather than toxic, and toxic responses may themselves be persistent or rapidly reversible
- The difficulty of predicting the onset of adverse effects that may disrupt entire body systems (e.g. neurological effects).
- The need to appreciate the impact of heritable and environmental factors on inter-individual differences to toxicity among human populations
- The acceptance of new science by regulators.

III. ROUD TABLE AND INITIAL THOUGHTS (28/04/08)

It was agreed that opportunities probably do exist to design alternative approaches to toxicity testing if advances in complementary fields can be effectively brought together with toxicology:

- The application of stem cells to toxicology is a real possibility, in particular to generate a variety of differentiated cell types which could be used to study the metabolism of compounds of interest and

to generate sufficient quantities of "interesting" metabolites to be evaluated

- The use of synthetic receptors working at the interface of biology and electronics
- Harnessing the big strides being made in computational chemistry, databases and informatics to give better models
- Achieving the right balance between theoretical and practical genetics/epigenetics – opportunities for bringing other disciplines much more fully into toxicology

- More intelligent use of classical genetics to better understand mechanisms
- Building on the genome project and systems biology to generate the virtual patient, building on the progress in sequencing and modelling

A number of complementary issues were also raised:

- 3Rs research does not have to be 2nd rate, there is good science being initiated for example by the National Centre for 3Rs (NC3Rs) in UK, working with the Medical Research Council (MRC). Today's regulatory toxicology is regarded by some as a largely observational science but there is scope to use modern tools and cutting edge science to make it more predictive and reduce the dependence on animals.
- It is commonly recognised that not all animals used in current regulatory testing may be necessary. Test data are not always scientifically relevant and some animal models are poor. However, safety testing without the use of animals seems a long term goal, which may not be expected to be reached within only a few years of research.
- One of the issues for introducing alternatives in regulatory science is the time to validation and achieving appropriate acceptance. New routes to validation and regulatory acceptance will be needed if approaches based on new science are developed.
- There are essentially two questions to be answered: how to improve toxicology and how to better implement the 3Rs.
- Truly innovative approaches to testing and risk assessment are needed for safe development of new products without recourse to animal experiments. At present, existing *in vitro* approaches have the potential to reduce animal use significantly when used in a focussed screening approach but, given issues around false positives/negatives and the

lack of methodologies to study interaction of different cell types/tissues, currently available approaches are unlikely to fully replace animal usage.

- Distinguishing hazard identification as primary screening from dose/response information that is essential for risk assessment. In particular, the difficulty involved in making an observation *in vitro* and then extrapolating this to toxicity *in vivo* is substantial.
- The types of questions facing toxicologists are changing. New concerns focus on the possible effects of factors in the environment which may for instance be responsible for a perceived growing incidence of cancer, of autoimmune and atopic diseases. The environment is complex with many factors potentially working in concert and individuals having very different exposures and genetically-determined responses.
- The need for cost/ benefit assessment for all substances to complement the safety data and support more informed decision making.
- Political deadlines do not make sense if the science solutions are lacking. There was a strong sense that we should invest in Replacement today whilst recognising that animals will be needed in the medium term. The key question is where to invest in this research and this is a task that will be undertaken by Work Group 2 of EPAA building on the workshop's recommendations.

It was agreed that the workshop should discuss these possibilities and issues.

Value was seen in sharing an example of where progress has been made with animal alternatives. Specific discussion was also needed on toxicogenomics, genetics and systems biology, use of stem cells, computational chemistry, synthetic receptors, and changing the ways animals are used in research.

IV. OPEN SESSION FOR EPAA STAKEHOLDERS (28/04/08)

This was attended by the Workshop participants and by more than 50 invited

stakeholders in the EPAA process. The objectives were to provide a political and

institutional context for the Workshop and to brief the stakeholders on the Workshop process and engage them in it.

