Lush Prize 2016

Science Prize

Research Paper

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Note from Lush Prize: This Research Paper was commissioned to help identify potential science projects that may be suitable for nomination for the Lush Prize 2016, but which may not have been aware of the Lush Prize through our outreach activities.

Additional publications research discovered that some of the projects highlighted in this paper did not meet the strict Eligibility Criteria for the Lush Prize (http://lushprize.org/awards/eligibility/); those that did were contacted and it was suggested that they submit a nomination.

All nominations firstly go through a shortlisting process before being passed to the independent panel of judges who select winners of the Lush Prize 2016.

1. Executive Summary

1.1 What is the Lush Science Prize?

Now in its fifth year, the Lush Prize supports animal-free testing by awarding money prizes totalling £250,000 to the most effective projects and individuals who have been working towards the goal of replacing animals in product or ingredient safety testing.

Prizes are awarded for developments in five strategic areas: science; lobbying; training; public awareness; and Young Researchers. Should there be a major breakthrough in 21st Century Toxicology - the area which holds out most hope for a 'Eureka' moment leading to the replacement of animal tests – a Black Box Prize equivalent to the entire annual fund of £250,000 will be awarded to the individual(s) or team(s) responsible. In 2015, the judges awarded a Black Box prize for the development of the skin sensitisation AOP and associated, approved, *in vitro* assays.

The Science Prize is awarded to the group (or groups) whose work the judging panel believe has made the most significant contribution(s) to the replacement of animal testing in the preceding year. This 2016 Science Background paper identifies 29 pieces of work that we believe constitute potential candidates for the Judge's shortlist.

1.2 Methodology

In order to obtain an overview of developments in the field of animal replacement in toxicity pathway research, we firstly reviewed the recent work of the relevant scientific institutions and projects in this area, including the OECD; CAAT; Hamner Institutes; Human Toxome Project; ECVAM; UK NC3Rs; US Tox21 Programme; the ToxCast programme; the Human Toxicology Project Consortium; and EU-ToxRisk (see section 4). We also assessed recent developments in toxicity testing research by reviewing the relevant literature (see section 5 for some highlights).

In our search for candidate prize winners, we identified conferences focusing on animal replacement in toxicity testing that have been held in the preceding 12 months. These included the EUSAAT-Linz conference, the Society of Toxicology annual conference, and the SEURAT-1 annual conference. There were a total of 2930 abstracts from oral and poster presentations from these conferences, but many were not relevant to the Lush Science prize. We then performed literature searches using PubMed, Google Scholar and Terkko Feed Navigator to identify projects describing recent advances in toxicity testing research. Three additional relevant projects were identified directly from the Tox21, ToxCast, and Human Toxicology Project Consortium websites. In all, searches yielded in excess of 3,100 projects which we assessed as described in Section 3. Relevant abstracts were scored using the system derived in previous years (see section 3 for details) in which 3 points are awarded for projects identifying new toxicity pathways, assays, or biomarkers; 2 points for reporting new knowledge or tools; and 1 point for abstracts which stand out in some other way.

Overall, from 85 abstracts which scored 1 or more: 5 scored 1; 51 scored 2; 7 scored 2 + 1 (i.e. total 3); and 17 scored 3 because they appeared to be reporting a new toxicity pathway, assay, or biomarker. Finally, 5 projects scored the maximum possible marks of 3 for reporting a new toxicity assay, pathway, or biomarker, plus an additional mark for standing out as well. The titles and authors of those abstracts scoring 1 or more are shown in section 6.3, whilst full abstracts of those projects scoring at least 3 (either as 2+1, 3, or 3+1) are given in Section 6.4.

1.3 Projects recommended for the shortlist

There were 29 projects which received scores of at least 3 for reporting new pathways, assays, or biomarkers of toxicity. The full abstracts are given in Section 6.4. We consider all to be worthy for consideration by the judges as potential prize winners.

2. Background

There are two compelling reasons for ending animal testing: it is cruel and it is ineffective[1]. The objectives of 21st Century Toxicity Testing (tt21c) research can be summarised as: improving safety testing of chemicals and contaminants by using more relevant and predictive human models; and simplifying and automating tests so that many more chemicals can be tested for safety. The Science background papers for the 2012, 2013, and 2014 Lush Prizes provide an overview, and links to further resources, describing the concept of 21st Century Toxicology.

The Lush Prizes aim to focus attention on toxicity testing for consumer products and ingredients, in a way which complements those projects which address the use of animals in medical testing. The Lush Science Prize seeks to reward those researchers making 'outstanding contributions' to tt21c research. In previous years, the focus of the award has been on research aimed at elucidating key pathways in which perturbation results in toxicity. This approach was continued in 2015, when it was further extended to cover additional aspects of tt21c, beyond toxicity pathways. In particular, in 2015, we also sought to identify significant contributions to the development of new *in vitro* assays that advance tt21c, and to the discovery of biomarkers that signal early activation of toxicity pathways. Here, for the 2016 Science Prize paper, we have repeated this three-pronged approach to identify projects that are candidates for the Lush Science Prize.

The brief for prize applicants, taken from the Lush prize website http://www.lushprize.org/ was as follows:

Science Prize

For individuals, research teams or institutions for work conducted on relevant toxicity pathways. Outstanding research producing an effective non-animal safety test based on an approach other than toxicity pathways, where none existed before, may also be considered.

There is a £50,000 prize fund shared between all the winners of the Science Prize.

21st Century Toxicology is a new approach to safety testing which is exciting regulators, toxicologists, campaigners and companies around the world. It has become possible because of advances in biology, genetics, computer science and robotics.

It offers better relevance to humans (rather than using mice, rats and rabbits), and will explain the underlying causes of toxicity. Unlike animal methods, the new tests will help predict human variability and differential effects on embryos, children and adults. And as the superior scientific basis of the new approach is recognised, outdated animal tests will be replaced.

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1   http://www.lushprize.org/background/animal-testing/

3. Methodology

The main aim of this report is to assist the Lush Prize judging panel by identifying key projects that are making major contributions to the field of animal-free toxicology research. From these, the panel may select the winners for the 2016 Lush Science Prize. In this section we describe how we identified projects that might be worthy of consideration as potential prize winners, and then how we scored each project to create a shortlist for the panel's consideration.

Of the "3 Rs", Lush's interest focuses primarily on Replacement, so our search for potential prize winners targeted projects working towards the replacement of animals in product testing, and we excluded research aimed at either Refining or Reducing the use of animals in experimentation. Since the focus of the Lush prize is on general pathways of compound safety testing, we excluded research that focuses on specific diseases, including cancer. We considered projects based anywhere in the world, but only considered work written in the English language. As far as possible, we restricted the search to work reported in the year preceding the award (i.e. June 2015 – June 2016).

In the identification of key developments in the area of toxicology research, and in the search for candidate prize winners, we followed three separate strands of investigation. We started firstly by reviewing the recent research of some key institutions and collaborative projects working in the area of animal replacement in toxicity pathway research. These included the OECD; CAAT; Hamner Institutes; Human Toxome Project; ECVAM; UK NC3Rs; US Tox21 Programme; the ToxCast programme; the Human Toxicology Project Consortium; and EU-ToxRisk (see section 4).

Secondly, we identified relevant conferences held in the preceding 12 months and assessed abstracts, where available, for oral and poster presentations. Scientific conferences provide the forum in which the most up-to-date science is shared, reporting on recent developments and work-in-progress, without the lag time required for formal presentation as a journal publication. The relevant conferences for 2015 – 2016, for which abstracts were available, included: the 16th annual congress of EUSAAT (the European Society for Alternatives to Animal Testing), held in September 2015 in Linz; the EU SEURAT-1 Project's Symposium held in December 2015 in Brussels; and finally The Society of Toxicology 55th Annual Meeting held in March 2016, in New Orleans, Louisiana.

Thirdly, we conducted a review of the recent literature. For this we used three separate sources. Firstly, we searched PubMed for research published from 01/06/2015 to 17/06/2016, combining search terms "toxicity pathway," "toxicity assay" and "toxicity biomarker", excluding any review articles and clinical trials, and restricting the subject matter to "humans". As a second literature source, we searched Google Scholar for relevant papers published in the period 2015 to 2016, combining search terms "toxicity pathway," "toxicity assay" and "toxicity biomarker", and restricting the subject matter to "humans". As a final literature source, we searched Terkko Feed Navigator, combining search terms "toxicity pathway," "toxicity assay" and "toxicity biomarker".