The session was chaired by Bernward Garthoff, EPAA co-chair from industry and addressed by Cornelis Brekelmans on behalf of Georgette Lalis co chair from Commission, Zoran Stancic on behalf of Commissioner Potočnik and by Sir Colin Berry.

The meeting was reminded that animal welfare is enshrined in European Law (Brekelmans) and specifically within new legislation on laboratory animals, Cosmetics, Chemicals (REACH) and pesticides, as well as Directive 86/609. This provides the context for focused discussion on the 3Rs between the Commission and a broad range of industries within the partnership EPAA for promoting alternative approaches to meet regulatory requirements. The EPAA's programme addresses research, validation, regulatory acceptance as well as dissemination and training. Through the creation of a "Mirror Group" comprising other stakeholders it engages the wider community including Academia and the NGOs. Of particular relevance to the Workshop, EPAA is seeking to refine the alternatives research priorities for industry and the Commission. The Workshop is thus an important part of this process, leading up to the 2008 EPAA Annual conference where the main theme is research.

The role of the Framework Programmes 6 & 7 to provide science support to policy was emphasized (Stancic). Spending on Alternatives research since the mid 1980's is substantial and totals €150 million. This work is high on the political agenda as there is a strong desire to implement alternative approaches, which can continue to assure human safety. Currently €30 million is being spent on alternatives to speed drug discovery and €5 million on environmental risk assessment. It was recognized that the progress made to date has essentially focused on 2Rs: Refinement and Reduction, and that the biggest challenge remains Replacement. This was the focus of the Workshop and there was a hope that it would provide new research directions and orientations. This was entirely consistent with Commissioner Potočnik's message to the 2007

EPAA Annual Conference – "we can and must do better".

Regulatory toxicology is a rational response to public concern about safety on which politicians act, placing demands on the science (Berry). It is however important that this moves from an observational to a predictive science. Berry expressed a concern that collecting too much data will inevitably lead to false positives and may result in further animal testing to explain these. Much could be achieved by looking critically at animal use today. Berry doubted whether the extensive cancer testing in animals has value for humans at expected exposures. He felt that building a good understanding of toxicokinetics *in vitro* was much more rational than arbitrary 10 times and 100 times protocols which simply swamp the defensive mechanism or lead to quite different modes of action (and yet more explanatory animal testing). We probably need to move to testing strategies specific to each compound to avoid the vast amount of unnecessary testing carried out today.

He was concerned that current hazard-led approaches fail to take account of the balance of risks and benefits so that potentially valuable (drug) developments are too easily discarded. It is also important to take account of human behaviour. For instance it has taken 7 years for an alternative skin corrosion test to be validated and accepted for regulatory use even though all the stakeholders had the same goal. He also noted that even where we have good markers of individual susceptibility (e.g. to Warfarin) doctors don't always use them and often risk patients' lives by relying on outdated and flawed but familiar monitoring procedures.

He felt that Pharmacokinetics and pharmacodynamics need to be better understood and more account taken of the variability between individuals. Unfortunately what is seen today in toxicology is not relevant to pharmacology as the conditions for use are so different – environmental levels of chemicals are simply too low. He agreed that stem cells are a fertile avenue of development.

V. SCIENCE DISCUSSION (29/04/08)

1. Lessons from the successful development of the Local Lymph Node Assay

The mouse Local Lymph Node Assay (LLNA) was cited as an example of where an investment in, and exploitation of, research has paid dividends with respect to improvements in safety testing while delivering animal welfare benefits (Kimber).

The LLNA is a method for the identification of chemicals that can cause skin sensitisation. The method is based upon an understanding that the acquisition of sensitisation is causally and quantitatively related to the stimulation of lymphocyte proliferation in lymph nodes draining the site of exposure to the chemical. The development of the LLNA has improved our ability to identify skin sensitising chemicals, and has provided important animal welfare benefits with respect to both the number of animals required for safety testing, and a refinement of the way in which they are used. The take home messages were:

- An investment in research pays dividends with new opportunities to develop alternative methods.
- Evaluation and validation of new methods can be a long, and sometimes difficult, journey.
- Validation does not necessarily equate with acceptance, and application - at least not automatically.

2. Toxicogenomics

The potential of toxicogenomic approaches to predictive toxicology was outlined (Daston). He noted that the toxicogenomic responses for comparable cells seen *in vitro* and *in vivo* were comparable. These technologies allow the investigator to determine the responses of every gene in responsive tissues, not just those for which there are pre-existing hypotheses. Gene expression signatures:

- might be indicative of the start of a possible toxic response

- are characteristic of chemical modes of action
- can be diagnostic and in some cases can be causally related if there is knowledge of mechanism.

Data on oestrogens in a uterine cancer cell line were presented showing the characteristic gene expression signatures at 'non-oestrogenic' targets. This showed the absence of low dose effects that have been claimed by others in controversial animal experiments. The Health and Environment Sciences Institute (HESI) of the International Life Sciences Institute (ILSI) has developed and tested well characterized models of liver toxicity. Applied to human cells, these toxicogenomic-based tests predicted well the toxic effects observed by traditional methods.

In the ensuing discussion it was stressed and agreed that analyses in toxicogenomics need to move from correlation to causation. There is a need for understanding of the mechanisms of toxic response to distinguish what is and what is not important, if the potential of the approach for Replacement is to be achieved.

The difficulty of reading the signature was questioned; which part of it relates to toxicity and which part to biology or adaptation? It was agreed that this is a key issue, highlighting the need to understand the underlying biology not only qualitatively but also quantitatively. Even with Paracetamol it is not possible from current data to understand which part of the gene response relates to hepatotoxicity.

This also related to another key issue. A strong correlation may have nothing to do with cause. A weak effect may be the real relevant one. This is important given that the events that toxicologists are looking for are by definition very rare and complicated by other factors such as co-morbidity/age and other environmental factors.

Two issues were highlighted with respect to data; the quality of databases and the issue of whether they are all open-access; and the problem of gene ontology – different groups

classify genes differently. It was noted that most drug failures occur in the liver despite the extensive testing. The issue is variable individual susceptibility; the challenge is how to share and access accumulated failure data.

Several participants pointed to the need to look for signatures of very early response, not the final cell damage e.g. early inflammatory responses. Consideration should also be given to the use of cells known to be susceptible and cells known to be healthy in order to differentiate the toxicogenomic responses. One potential problem is that in vitro genomic models are not 100% sensitive which may complicate validation.

There was general agreement that Toxicogenomic approaches will become important in Regulatory Toxicology.

Questions that need to be addressed for Toxicogenomics include:

- More experiments to catalogue the gene expression responses to known toxicants that take dose, time and normal changes over time into account, and to anchor these responses to phenotypic changes and adverse effects
- The development of large, well-curated databases that facilitate the querying of all gene expression data in the public domain
- Basic investigations into the underlying role of gene expression in the toxicological response, so that the changes that are integral to toxic responses can be identified
- Toxigenomics typically refers to an evaluation of gene expression (i.e., mRNA levels) and there is a need for research focused on a holistic approach to genomics.

3. Stem Cells

Various types of stem cells are becoming available that could have real value for toxicology (Pedersen). Pluripotent stem cells derived from mature tissues can offer sources of well-controlled human cells which are otherwise not available such as neural tissue. Different types of neural cells can be made and technology is continuously improving;

skin and cardiomyocytes are close. Epi-blast mouse stem cells are also very promising with close similarity to human cells as regards morphology, responses to growth factors and environments. Both types could be used for toxicology with the caveats that cell assays do not mimic the whole organism and that we lack a good understanding of pharmacokinetics,-dynamics or metabolism.

It was pointed out that more work needs to be done to characterise these types of cells to enable them to be useful for toxicology. At the current stage of development, the responses to growth factors of Epi-blast stem cell derived neural cells and of true adult neural cells differ significantly.