As in previous years, our selection procedure was a three stage process. At each stage of our search, research projects were carefully excluded based on our selection criteria, in order to achieve a manageable shortlist of excellent work which fully met the prize brief. In the first stage, we reviewed the title of the work and rejected any which were clearly reviews or which were obviously unsuitable through either using animal models or through being overly focused on disease. We also rejected any which were

not written in the English language, and those for which abstracts were unavailable. In the second stage, we assessed the abstracts of projects which passed the initial filter and further eliminated those which reported findings from clinical trials, those focusing on disease research, and all research that included animal subjects.   In the third stage, projects identified as potentially relevant based on the abstract were scored using a system devised and successfully applied in previous years. In this system, points are awarded according to the following criteria:

Does the work appear to be reporting: discovery of a new pathway; a significant advance in assay technology or approach; or a new biomarker for early activation of toxicity?   *Score 3*

If it is working with an apparently previously understood pathway, assay technology, or biomarker, does it bring new knowledge or tools?   *Score 2*

Does it stand out in any other way?   *Score 1*

Projects awarded a score of 2 or 3 could also receive an extra 1 point if they also stood out in some other way, so the maximum possible score is 4.

4. Significant Institutional and Project Developments

This section summarises significant events or news focussing on 21st century toxicology from selected Institutions and major collaborative projects, reported within the last year.

4.1 Tox21

Tox21 is a collaborative effort among the National Institutes of Health (NIH), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA). NIH partners include NCATS (National Center for Advancing Translational Sciences) and the National Toxicology Program, administered by the National Institute of Environmental Health Sciences. The Tox21 initiative is designed to improve current toxicity assessment methods, which are slow and costly, by utilising robotic systems and high-throughput technologies to screen thousands of compounds.

In January 2016, Tox21 launched the Tox Testing Challenge[2], with an award fund of up to $1 million to improve the relevance and predictivity of data generated from automated chemical screening technology used for toxicity testing. The Challenge is co-sponsored by the Tox21 consortium members. In May 2016 Tox21 announced that ten selected semi-finalists will each receive $10,000 to help develop prototypes of their ideas.

4.2 ToxCast

The US EPA's ToxCast (Toxicity Forecaster) programme aims to use high-throughput screening tools to test many thousands of chemicals in in vitro assays. It is organised under the umbrella of Tox21, and has made some significant progress in the last year[3].

- A 17-centre, transatlantic, collaborative, large-scale modelling project has demonstrated the efficacy of using predictive computational models trained on high-throughput screening data to evaluate over 32,000 chemical structures for oestrogen receptor activity.

- Updates of the ToxCast and Tox21 high-throughput *in vitro* database, available to download, with associated summary files, for thousands of chemicals and hundreds of assays.

- Publication of a network model of oestrogen receptor activation, based on integration of data from 18 *in vitro* assays for various aspects of oestrogen receptor function[4].

4.3 EU-ToxRisk

EU-ToxRisk is a consortium of 39 partner organisations from academia, SMEs, large corporations, and regulatory bodies, co-funded by Horizon 2020 and Cosmetics Europe. The project has a budget of €30 million, to be spent over six years, and is the

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2   http://transformtoxtesting.com/

3   https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data

4   Judson *et al* Integrated model of chemical perturbations of a biological pathway using 18 *in vitro* high-throughput screening assays for the estrogen receptor. Toxicol Sci 148, 137-54 doi: 10.1093/toxsci/kfv168

successor to the SEURAT-1 programme. SEURAT-1's budget was €50 million, spent over five years, so EU-ToxRisk is funded at half the level of SEURAT-1.   EU-ToxRisk aims to integrate new concepts for efficient, animal-free regulatory chemical safety assessment. The project was started in January 2016 and is co-ordinated by Bob van de Water, Professor of Toxicology at Leiden University, The Netherlands.

4.4 The Organisation for Economic Co-operation and Development (OECD)

Animal-free Test Guidelines published by the OECD since the 2015 Lush Science Prize research paper was prepared include:

- TG 491 *in vitro* test for identifying chemicals inducing serious eye damage using an established rabbit cornea cell line;

- TG 492 *in vitro* test for identifying chemicals NOT requiring classification for eye irritation or serious eye damage, using reconstructed human cornea-like epithelium (RhCE). We have included the Kaluzhny *et al* paper describing the validation of this test in our list of potential Lush Prize winners, with a score of score 3+1;

- TG 493 describes *in* vitro binding assays for identifying human oestrogen receptor binding chemicals;

- The hCLAT test, recommended for approval by EURL ECVAM last year, has been approved as a test guideline by the OECD Working Group (April 2016), although the official publication of the Test Guideline (TG 442E) has not yet appeared.

4.5 Hamner Institutes

The Hamner Institutes suddenly and unexpectedly closed on 31st December 2015, after more than 40 years of non-profit research into chemical safety testing.   One division, the Institute for Chemical Safety Sciences, was sold to SciMetrika, when the rest of the Hamner Institutes were closed down. The Hamner Institutes' website is still running, and makes no mention of the Institutes' closure. It is not clear what has happened to the DILIsym project, although its website is still running.

4.6 Cosmetics Europe

Cosmetics Europe is the trade association for the European cosmetics industry. In addition to its support for the EU-ToxRisk programme, mentioned above, Cosmetics Europe has independently created a Long Range Science Strategy that will fund research focussed on repeat dose toxicity, bioavailability (ADME), and systemic toxicity. The primary goal of the strategy is animal-free prediction of risk of human toxicity (for cosmetic ingredients and products) with broad scientific and regulatory acceptance. Currently funding (amount unspecified) is allocated for the period 2016 to 2020.

4.7 European Union Reference Laboratory for alternatives to animal testing (**EURL ECVAM**)

The role of EURL ECVAM is the validation of methods which reduce, refine, or replace the use of animals in safety testing and in efficacy/potency testing of chemicals, biologicals and vaccines.

In November 2015, an expert meeting arranged by EURL ECVAM determined that negative data from approved *in vitro* skin sensitisation assays should be accepted as evidence of low risk, unless there was additional information to suggest that a chemical is likely to be a pro-hapten[5].

In February 2016, EURL ECVAM conducted a public survey to identify knowledge sources relevant to 3Rs. The results of the survey will be available by the end of 2016.

In April 2016, EURL ECVAM announced that EU member states had modified REACH regulations to make the newly approved *in vitro* skin sensitisation assays TG 442-C, -D, and the hCLAT assay, the default sources of information for skin sensitisation risk under REACH, rather than the animal-based LLNA that was previously the default test.

4.8 HSI-Human Toxicology Project Consortium (HTPC)

The HSI-Human Toxicology Project Consortium is a group of stakeholders currently drawn from the corporate and public interest communities that share the objective of accelerating the implementation of a biological pathway-based approach to toxicology, as described in the National Research Council's 2007 report on "Toxicity Testing in the 21st Century."   Its work in the 2015/2016 period has focussed mainly on dissemination of advances in assays and technologies for tt21c, such as the development of brain organoids, and high content screening tools for compound safety testing. The HTPC website highlighted two interesting papers, discussed in Section 5.

4.9 National Centre for the Replacement, Refinement, & Reduction of Animals in Research (NC3Rs)

In November 2015, NC3Rs published a roadmap for the UK on developing and applying non-animal technologies (NATs) to improve the methods and tools available for chemical safety testing[6]. The roadmap was prepared in collaboration with the MRC, BBSRC, EPSRC, Innovate UK, and the Defence Science & Technology Laboratory. It outlines plans for how the UK might invest in NATs over a 15 year period to 2030. While the roadmap offers direction for UK investment, it does not accurately reflect the current state of regulatory approvals, highlighting, as it does, that there are no non-animal OECD test guidelines for skin sensitisation. Such OECD guidelines were published in February 2015 and had been recommended for adoption before that.

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5   https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-status-reports/pre-prohapten-workshop-report

6   https://www.nc3rs.org.uk/sites/default/files/documents/NonAnimalTechCO082_RYE_4_nrfinal2.pdf

5. Literature Highlights

We have undertaken an extensive literature search to identify potential Lush Science Prize nominees. Details of the search and our results are given elsewhere in this document (see Sections 3 and 6). In the course of the research for this Science Prize paper, we noted some trends in the nature of research undertaken to develop alternatives for animal tests, which the Lush Prize organisation might consider relevant to the future scope of the Science Prize.