What is missing is the ability to scale up to differentiated cells; converting stem cells into liver cells was for instance very difficult. It is the progeny of the stem cells that would be of particular interest as stem cells are not representative of adult cells. A short coming of induced pluripotent stem cells is that we don't understand their regulating signals or how to use mixtures of cells or 3d clusters /engineered systems. Almost nothing is known about metabolism in human stem cells. It was also asked on whether stem cells provide a route to organs for which we don't have presently models.

A rather different point was also raised relating to data and findings from such a novel and still immature area of toxicology. How early results should be shared with regulators was questioned There appears to be a need for data to be ring fenced in a "safe harbour" until such time as responses are better understood. This raises questions in relation to freedom of information (Aarhus Convention).

The meeting recognised the status of the stem cell research today but felt that toxicology is the most realistic mid-term clinical deliverable provided that support was given to the larger production of these cells. The Commission is funding three programmes in this and related areas, but there probably is a need for toxicologists to inform this work better.

Some identified needs are:

- to obtain and evaluate the response to toxicants of differentiated cell types

- that represent important toxicity targets.
- to generate a variety of differentiated cell types which could be used to study the metabolism of compounds of interest and to generate sufficient quantities of "interesting" metabolites to be evaluated
- to develop multi-cell cultures that better approximate *in vivo* tissues
- With bioengineering support, to develop fluidic devices by which different cell types can be exposed to the test agent in a sequential manner (e.g., liver [as a metabolizing tissue] with a target tissue)

4. Chemistry and Chemi-informatics

Computational chemistry is rapidly becoming more powerful, quicker, faster and today has almost unlimited power such that computing no longer rate-limits Structure Activity Relationship (SAR) studies (Richards). Good tools for modelling, pattern recognition and for receptor/ protein binding are available. Techniques are in sight to do this with non-crystallising proteins. What is needed is a better understanding of the biology behind the responses. It is possible to envisage the modelling of virtual cells and even the liver in the foreseeable future.

The need to be able to better predict the metabolites of compounds was repeated throughout the meeting; metabolites are responsible for many of the toxic responses we see. Care is needed to ensure that the right structures are addressed and that data and models are publically available.

There was agreement that until now the best chemists might not have been interested in toxicology work because there is too little intellectual enjoyment (correlations). There would be, however, plenty of room to revisit SAR, to bring a new professionalism to it, especially if exciting new collaborations between chemists and toxicologists were established focussed on systems biology, mechanism and causality.

Overall this was judged to be a particularly potentially fruitful way of moving forward. Needs are:

- Extending our understanding of the structure of proteins in membranes
- Further populating existing chemical databases and linking them such that they can be queried simply via a common portal
- development of computer-based metabolic predictions based on better understanding of physical chemistry and species-specific cyp affinities. The key example was using SAR to predict p450 metabolism.
- Development of predictive models of chemical reactivity and other chemicals activities by relying on first-principles of chemical action
- Application of modern computational chemistry linked with the engagement of talented chemists to revolutionise (Q)SAR research

5. Bioengineering

The meeting was introduced to a range of new sensors which can interface with biological systems in different ways (Turner). These could be direct or indirect, involving imaging/molecular mirrors, i.e. surfaces with molecular recognition possibilities, various sensors and arrays, and nanowires which could be placed inside cells or animals to obtain information in real time. Other possibilities would be the development of 3-D matrices which include multiple cell types plus synthetic sensing elements to bring the system closer to an artificial organ. Population of a 3-D structure with fluidic flows could enable the creation of an artificial liver.

The participants immediately saw applications e.g. *in vivo* sensing and in the possible marriage of these techniques with stem cells.

There was a consensus that such bioengineering technologies were looking for applications and could easily be applied in toxicology. They could facilitate the development of better culture methods and better biological readouts for organ toxicity. These include:

- High-content protein and other arrays that will allow the detailed investigation of cellular responses.