Firstly, two papers[7,8] highlighted the potential for tt21c techniques and assays to be extended to drug and food ingredient safety testing. While the Lush Prize focusses on "toxicity testing for consumer products and ingredients, to complement projects addressing the use of animals in medical testing", the reality is that many more animals are used in preclinical drug development and medical research than in chemical testing. Steps to extend the principles and accumulated knowledge of tt21c to other areas and industries where animals are widely used are to be welcomed.

Secondly, we came across very many reports and descriptions of specific *in vitro* models for detecting chemical-induced perturbations in cellular systems. These range from newly developed cell lines, through 3D culture models and perfusion systems, to organs-on-a-chip and even humans-on-a-chip. Such systems are based on ideas that have been developed over quite some time, and incremental changes are being made. These tools are developed by research teams and companies approaching the problems in different ways, and it is very difficult to predict which systems will be successful in the long-term. As an example, hiPSCs can be differentiated to hepatocyte-like cells. Unfortunately, these cells express a foetal hepatocyte phenotype and are therefore not suitable for testing chemical hepatotoxicity in adults. Growing hiPSC-hepatocytes in a 3D culture system shifts the phenotype towards a more adult pattern, but only incompletely. One of the reports that we have proposed be considered for a prize[9] noted that hepatocytes only acquire an adult phenotype post-partum. Avior *et al* treated hiPSC-hepatocytes with nutritional factors and successfully induced an adult hepatocyte phenotype. Significant as such work appears to be, it will be difficult to assess its impact on tt21c for some time. The process of incremental improvements in technology, following significant leaps such as the development of iPSCs, make it difficult to identify individual steps as worthy of awards without the benefit of hindsight.

Finally, and depressingly, we were struck by the number of papers that continue to conduct comparative studies between animal and human models – examples include comparing mouse and human neuroprogenitor cells during assay developemnt[10], comparing human and rat lung slices for testing the toxicity of cigarette smoke[11], and

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7   Langley *et al*, Lessons from Toxicology: Developing a 21st-Century Paradigm for Medical Research. Environ Health Perspect. 2015 Nov;123(11):A268-72. doi: 10.1289/ehp.1510345

8   Rovida *et al*, Toxicity testing in the 21st century beyond environmental chemicals. ALTEX. 2015;32(3):171-81. doi: 10.14573/altex.1506201.

9   Avior et al, Microbial-derived lithocholic acid and vitamin K2 drive the metabolic maturation of pluripotent stem cells-derived and fetal hepatocytes. Hepatology. 2015 Jul;62(1):265-78. doi: 10.1002/hep.27803 Score 2+1

10 Druwe *et al*, Sensitivity of neuroprogenitor cells to chemical-induced apoptosis using a multiplexed assay suitable for high-throughput screening. Toxicology. 2015 Jul 3;333:14-24. doi: 10.1016/j.tox.2015.03.011

11 Obernolte *et al* EUSAAT 2015, Abstract 161

comparing transported activities in primary human and Cynomolgus monkey hepatocytes[12].   Clearly there remains a significant drive to fund and conduct animal experiments without prior stringent ethical review.

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12 Ullrich *et al* EUSAAT 2015, Abstract 59

6.   Toxicity Pathway Abstracts

6.1 Conference Abstract Selection

As described in the Methodology, we reviewed abstracts from the 16th annual congress of EUSAAT, the EU SEURAT-1 Project's 2015 Brussels Symposium, and The Society of Toxicology's 55th Annual meeting, 2016.

From the 333 abstracts which comprised the EUSAAT 2015 conference presentation and poster proceedings, we identified 18 abstracts which passed through our selection process to the final (3rd) scoring stage. Of these, 12 scored 1 or more.

From the SEURAT-1 2015 meeting, 40 abstracts were available, of which 7 passed to the scoring stage. Of these, 6 scored 1 or more.

From the total of 2557 abstracts presented at the Society of Toxicology's 2016 meeting, and identified as potentially relevant based on the Abstract book keyword index, 96 were identified by the keyword "biomarker", of which 3 were scored; 61 were identified by the keyword "mechanism", of which 2 were scored; and 117 were identified by the keywords "alternatives to animals," of which 12 were scored. Of the total 17 abstracts which passed through to the scoring, stage, 11 scored 1 or more.

6.2 Published Abstract Selection

From the PubMed search, we identified 1842 relevant titles from the "Toxicity assay" search, a further 114 relevant projects from the "Toxicity biomarker" search, and finally an additional 173 titles from the "toxicity pathway" search: a combined total of 2129 articles.

Stages 1 and 2 of the selection process (review of titles and, if necessary abstracts, to reject review articles, articles not written in English, results of clinical trials, articles reporting use of animal subjects, or those focused on cancer research or other disease), reduced these 2129 titles to just 80 abstracts: 58 from the Toxicity assay search; 7 from the Toxicity biomarker search; and 15 from the Toxicity pathway search. These final 80 abstracts were passed to the third assessment stage, to be scored as potential prize winners. Of these, 45 scored 1 or more (36 from the Toxicity assay search, 6 from the Toxicity biomarker search, and 3 from the Toxicity pathway search).

The Google Scholar search for the period 2015 to 2016 identified 383 possibly relevant abstracts additional to those identified by PubMed described above. Of these, 329 were identified by the Toxicity pathway search, a further 14 from the Toxicity biomarker search, and 40 from the Toxicity pathway search. Of the 383, only 9 survived selection stages 1 and 2 (4 from the Toxicity assay search and 5 from the Toxicity pathway search) and were passed on to the scoring stage. Of these 9, a final 6 scored 1 or more.

The Terkko FeedNavigator search yielded no further relevant papers beyond those already identified by the other literature searches.

We also identified three further relevant projects, directly from the Tox21, ToxCast, and HTPC websites, which scored 1 or more.

6.3 Scores

From the three separate sources of potential shortlisted projects, we identified a total of 85 abstracts describing work which scored at least one point according to our given criteria. Of these, 5 scored 1 for standing out in some way (for example by providing opportunities for data sharing, or for combining methodologies to give "added value"); 51 scored 2 for bringing new knowledge or tools to a previously identified pathway, assay, or biomarker of toxicity; 7 scored 2 for bringing new knowledge or tools to a previously identified pathway, assay, or biomarker of toxicity but with an additional 1 (i.e. total 3) because they stood out in some other way; and 17 scored 3 because they appeared to be reporting a new toxicity pathway, assay, or biomarker. Only 5 scored the maximum possible 4 marks, with 3 awarded for reporting a new toxicity pathway, assay, or biomarker plus an additional mark for standing out in some other way.

The Table lists details (Title, Authors, contribution (pathway, assay, or biomarker), source, and score) of all the abstracts scoring 1 or more. The Table is ordered by source of abstract – websites, PubMed, Google Scholar, EUSAAT, SEURAT-1, and SoT. All of the abstracts for those projects scoring a total of 3 or more are shown in full in Section 6.4. For abstracts identified in the PubMed searches, the abstract title is a hyperlink to the PubMed index for that paper.

Title	Authors	Category	Source	Score
Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization	Huang *et al*	Assay	Tox21	3+1
High-throughput imaging-based nephrotoxicity prediction for xenobiotics with diverse chemical structures	Su *et al*	Assay	HTPC	3

Title	Authors	Category	Source	Score
Integrated Model of Chemical Perturbations of a Biological Pathway Using 18 In Vitro High-Throughput Screening Assays for the Estrogen Receptor	Judson *et al*	Assay	ToxCast	2+1
Characterization of the modes of action of deoxynivalenol (DON) in the human Jurkat T-cell line	Katika *et al*	Pathway	PubMed	3
Toxicological responses of environmental mixtures: Environmental metal mixtures display synergistic induction of metal-responsive and oxidative stress genes in placental cells	Adebambo *et al*	Biomarker	PubMed	2
Exposure to coplanar PCBs induces endothelial cell inflammation through epigenetic regulation of NF-κB subunit p65	Liu *et al*	Pathway	PubMed	3
Identification of TGF-β receptor-1 as a key regulator of carbon nanotube-induced fibrogenesis	Mishra *et al*	Pathway	PubMed	2
Roles of oxidative stress and the ERK1/2, PTEN and p70S6K signaling pathways in arsenite-induced autophagy	Huang *et al*	Pathway	PubMed	2
Action of methyl-, propyl- and butylparaben on GPR30 gene and protein expression, cAMP levels and activation of ERK1/2 and PI3K/Akt signaling pathways in MCF-7 breast cancer cells and MCF-10A non-transformed breast epithelial cells	Wróbel & Gregoraszczuk	Pathway	PubMed	2
Cadmium delays non-homologous end joining (NHEJ) repair via inhibition of DNA-PKcs phosphorylation and downregulation of XRCC4 and Ligase IV	Li *et al*	Pathway	PubMed	2
Tributyltin induces mitochondrial fission through NAD-IDH dependent mitofusin degradation in human embryonic carcinoma cells	Yamada *et al*	Pathway	PubMed	2
Molecular mechanisms of human thyrocyte dysfunction induced by low concentrations of polychlorinated biphenyl 118 through the Akt/FoxO3a/NIS pathway	Guo *et al*	Pathway	PubMed	2
Concurrent acetylation of FoxO1/3a and p53 due to sirtuins inhibition elicit Bim/PUMA mediated mitochondrial dysfunction and apoptosis in berberine-treated HepG2 cells	Shukla *et al*	Pathway	PubMed	2
The plasticizer BBP selectively inhibits epigenetic regulator sirtuins.	Zhang *et al*	Pathway	PubMed	3+1