- Microfluidics devices that can facilitate the culture of multiple tissues in a flow-coupled system
- Nanosensors that can evaluate the responses of individual cells.
- There is a need for appropriate funding calls to support this multi-disciplinary research

6. Genome, Modelling and Systems Biology

The importance of modelling in systems biology to be able to understand which correlations are causative was underlined (Lehrach). The importance of this work being quantitative was emphasised. Life is a complex network of interacting systems, each component interacting with many others, such that disturbances and perturbations have wide effect. Second-generation sequencing is very efficient and it is now much easier to generate reliable genomic information. Novel genetics research was described, involving replacement of chromosomes one at a time between two quite distant strains of mice. This powerful genetics tool can inform models. Classical genetics, together with toxicogenomics, could enable the modelling of processes directed towards prediction, for instance to predict a particular patient's response to a drug or chemical exposure. It was recognised that whilst much of this may appear fairly remote today, systems biology needs a real practical opportunity to address such as toxicology.

It was noted that models had so far failed to closely mimic real life and that modelling needs to keep a foot in the real world. This led to a discussion about the importance of good quality databases and the loss or ignoring of historical phenotyping. Primary data needs to be of the highest quality for modelling to be helpful although it was also pointed out that modelling can reveal where primary data is incorrect or missing.

There is a need to create a suitable environment where the experimentalists and modellers can work together as was the case in climate change. The advantage would be that biological modelling will be more reliable than climate modelling because we know what we are modelling – in contrast to climate models!

The needs are:

- Development of models of target tissues and their toxicological responses, first as a means of prioritizing hypotheses about potential mechanisms of toxic action, and ultimately as predictors of toxic response (when coupled with other technologies like chemi-informatics, in vitro toxicogenomics and others).
- Research focused on a holistic approach to genomics, i.e., integrating expression data with, an understanding of the roles of non-coding RNAs (e.g., miRNAs which affect the half-life of mRNA), the epigenome (i.e., DNA methylation, histone code, non-coding RNAs), post-translational modifications, protein levels and metabolite levels. Progress is being made, slowly, in very difficult area
- Making progress on the question of how to marry the different approaches.

7. 3Rs and Animal Use in Toxicity Testing

Today's standard animal test protocols look at a number of assays over a number of times periods to assess acute and chronic effects covering eye/skin irritation, mutagenicity, immunotoxicity, reproductive and developmental toxicity, and neuro-behavioural effects (Eaton). The largest numbers of animals are used for multi-generational developmental testing. The progress made by ILSI/ HESI in the development of protocols for pesticides which obviated the need for multigenerational studies drastically reducing animal use was noted.

An evaluation of data on 90 day and 1 year for tests using dogs was described. It took a major scholarly endeavour to show that the 1 year test gave no valuable additional information and to be able to argue that regulatory standards were not dropping by the removal of this late time point. It was recognised that there are often other non-scientific, commercial interests in play when proposals replacing existing animal tests are made that need to be taken into account.

8. Genetic Models

Research on site-directed cell-specific temporally controlled [cre-lox] mice models and their potential in toxicology was described (Chambon). These can be valuable in terms of understanding mechanisms of toxicity and for testing chemicals and the doses likely to cause cancers that metastasise. But it is necessary to define exactly which genes should be targeted in order to provide relevant information, and also to consider downstream events related to mechanism; this major test would not be for first screening.

This led to a discussion about the controversy of using genetic models which theoretically help speed up testing in toxicology and in carcinogenesis. Some asked rhetorically "Why would you replace a poor mouse test with one validated against that test?"

Sensible group sizes were questioned and it was pointed out that mouse genetic variability is comparable to humans. Inbred mice may therefore miss the unexpected/rare events. On the other hand, the use of several mouse genetic models exhibiting a variety of genetic backgrounds may reveal the unexpected/rare events in humans.