Title	Authors	Category	Source	Score
Towards in vitro DT/DNT testing: Assaying chemical susceptibility in early differentiating NT2 cells.	Menzner *et al*	Assay	PubMed	3
A fluorescence high throughput screening method for the detection of reactive electrophiles as potential skin sensitizers.	Avonto *et al*	Assay	PubMed	2
Development of an in Silico Profiler for Mitochondrial Toxicity.	Nelms *et al*	Assay	PubMed	2+1
Transcriptomic Analysis of Human Primary Bronchial Epithelial Cells after Chloropicrin Treatment	Pesonen *et al*	Pathway	PubMed	2+1
Eye Irritation Test (EIT) for Hazard Identification of Eye Irritating Chemicals using Reconstructed Human Cornea-like Epithelial (RhCE) Tissue Model	Kaluzhny *et al*	Assay	PubMed	3+1 – new OECD test
Integrating High-Dimensional Transcriptomics and Image Analysis Tools into Early Safety Screening: Proof of Concept for a New Early Drug Development Strategy	Verbist *et al*	Assay	PubMed	1
A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation and optimization for histone deacetylase inhibitors	Rempel *et al*	Assay	PubMed	2+1
Human Pluripotent Stem Cell Based Developmental Toxicity Assays for Chemical Safety Screening and Systems Biology Data Generation	Shinde *et al*	Assay	PubMed	3
Morphological and Functional Characterization and Assessment of iPSC-Derived Hepatocytes for In Vitro Toxicity Testing	Lu *et al*	Assay	PubMed	1
Selective Sensitization of Zinc Finger Protein Oxidation by Reactive Oxygen Species through Arsenic Binding	Zhou *et al*	Pathway	PubMed	2
Nephron Toxicity Profiling via Untargeted Metabolome Analysis Employing a High Performance Liquid Chromatography-Mass Spectrometry-based Experimental and Computational Pipeline	Ranninger *et al*	Assay	PubMed	2+1
Non-animal photosafety screening for complex cosmetic ingredients with photochemical and photobiochemical assessment tools	Nishida *et al*	Assay	PubMed	2

Title	Authors	Category	Source	Score
Mimicking enzymatic effects of cytochrome P450 by an efficient biosensor for in vitro detection of DNA damage	Jalalvand *et al*	Assay	PubMed	3
Toxicogenomic responses of human liver HepG2 cells to silver nanoparticles	Sahu *et al*	Pathway	PubMed	2
Development of the adverse outcome pathway "alkylation of DNA in male premeiotic germ cells leading to heritable mutations" using the OECD's users' handbook supplement	Yauk *et al*	Pathway	PubMed	3 – new AOP
Gene Expression Regulation and Pathway Analysis After Valproic Acid and Carbamazepine Exposure in a Human Embryonic Stem Cell-Based Neurodevelopmental Toxicity Assay	Schulpen *et al*	Pathway	PubMed	2
Mechanism of neutrophil activation and toxicity elicited by engineered nanomaterials	Johnston *et al*	Pathway	PubMed	2
Investigation of acetaminophen toxicity in HepG2/C3a microscale cultures using a system biology model of glutathione depletion	Leclerc *et al*	Pathway/ Assay	PubMed	2
Multiparametric assay using HepaRG cells for predicting drug-induced liver injury	Tomida *et al*	Assay	PubMed	2
Extension of the Dermal Sensitisation Threshold (DST) approach to incorporate chemicals classified as reactive	Safford *et al*	Assay	PubMed	2
Arsenic Inhibits DNA Mismatch Repair by Promoting EGFR Expression and PCNA Phosphorylation	Tong *et al*	Pathway	PubMed	2
Particle-induced cell migration assay (PICMA): A new in vitro assay for inflammatory particle effects based on permanent cell lines	Westphal *et al*	Assay	PubMed	3
Endoplasmic Reticulum Stress and Store-Operated Calcium Entry Contribute to Usnic Acid-Induced Toxicity in Hepatic Cells	Chen *et al*	Pathway	PubMed	2
Evaluation of an optimized protocol using human peripheral blood monocyte derived dendritic cells for the in vitro detection of sensitizers: Results of a ring study in five laboratories	Reuter *et al*	Assay	PubMed	3
Perfluorooctanoic acid (PFOA) affects distinct molecular signalling pathways in human primary hepatocytes	Burhke *et al*	Pathway	PubMed	2

Title	Authors	Category	Source	Score
The Myeloid U937 Skin Sensitization Test (U-SENS) addresses the activation of dendritic cell event in the adverse outcome pathway for skin sensitization	Piroird *et al*	Assay	PubMed	2
Microbial-derived lithocholic acid and vitamin K2 drive the metabolic maturation of pluripotent stem cells-derived and fetal hepatocytes	Avior *et al*	Assay	PubMed	2+1
Contribution of membrane trafficking perturbation to retinal toxicity	Khoh-Reiter *et al*	Pathway	PubMed	2
Development of a toxicogenomics signature for genotoxicity using a dose-optimization and informatics strategy in human cells	Li *et al*	Pathway	PubMed	2
Genes specifically modulated in sensitized skins allow the detection of sensitizers in a reconstructed human skin model. Development of the SENS-IS assay	Cottrez *et al*	Assay	PubMed	2+1
Use of HPLC/UPLC-spectrophotometry for detection of formazan in in vitro Reconstructed human Tissue (RhT)-based test methods employing the MTT-reduction assay to expand their applicability to strongly coloured test chemicals	Alépée *et al*	Assay	PubMed	2
Successful validation of genomic biomarkers for human immunotoxicity in Jurkat T cells in vitro	Schmeits *et al*	Assay	PubMed	3
Identification of cornifelin and early growth response-1 gene as novel biomarkers for in vitro eye irritation using a 3D reconstructed human cornea model MCTT HCE™	Choi *et al*	Biomarker/assay	PubMed	2
High-content screening of drug-induced mitochondrial impairment in hepatic cells: effects of statins	Tolosa *et al*	Assay	PubMed	3
Development of an in vitro test to identify respiratory sensitizers in bronchial epithelial cells using gene expression profiling	Dik *et al*	Pathway	Google Scholar	2
Contact dermatitis: in pursuit of sensitizer's molecular targets through proteomics	Guedes *et al*	Pathway	Google Scholar	2
A fluorescence high throughput screening method for the detection of reactive electrophiles as potential skin sensitizers	Avonto *et al*	Assay	Google Scholar	2
MicroRNA-122: A Novel Hepatocyte-Enriched in vitro Marker of Drug-Induced Cellular Toxicity	Kia *et al*	Pathway	Google Scholar	2

Title	Authors	Category	Source	Score
Assessment of the eye irritation potential of chemicals: A comparison study between two test methods based on human 3D hemi-cornea models	Tandon *et al*	Assay	Google Scholar	3
RNA transcripts for the quantification of differentiation allow marked improvements in the performance of embryonic stem cell test (EST)	Romero *et al*	Assay	Google Scholar	2
Conversion of the draft OECD guideline for the testing of chemicals: the Cytosensor Microphysiometer test method	Eggert *et al*	Assay	EUSAAT 2015 #39	2
CERST-NRW – a platform for developing human in vitro methods for (neuro)-developmental toxicity testing	Goniwiecha *et al*	Assay	EUSAAT 2015 #87	1
Stem cell-derived human dorsal root ganglia-like cells to identify peripheral neurotoxicants	Hoelting *et al*	Assay	EUSAAT 2015 #136	3
Development, optimization and standardization of an in vitro skin irritation test for medical devices using the reconstructed human tissue model EpiDerm	Kandarova *et al*	Assay	EUSAAT 2015 #233	2
Identification of transcriptome signatures and biomarkers specific for migration-inhibiting potential developmental toxicants in human neural crest cells	Pallocca *et al*	Biomarker	EUSAAT 2015 #188	2
BoNT activity determination by stimulus-dependent release of neuro-secretory vesicle-targeted luciferase from neuronal cell lines	Pathe-Neuschäfer-Rube *et al*	Assay	EUSAAT 2015 #16	3+1
Evaluation of developmental neurotoxicity triggered by N-methyl-D-aspartate receptor (NMDAR) antagonists based on the Adverse Outcome Pathway (AOP) concept	Price *et al*	Pathway	EUSAAT 2015 #63	2
Intra- and inter-laboratory validation of LuSens: a reporter gene-cell line to detect keratinocyte activation by skin sensitizers	Ramirez *et al*	Biomarker	EUSAAT 2015 #194	2
Metabolomics in vitro: a new approach for systemic toxicity – first applications for mode of action identification and chemical grouping	Ramirez *et al*	Assay	EUSAAT 2015 #196	1