A further suggestion emerged combining targeted use of transgenic mice for mechanistic evaluation in combination with studies of lower organisms. The meeting mostly agreed that for suitable endpoints, model organisms like zebra fish could prove useful in toxicology studies to replace higher animals, but only in defined areas (known metabolites) where we are looking at early gene events known to occur in human toxicity. Zebra fish are surprisingly poorly characterised genetically. As in all cases of new testing, the validation would need to be against human data derived from clinical collaborations.

9. General Points

- The meeting questioned whether research funders and journals such as Nature should force better database behaviour given this was a recurring theme throughout the workshop. It was agreed that there is work to do on databases; on how to stimulate them, to take advantage of the

nucleii already started, making them publically available and encouraging people to share to get to a single searchable database on ~omics.

- The EPAA research workgroup will review the ideas and suggestions from the Workshop in preparing research priorities to be funded through the Commission and/or industry and industry associations. The Participants felt that there was a need for follow-up workshops to delve further into many of the topics raised.
- All agreed that there is a need to recognise that adequate time will be needed to move from the science to accepted tests in the area of repeat dose toxicology. It is not possible now. The science is simply too immature to set a time line at this research stage.
- Some worried about the lead time to regulatory approval for new methods. Others were less concerned as it should be possible to get over the barriers to approval if the new approaches give better predictions than animal tests.
- The EPAA was commended as powerful linking industry, academia and governments to promote the field of Alternatives.

VI. CONCLUSIONS

The Participants agreed that there is considerable value in reconsidering the science base for regulatory testing in the field of repeat dose systemic toxicity.

The time is right to harness more effectively the very substantial achievements that have been witnessed in biology and chemistry during the last 10 years. Many seminal discoveries and technological advances have the potential to impact substantially on the development of alternative approaches. Funding at the nexus of the disciplines of toxicology, biology chemistry and mathematics was recommended.

More specifically, the Workshop identified opportunities in computational chemistry, in mathematical modelling, in stem cell biology and through harnessing the power of various 'omics' technologies which could each be developed in tandem with systems biology and bio-engineering. The opportunity exists to re-invigorate alternatives research.

The provision of truly innovative approaches that will allow the safe development of new products without recourse to animal experiments is amongst the great scientific challenges and should be promoted as such by EPAA.

It is important however that sight is not lost of the other 2Rs – refinement and reduction given the likely timelines to replacement. Consideration is needed for approaches using lower organisms linked with highly targeted transgenic animals.

Recommendations were made for future, subject focused workshops and for steps to ensure greater access and compatibility to databases.

In summary and in the context of the meeting objectives it was agreed that the meeting succeeded in identifying key areas of complementary science which have the capacity to revolutionise the science of safety assessment. Successful engagement of the wider science community in this area holds the key to this and will help legitimise alternatives research. The provision of truly innovative approaches to testing that will allow the safe development of new products without recourse to animal experiments is amongst the great scientific challenges. Whilst achieving that goal is over the horizon, it doesn't make the challenge any less intriguing, or any less worthwhile.

APPENDIX 1: WORKSHOP MODERATOR AND PARTICIPANTS

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APPENDIX 2: SUMMARY & RELEVANCE OF PAPERS DISTRIBUTED BEFORE THE WORKSHOP

The purpose of the workshop is to solicit your ideas about future research directions that may be fruitful to us as we develop non-animal alternatives for repeated-dose toxicity testing. Although we intend for the workshop to be an open, unconstrained discussion of what might be possible, we thought it would be helpful for you to have copies of papers that give an idea of what others have been thinking about possible approaches to 1) understanding the critical molecular interactions that underlie toxicity; 2) approaches to predicting responses at higher levels of biological organization from responses observed in simpler systems.

Toxicity testing is now at a crossroads. The field of toxicology has long since moved past the point of being an observational science and is now dominated by research into the mechanistic underpinnings of adverse responses. However, the testing of new chemical entities is still largely an observational exercise in which the chemical is administered to intact animals, and the responses are observed at the tissue and organ level. There are a number of reasons why testing continues to be done in this way. First, the outcomes from these studies are typically disease states or indicators of disease states (e.g., clinical chemistry biomarkers) that are relevant to humans. Furthermore, the process of extrapolating the dosage that produced an adverse effect in animals to a predicted safe exposure level for humans is a relatively straightforward procedure. Secondly, toxicity can occur by any of a large number of mechanisms of action; we have identified a great many of these mechanisms, but by no means all of them. Using animal models that are phylogenetically close to humans provides some assurance that the model contains the vast majority of mechanisms that are relevant for human toxicity, even if we don't know what all the possible mechanisms are. Thirdly, the intact organism serves as an integrator of pharmacokinetics, metabolism, and interaction between different tissues and organs in a way that is difficult to model in simpler systems. These are the obstacles that need to be overcome in order to replace

animal testing as a predictor of the potential for repeated exposures to a chemical to cause systemic toxicity.

Technologies are now becoming available that allow us to address these sticking points. On the subject of identification of mechanisms, genome-wide analysis of gene expression holds promise in identifying the initial range of responses of the organism, at least at the molecular level. After several years of investigation, it is now clear that virtually all (except the most acute) toxic responses are accompanied by changes in gene expression. In some instances the gene expression changes are integral to the adverse response, in others they are secondary and represent compensatory changes. In either case, analysis of gene expression can provide valuable information about the nature of toxicity, while providing confidence that the range of likely mechanisms has been identified. The papers by Boverhof and Zacharewski (2006) and Daston (2006) provide a brief overview of the possible applications of genomics technology in toxicology.

Genomics technologies may also make it possible to address questions in toxicology that we have been unable to address, except in rare circumstances, in the past. One of these is the question of the range of variability of susceptibility to a toxic insult, across the human population. We know that not everyone has the same susceptibility to a given insult (consider, for example, the occasional cigarette-smoking centenarian), and that susceptibility/resistance is attributable to the interactions of the toxicant with a number of gene products, each of which confer a slight increase or decrease in the risk of developing a disease in response to the insult. Genomics technologies are capable of helping us identify the genotypes that confer susceptibility, a concept reviewed in Orphanides and Kimber (2003).

The potential for an untested chemical entity to exert toxicity can sometimes be predicted by comparing its chemistry to other

substances that have been evaluated for toxicity. The rigor with which these comparisons can be made has improved markedly over the past several years because of two advances, one being the rapid evolution of computing power and the other being the development of large relational databases of chemical structures coupled with information on the physics, chemistry, pharmacology and toxicology of chemicals. The potential to use these databases to predict toxicity is described by Richard (2006).

Computational power will also provide us with the capability to develop sophisticated chemical-biological interactions at a subcellular and cellular level. These will be important if we wish to understand the quantitative relationships between dosage and effect, and temporal aspects that may separate exposure from response. An example of this kind of modeling is presented in Shankaran et al. (2007). This is not a toxicological paper per se, but does describe the interaction of a ligand with its receptor, which is how many toxicants act.

In order to make progress in replacing animal models, it is likely that we will need to develop models that describe the interactions of a chemical with its biological target at the molecular or cellular level. Toxicity, however, is expressed at the tissue or organ level and can be considered to be the summation of a large number of responses at these less complex levels. Toxicologists have been taking a number of approaches to integrate responses across biological organization, approaches that have been called

biologically-based dose-response modelling (e.g., Conolly et al., 2004) or systems biology/toxicology (e.g., Ekins et al., 2005). The substance of these papers is probably more than we are likely to get into in our discussions, but they provide an important reminder of the need to be able to provide not just a yes/no prediction about toxicity potential but more quantitative information about the likelihood of response and the dose at which it can occur, in order for models to be useful for toxicological assessment. We have included one more paper in this category, Davidson's (2006) review on the control of animal body plans by gene regulatory networks. It describes a different way of thinking about how to organize gene expression in formation in a way that describes biology at the organismal level.

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