Title	Authors	Category	Source	Score
Micellar liquid chromatography as an alternative for in vivo and expensive in vitro tests commonly used in prediction of human intestinal absorption	Shokry *et al*	Assay	EUSAAT 2015 #54	3
In vitro assays for the potency determination of botulinum neurotoxin serotypes A and B	Wild *et al*	Assay	EUSAAT 2015 #17	3+1
Evaluation of an in vitro human dermal Sensitization test for use with medical device extracts	Willoughby	Assay	EUSAAT 2015 #315	2
In silico modeling for the prediction of dose and pathway related adverse effects in humans from *in vitro* repeated-dose studies	Klein *et al*	Assay	SEURAT-1 2015 NOTOX #2	2
A 3D *in vitro* HepaRG model for the identification and study of compounds with cholestatic liability	Puigvert *et al*	Pathway	SEURAT-1 2015 NOTOX #5	2
A High Content Imaging (HCI) BAC-GFP Toxicity Pathway Reporter Platform for Mechanism-based Assessment of Drug-Induced Liver Injury (DILI)	Wink *et al*	Assay	SEURAT-1 2015 Detective #1	2
In vitro verification of an adverse outcome pathway of cholestatic liver injury using "omics" technologies	Wink *et al*	Assay	SEURAT-1 2015 Detective #2	2
In vitro based prediction of human hepatotoxicity	Stöbe *et al*	Assay	SEURAT-1 2015 Detective #3	2
Identification of genomic biomarkers for anthracycline-induced cardiotoxicity in human iPSC-derived cardiomyocytes: an *in vitro* repeated exposure toxicity approach for safety assessment	Chaudhari *et al*	Biomarkers	SEURAT-1 2015 Detective #4	2
Migration of Human Neural Crest Cells as Functional Endpoint to Screen for Developmental Neurotoxicity	Nyffeler *et al*	Assay	SoT 2016 #1171	3
Human Relevant Intra-Laboratory Validated In Vitro Vasculo/Angiogenesis Test	Heinonen *et al*	Assay	SoT 2016 #2176	3

Title	Authors	Category	Source	Score
High Density Human Stem Cell Micromass to Model Teratogen-Induced Limb Malformations	Yu *et al*	Assay	SoT 2016 #2178	3
Quantifiable 3D In Vitro Vasculogenesis Assay Emphasizes Importance of Cell-Matrix Interactions When Screening Putative Developmental Toxins	Thiede *et al*	Assay	SoT 2016 #2180	2
Multivariate Models for Prediction of Human Skin Sensitization Hazard	Strickland *et al*	Assay	SoT 2016 #2202	1
Development of Photo-Direct Peptide Reactivity Assay	Nishida *et al*	Assay	SoT 2016 #2204	2
Identification of Compounds That Modulate Retinol Signaling Using a Cell-Based qHTS Assay	Chen *et al*	Assay	SoT 2016 #2462	2
Development of an In Vitro Test for Allergenic Potency of Proteins	Tsukumo *et al*	Assay	SoT 2016 #2471	2
In Vitro Model Development for Safety Assessment of Acutely Ingested Proteins	Wezalis *et al*	Assay	SoT 2016 #3064	2
In Vitro Developmental Neurotoxicity Testing Models Using HiPSC-Derived Neural Stem Cells	Yi *et al*	Assay	SoT 2016 #3074	2
Thymic Stromal Lymphopoietin Induced by Protein and Chemical Allergens in Cultured Human Keratinocytes: A Potential Biomarker for Immediate-Type Hypersensitivity	Kuroda *et al*	Biomarkers	SoT 2016 #2732	2
Bile Canaliculi Dynamics Alterations as Early Preclinical Predictive Markers of Drug Induced Cholestasis	Burbank *et al*	Biomarkers	SoT 2016 #1970	2
In Vitro Toxicity and Resilience of A 3D Human Dopaminergic Model to Rotenone	Harris *et al*	Pathway	SoT 2016 #2340	2

6.4 Nominated Abstracts

This year 29 projects received the highest scores of either 3 or 4 for reporting new pathways, assays, or biomarkers of toxicity. The 29 abstracts are given below. For abstracts from published work, we provide the digital object identifier (DOI) which allows the original document to be located online. For conference abstracts, we give the abstract or poster number for identification.

We consider all worthy of being considered by the judges as potential prize winners.

Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization

R Huang, M Xia, S Sakamuru, J Zhao, S A Shahane, M Attene-Ramos, T Zhao, C P Austin, A Simeonov.

Division of Pre-clinical Innovation, National Center for Advancing Translational Sciences, National Institutes of Health, 9800 Medical Center Drive, Rockville, Maryland 20850, USA.

Score 3 + 1 (New assay/pathway + scale)

Abstract

Target-specific, mechanism-oriented in vitro assays post a promising alternative to traditional animal toxicology studies. Here we report the first comprehensive analysis of the Tox21 effort, a large-scale in vitro toxicity screening of chemicals. We test   10,000 chemicals in triplicates at 15 concentrations against a panel of nuclear receptor and stress response pathway assays, producing more than 50 million data points. Compound clustering by structure similarity and activity profile similarity across the assays reveals structure-activity relationships that are useful for the generation of mechanistic hypotheses. We apply structural information and activity data to build predictive models for 72 in vivo toxicity end points using a cluster-based approach. Models based on in vitro assay data perform better in predicting human toxicity end points than animal toxicity, while a combination of structural and activity data results in better models than using structure or activity data alone. Our results suggest that in vitro activity profiles can be applied as signatures of compound mechanism of toxicity and used in prioritization for more in-depth toxicological testing.

High-throughput imaging-based nephrotoxicity prediction for xenobiotics with diverse chemical structures

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Score 3 (Assay)

Abstract

The kidney is a major target for xenobiotics, which include drugs, industrial chemicals, environmental toxicants and other compounds. Accurate methods for screening large numbers of potentially nephrotoxic xenobiotics with diverse chemical structures are currently not available. Here, we describe an approach for nephrotoxicity prediction that combines high-throughput imaging of cultured human renal proximal tubular cells (PTCs), quantitative phenotypic profiling, and machine learning methods. We automatically quantified 129 image-based phenotypic features, and identified chromatin and cytoskeletal features that can predict the human in vivo PTC toxicity of 44 reference compounds with ~82 % (primary PTCs) or 89 % (immortalized PTCs) test balanced accuracies. Surprisingly, our results also revealed that a DNA damage response is commonly induced by different PTC toxicants that have diverse chemical structures and injury mechanisms. Together, our results show that human nephrotoxicity can be predicted with high efficiency and accuracy by combining cell-based and computational methods that are suitable for automation.

Integrated Model of Chemical Perturbations of a Biological Pathway Using 18 In Vitro High-Throughput Screening Assays for the Estrogen Receptor.

R S Judson[1], F M Magpantay[2], V Chickarmane[3], C Haskell[4], N Tania[5], J Taylor[6], M Xia[7], R Huang[7], D M Rotroff[8], D L Filer[9], K A Houck[10], M T Martin[10], N Sipes[11], A M Richard[10], K Mansouri[9], R W Setzer[10], T B Knudsen[10], K M Crofton[10], R S Thomas[10].

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[10]U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711;

[11]NIH National Toxicology Program, Research Triangle Park, North Carolina 27711.

Score 2+1   (Assays + scale)

Abstract

We demonstrate a computational network model that integrates 18 in vitro, high-throughput screening assays measuring estrogen receptor (ER) binding, dimerization, chromatin binding, transcriptional activation, and ER-dependent cell proliferation. The network model uses activity patterns across the in vitro assays to predict whether a chemical is an ER agonist or antagonist, or is otherwise influencing the assays through a manner dependent on the physics and chemistry of the technology platform ("assay interference"). The method is applied to a library of 1812 commercial and environmental chemicals, including 45 ER positive and negative reference chemicals. Among the reference chemicals, the network model correctly identified the agonists and antagonists with the exception of very weak compounds whose activity was outside the concentration range tested. The model agonist score also correlated with the expected potency class of the active reference chemicals. Of the 1812 chemicals evaluated, 111 (6.1%) were predicted to be strongly ER active in agonist or antagonist mode. This dataset and model were also used to begin a systematic investigation of assay interference. The most prominent cause of false-positive activity (activity in an assay that is likely not due to interaction of the chemical with ER) is cytotoxicity. The model provides the ability to prioritize a large set of important environmental chemicals with human exposure potential for additional in vivo endocrine testing. Finally, this model is generalizable to any molecular pathway for which there are multiple upstream and downstream assays available.

Characterization of the modes of action of deoxynivalenol (DON) in the human Jurkat T-cell line.

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RIKILT-Institute of Food Safety, Wageningen University & Research Centre, Wageningen, The Netherlands .

Score 3 (Pathway)

Abstract

Deoxynivalenol (DON) is one of the most abundant mycotoxins worldwide and mostly detected in cereals and grains. As such, DON poses a risk for many adverse health effects to human and animals. In particular, immune cells are very sensitive to DON, with the initiating step leading to toxicity being a binding to the eukaryotic 60S ribosomal subunit and induction of ribotoxic stress. The present study aimed to: (1) extend insight into the mechanism of action (MOA) of DON in immune cells; and (2) understand why immune cells are more sensitive to DON than most other cell types. Previously published microarray studies have described the effects of DON on immune cells. To build upon these findings, here, immunocytological and biochemical studies were performed using human T-lymphocyte Jurkat cells that were exposed for 3 h to 0.5 µM DON. Induction of ER stress by DON was confirmed by immunocytology demonstrating increased protein expression of two major ER stress markers ATF3 and DDIT3. T-cell activation was confirmed by induction of phosphorylation of protein kinases JNK and AKT, activation of NF-κB (p65), and increased expression of NFAT target gene NUR77; each of these are known inducers of the T-cell activation response. Induction of an oxidative stress response was also confirmed by monitoring the nuclear translocation of major oxidative stress markers NRF2 and KEAP1, as well as by changes (i.e. decreases) in cell levels of reduced glutathione. Lastly, this study showed that DON induced cleavage of caspase-3, an event known to mediate apoptosis. Taken together, these results allowed us to formulate a potential mechanism of action of DON in immune cells, i.e. binding to eukaryotic 60S ribosomal subunit → ribotoxic stress → ER stress → calcium release from the ER into cytoplasm → T-cell activation and oxidative stress → apoptosis. It is proposed that immune cells are more sensitive to DON than other cell types due to the induction of a T-cell activation response by increased intracellular calcium levels.

Keywords: Apoptosis; ER stress; Jurkat cells; NF-κB; NFAT; deoxynivalenol; immunotoxicity; oxidative stress

Exposure to coplanar PCBs induces endothelial cell inflammation through epigenetic regulation of NF-κB subunit p65.

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Score 3 (Pathway)

Abstract

Epigenetic modifications of DNA and histones alter cellular phenotypes without changing genetic codes. Alterations of epigenetic marks can be induced by exposure to environmental pollutants and may contribute to associated disease risks. Here we test the hypothesis that endothelial cell dysfunction induced by exposure to polychlorinated biphenyls (PCBs) is mediated in part though histone modifications. In this study, human vascular endothelial cells were exposed to physiologically relevant concentrations of several PCBs congeners (e.g., PCBs 77, 118, 126 and 153) followed by quantification of inflammatory gene expression and changes of histone methylation. Only exposure to coplanar PCBs 77 and 126 induced the

expression of histone H3K9 trimethyl demethylase jumonji domain-containing protein 2B (JMJD2B) and nuclear factor-kappa B (NF-κB) subunit p65, activated NF-κB signalling as evidenced by nuclear translocation of p65, and up-regulated p65 target inflammatory genes, such as interleukin (IL)-6, C-reactive protein (CRP), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and IL-1α/β. The increased accumulation of JMJD2B in the p65 promoter led to a depletion of H3K9me3 repression mark, which accounts for the observed up-regulation of p65 and associated inflammatory genes. JMJD2B gene knockdown confirmed a critical role for this histone demethylase in mediating PCB-induced inflammation of the vascular endothelium. Finally, it was determined, via chemical inhibition, that PCB-induced up-regulation of JMJD2B was estrogen receptor-alpha (ER-α) dependent. These data suggest that coplanar PCBs may exert endothelial cell toxicity through changes in histone modifications.

The plasticizer BBP selectively inhibits epigenetic regulator sirtuins.

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Score 3 + 1 (Pathway + combination of human *in vitro* assay and *in silico* molecular binding analysis)

Abstract

The plasticizer benzyl butyl phthalate (BBP) is a well-known endocrine disruptor. Widespread human exposure to phthalates has raised substantial public concern due to its detrimental health effects. However, molecular mechanisms of the phthalates effect require elucidation. In this study, we analyzed: 1) the binding interaction of several phthalates and persistent organic pollutants with epigenetic regulator sirtuins and 2) the effect of BBP on the sirtuins in HepG2 cells. AutoDock molecular docking analysis showed that BBP binds to Sirt1 and Sirt3 proteins similarly to the native ligands with shortest binding free energies (ΔGb) of -7.35 and -8.3 kcal/mol, respectively; and inhibition constants (Ki) of 4.07 μM and 0.82 μM, respectively. Furthermore, BBP was superimposed onto the co-crystallized ligands within the least root-mean-square deviation (RMSD) of 0.96Å and 1.55Å for Sirt1 and Sirt3, respectively, and bound into the sites with a sufficient number of hydrogen bonds, implying the best fit compared to other sirtuins. In HepG2 cells, BBP significantly down-regulated Sirt1 and Sirt3 ($p < 0.05$) gene expression at a concentration as low as 10nM; other sirtuins remained unaffected. Consistent with decreased gene expression, Sirt1 and Sirt3 protein levels were significantly decreased at 48 h ($p < 0.05$). In addition, mitochondrial biogenesis regulators PGC-1α, NRF-1, and NRF-2, were decreased ($p < 0.05$). SiRNA studies showed that BBP did not regulate PGC-1α via sirtuin and BBP requires sirtuin's presence to regulate NRF-1 or NRF-2. BBP significantly increased ROS production ($p < 0.05$) and ROS may be chiefly regulated by NRF-1 and NRF-2 in HepG2 cells under Sirt1 and Sirt3 silenced condition. This is the first report to demonstrate that BBP selectively disrupts specific sirtuins in HepG2 cells. In conclusion, our study suggests that BBP can impair two vital epigenetic regulators and mitochondrial biogenesis regulators in liver cells.

Towards in vitro DT/DNT testing: Assaying chemical susceptibility in early differentiating NT2 cells.

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Score 3 (Assay)

Abstract

Human pluripotent embryonal carcinoma (NT2) cells are increasingly considered as a suitable model for in vitro toxicity testing, e.g. developmental toxicity and neurotoxicity (DT/DNT) studies, as they undergo neuronal differentiation upon stimulation with retinoic acid (RA) and permit toxicity testing at different stages of maturation. NT2 cells have recently been reported to show specific changes in dielectric resistance profiles during differentiation which can be observed as early as 24h upon RA-stimulation. These observations suggest altered susceptibility to chemicals at an early stage of differentiation. However, chemical susceptibility of early differentiating NT cells has not yet been studied. To address this question, we have established a cell fitness screening assay based on the analysis of intracellular ATP levels and we applied the assay in a large-scale drug screening experiment in NT2 stem cells and early differentiating NT2 cells. Subsequent analysis of ranked fitness phenotypes revealed 19 chemicals with differential toxicity profile in early differentiating NT2 cells. To evaluate whether any of the identified drugs have previously been associated with DT/DNT, we conducted a literature search on the identified molecules and quantified the fraction of chemicals assigned to the FDA (Food and Drug Administration) pregnancy risk categories (PRC) N, A, B, C, D, and X in the hit list and the small molecule library. While the fractions of the categories N and B were decreased (0.81 and 0.35-fold), the classes C, D and X were increased (1.35, 1.47 and 3.27-fold) in the hit list compared to the chemical library. From these data as well as from the literature review, identifying large fractions of chemicals being directly (42%) and indirectly associated with DT/DNT (32%), we conclude that our method may be beneficial to systematic in vitro-based primary screening for developmental toxicants and neurotoxicants and we propose cell fitness screening in early differentiating NT2 cells as a strategy for evaluating chemical susceptibility at different stages of differentiation to reduce animal testing in the context of the 3Rs.

Development of an in Silico Profiler for Mitochondrial Toxicity.

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School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University , Byrom Street, Liverpool L3 3AF, U.K.

Score 2 + 1 (Assay + in silico tool for identifying MIEs)

Abstract

This study outlines the analysis of mitochondrial toxicity for a variety of pharmaceutical drugs extracted from Zhang et al. ((2009) Toxicol. In Vitro, 23, 134-140). These chemicals were grouped into categories based upon structural similarity. Subsequently, mechanistic analysis was undertaken for each category to identify the molecular initiating event driving mitochondrial toxicity. The mechanistic information elucidated during the analysis enabled mechanism-based structural alerts to be developed and combined together to form an in silico profiler. This profiler is envisaged to be used to develop chemical categories based upon similar mechanisms as part of the adverse outcome pathway paradigm. Additionally, the profiler could be utilized in screening large data sets in order to identify chemicals with the potential to induce mitochondrial toxicity.

Transcriptomic Analysis of Human Primary Bronchial Epithelial Cells after Chloropicrin Treatment.

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Score 2 + 1 (Pathway + gene analysis)

Abstract

Chloropicrin is a vaporizing toxic irritant that poses a risk to human health if inhaled, but the mechanism of its toxicity in the respiratory tract is poorly understood. Here, we exposed human primary bronchial epithelial cells (HBEpC) to two concentrations of chloropicrin (10-50 μM) for 6 or 48 h and used genomic microarray, flow cytometry, and TEM-analysis to monitor cellular responses to the exposures. The overall number of differentially expressed transcripts with a fold-change > ± 2 compared to controls increased with longer exposure times. The initial response was activation of genes with a higher number of up- (512 by 10 μM and 408 by 40 μM chloropicrin) rather than down-regulated transcripts (40 by 10 μM and 215 by 40 μM chloropicrin) at 6 h seen with both exposure concentrations. The number of down-regulated transcripts, however, increased with the exposure time. The differentially regulated transcripts were further examined for enriched Gene Ontology Terms (GO) and KEGG-pathways. According to this analysis, the "ribosome" and "oxidative phosphorylation" were the KEGG-pathways predominantly affected by the exposure. The predominantly affected (GO) biological processes were "protein metabolic process" including "translation," "cellular protein complex assembly," and "response to unfolded protein." Furthermore, the top pathways, "NRF2-activated oxidative stress" and "Ah-receptor signaling," were enriched in our data sets by IPA-analysis. Real time qPCR assay of six selected genes agreed with the microarray analysis. In addition, chloropicrin exposure increased the numbers of late S and/or G2/M-phase cells as analyzed by flow cytometry and induced autophagy as revealed by electron microscopy. The

targets identified are critical for vital cellular functions reflecting acute toxic responses and are potential causes for the reduced viability of epithelial cells after chloropicrin exposure.

Eye Irritation Test (EIT) for Hazard Identification of Eye Irritating Chemicals using Reconstructed Human Cornea-like Epithelial (RhCE) Tissue Model.

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Score 3 + 1 (Assay + new OECD test)

Abstract

To comply with the Seventh Amendment to the EU Cosmetics Directive and EU REACH legislation, validated non-animal alternative methods for reliable and accurate assessment of ocular toxicity in man are needed. To address this need, we have developed an eye irritation test (EIT) which utilizes a three dimensional reconstructed human cornea-like epithelial (RhCE) tissue model that is based on normal human cells. The EIT is able to separate ocular irritants and corrosives (GHS Categories 1 and 2 combined) and those that do not require labeling (GHS No Category). The test utilizes two separate protocols, one designed for liquid chemicals and a second, similar protocol for solid test articles. The EIT prediction model uses a single exposure period (30 min for liquids, 6 hr for solids) and a single tissue viability cut-off (60.0% as determined by the MTT assay). Based on the results for 83 chemicals (44 liquids and 39 solids) EIT achieved 95.5/68.2/ and 81.8% sensitivity/specificity and accuracy (SS&A) for liquids, 100.0/68.4/ and 84.6% SS&A for solids, and 97.6/68.3/ and 83.1% for overall SS&A. The EIT will contribute significantly to classifying the ocular irritation potential of a wide range of liquid and solid chemicals without the use of animals to meet regulatory testing requirements. The EpiOcular EIT method was implemented in 2015 into the OECD Test Guidelines as TG 492.

A transcriptome-based classifier to identify developmental toxicants by stem cell testing: design, validation and optimization for histone deacetylase inhibitors.

E Rempel[1], L Hoelting, T Waldmann, NV Balmer, S Schildknecht, M Grinberg, JA Das Gaspar, V Shinde, R Stöber, R Marchan, C van Thriel, J Liebing, J Meisig, N Blüthgen, A Sachinidis, J Rahnenführer, JG Hengstler, M Leist.

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Score 2 + 1 (Assay + use of informatics)

Abstract

Test systems to identify developmental toxicants are urgently needed. A combination of human stem cell technology and transcriptome analysis was to provide a proof of concept that toxicants with a related mode of action can be identified and grouped for read-across. We chose a test system of developmental toxicity, related to the generation of neuroectoderm from pluripotent stem cells (UKN1), and exposed cells for 6 days to the histone deacetylase inhibitors (HDACi) valproic acid, trichostatin A, vorinostat, belinostat, panobinostat and entinostat. To provide insight into their toxic action, we identified HDACi consensus genes, assigned them to

superordinate biological processes and mapped them to a human transcription factor network constructed from hundreds of transcriptome data sets. We also tested a heterogeneous group of 'mercurials' (methylmercury, thimerosal, mercury(II)chloride, mercury(II)bromide, 4-chloromercuribenzoic acid, phenylmercuric acid). Microarray data were compared at the highest non-cytotoxic concentration for all 12 toxicants. A support vector machine (SVM)-based classifier predicted all HDACi correctly. For validation, the classifier was applied to legacy data sets of HDACi, and for each exposure situation, the SVM predictions correlated with the developmental toxicity. Finally, optimization of the classifier based on 100 probe sets showed that eight genes (F2RL2, TFAP2B, EDNRA, FOXD3, SIX3, MT1E, ETS1 and LHX2) are sufficient to separate HDACi from mercurials. Our data demonstrate how human stem cells and transcriptome analysis can be combined for mechanistic grouping and prediction of toxicants. Extension of this concept to mechanisms beyond HDACi would allow prediction of human developmental toxicity hazard of unknown compounds with the UKN1 test system.

NOTE: The UKN1 human stem cell line used in this study seems to require differentiation using conditioned medium from mouse embryonic cells – check with authors whether these are primary cells or a cell line.

Human Pluripotent Stem Cell Based Developmental Toxicity Assays for Chemical Safety Screening and Systems Biology Data Generation.

V Shinde[1], S Klima[2], PS Sureshkumar[1], K Meganathan[1], S Jagtap[1], E Rempel[3], J Rahnenführer[3], JG Hengstler[4], T Waldmann[2], J Hescheler[1], M Leist[2], A Sachinidis [5].

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Score 3 (Assay)

Abstract

Efficient protocols to differentiate human pluripotent stem cells to various tissues in combination with -omics technologies opened up new horizons for in vitro toxicity testing of potential drugs. To provide a solid scientific basis for such assays, it will be important to gain quantitative information on the time course of development and on the underlying regulatory mechanisms by systems biology approaches. Two assays have therefore been tuned here for these requirements. In the UKK test system, human embryonic stem cells (hESC) (or other pluripotent cells) are left to spontaneously differentiate for 14 days in embryoid bodies, to allow generation of cells of all three germ layers. This system recapitulates key steps of early human embryonic development, and it can predict human-specific early embryonic toxicity/teratogenicity, if cells are exposed to chemicals during differentiation. The UKN1 test system is based on hESC differentiating to a population of neuroectodermal progenitor (NEP) cells for 6 days. This system recapitulates early neural development and predicts early developmental neurotoxicity and epigenetic changes triggered by chemicals. Both systems, in combination with transcriptome

microarray studies, are suitable for identifying toxicity biomarkers. Moreover, they may be used in combination to generate input data for systems biology analysis. These test systems have advantages over the traditional toxicological studies requiring large amounts of animals. The test systems may contribute to a reduction of the costs for drug development and chemical safety evaluation. Their combination sheds light especially on compounds that may influence neurodevelopment specifically.

NOTE: The UKN1 human stem cell line used in this study seems to require differentiation using conditioned medium from mouse embryonic cells – check with authors whether these are primary cells or a cell line.

Nephron Toxicity Profiling via Untargeted Metabolome Analysis Employing a High Performance Liquid Chromatography-Mass Spectrometry-based Experimental and Computational Pipeline.

C Ranninger[1], M Rurik[2], A Limonciel[3], S Ruzek[1], R Reischl[1], A Wilmes[3], P Jennings[3], P Hewitt[4], W Dekant[5], O Kohlbacher[2], CG Huber[6].

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Score 2 + 1 (Metabolomics assay + linked to proteomics & transcriptomics)

Abstract

Untargeted metabolomics has the potential to improve the predictivity of in vitro toxicity models and therefore may aid the replacement of expensive and laborious animal models. Here we describe a long term repeat dose nephrotoxicity study conducted on the human renal proximal tubular epithelial cell line, RPTEC/TERT1, treated with 10 and 35 µmol·liter(-1) of chloroacetaldehyde, a metabolite of the anti-cancer drug ifosfamide. Our study outlines the establishment of an automated and easy to use untargeted metabolomics workflow for HPLC-high resolution mass spectrometry data. Automated data analysis workflows based on open source software (OpenMS, KNIME) enabled a comprehensive and reproducible analysis of the complex and voluminous metabolomics data produced by the profiling approach. Time- and concentration-dependent responses were clearly evident in the metabolomic profiles. To obtain a more comprehensive picture of the mode of action, transcriptomics and proteomics data were also integrated. For toxicity profiling of chloroacetaldehyde, 428 and 317 metabolite features were detectable in positive and negative modes, respectively, after stringent removal of chemical noise and unstable signals. Changes upon treatment were explored using principal component analysis, and statistically significant differences were identified using linear models for microarray assays. The analysis revealed toxic effects only for the treatment with 35 µmol·liter(-1) for 3 and 14 days. The most regulated metabolites were glutathione and

metabolites related to the oxidative stress response of the cells. These findings are corroborated by proteomics and transcriptomics data, which show, among other things, an activation of the Nrf2 and ATF4 pathways.

Mimicking enzymatic effects of cytochrome P450 by an efficient biosensor for in vitro detection of DNA damage.

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Score 3 (Assay)

Abstract

A novel biosensor for detecting DNA damage induced by benzo(a)pyrene (BP) and its metabolite was presented in this work. The nafion-solubilized single wall carbon nanotubes-ionic liquid (SWCNTs-NA-IL) composite film was prepared and then horseradish peroxidase (HRP) and double-stranded DNA were alternately assembled on the composite film by the layer-by-layer method. The biosensor was characterized by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), differential pulse voltammetry (DPV), scanning electron microscopy (SEM) and computational methods. UV-vis spectrophotometry was also used to investigate DNA damage induced by BP and its metabolites in solution. The DNA biosensor was treated separately in BP, hydrogen peroxide (H2O2) and in their mixture, respectively. The EIS analysis showed a decrease in the charge transfer resistance at the DNA/HRP/SWCNTs-NA-IL/GCE incubated in a mixture of HRP and H2O2, because HRP in the presence of H2O2 could mimic enzymatic effects of cytochrome P450 (CYP450) to metabolize BP which could cause significant DNA damage and the exposed DNA bases reduced the electrostatic repulsion of the negatively charged redox probe and leads to Faradaic impedance changes. Finally, a novel biosensor for BP determination was developed and this method provided an indirect, and quantitative estimation of DNA damage in vitro.

Keywords: Benzo(a)pyrene; Biosensor; DNA damage

Development of the adverse outcome pathway "alkylation of DNA in male premeiotic germ cells leading to heritable mutations" using the OECD's users' handbook supplement.

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Score 3 (Pathway - new AOP)

Abstract

The Organisation for Economic Cooperation and Development's (OECD) Adverse Outcome Pathway (AOP) programme aims to develop a knowledgebase of all known pathways of toxicity that lead to adverse effects in humans and ecosystems. A Users' Handbook was recently released to provide supplementary guidance on AOP development. This article describes one AOP-alkylation of DNA in male premeiotic germ cells leading to heritable mutations. This outcome is an important regulatory endpoint. The AOP describes the biological plausibility and empirical evidence supporting that compounds capable of alkylating DNA cause germ cell mutations and subsequent mutations in the offspring of exposed males. Alkyl adducts are subject to DNA repair; however, at high doses the repair machinery becomes saturated. Lack of repair leads to replication of alkylated DNA and ensuing mutations in male premeiotic germ cells. Mutations that do not impair spermatogenesis persist and eventually are present in mature sperm. Thus, the mutations are transmitted to the offspring. Although there are some gaps in empirical support and evidence for essentiality of the key events for certain aspects of this AOP, the overall AOP is generally accepted as dogma and applies broadly to any species that produces sperm. The AOP was developed and used in an iterative process to test and refine the Users' Handbook, and is one of the first publicly available AOPs. It is our hope that this AOP will be leveraged to develop other AOPs in this field to advance method development, computational models to predict germ cell effects, and integrated testing strategies.

Keywords: adverse outcome pathway; alkylation; germ cell; heritable mutation; weight of evidence

Particle-induced cell migration assay (PICMA): A new in vitro assay for inflammatory particle effects based on permanent cell lines.

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Score 3 (Assay)

Abstract

Inflammation is a decisive pathophysiologic mechanism of particle toxicity and accumulation of neutrophils in the lung is believed to be a crucial step in this process. This study describes an in vitro model for investigations of the chemotactic attraction of neutrophils in response to particles using permanent cell lines. We challenged NR8383 rat macrophages with particles that were characterized concerning chemical nature, crystallinity, and size distribution in the dry state and in the culture medium. The cell supernatants were used to investigate migration of differentiated human leukemia cells (dHL-60 cells). The dose range for the tests was determined using an impedance-based Real-Time Cell Analyzer. The challenge of NR8383 cells with 32-96 µg cm(-2) coarse and nanosized particles resulted in cell supernatants which induced strong and dose-dependent migration of dHL-60 cells. Quartz caused the strongest effects - exceeding the positive control "fetal calf serum" (FCS) several-fold, followed by silica, rutile, carbon black, and anatase. $BaSO_4$ served as inert control and induced no cell migration. Particles caused NR8383 cells to secrete chemotactic compounds. The assay clearly distinguished between the particles of different inflammatory potential in a highly reproducible way. Specificity of the test is suggested by negative results with $BaSO_4$.

Keywords: Cell migration; NR8383 cells; Neutrophilic inflammation; Particles; dHL-60 cells

Evaluation of an optimized protocol using human peripheral blood monocyte derived dendritic cells for the in vitro detection of sensitizers: Results of a ring study in five laboratories.

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Score 3 (Assay)

Abstract

Allergic contact dermatitis is a delayed T-cell mediated allergic response associated with relevant social and economic impacts. Animal experiments (e.g. the local lymph node assay) are still supplying most of the data used to assess the sensitization potential of new chemicals. However, the 7th amendment to the EU Cosmetic Directive have introduced a testing ban for cosmetic ingredients after March 2013. We have developed and optimized a stable and reproducible in vitro protocol based on human peripheral blood monocyte derived dendritic cells to assess the sensitization potential of chemicals. To evaluate the transferability and the predictivity of this PBMDCs based test protocol, a ring study was organized with five laboratories using seven chemicals with a known sensitization potential (one none-sensitizer and six sensitizers, including one pro-hapten). The results indicated that this optimized test protocol could be successfully transferred to all participating laboratories and allowed a correct assessment of the sensitization potential of the tested set of chemicals. This should allow a wider acceptance of PBMDCs as a reliable test system for the detection of human skin sensitizers and the inclusion of this protocol in the toolbox of in vitro methods for the evaluation of the skin sensitization potential of chemicals.

Keywords: Human dendritic cells; In vitro test system; Peripheral blood monocyte derived dendritic cells; Ring study; Skin sensitization

Microbial-derived lithocholic acid and vitamin K2 drive the metabolic maturation of pluripotent stem cells-derived and fetal hepatocytes.

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Score 2 + 1 (Assay + significant advance in use of hPSCs for hepatocyte model)

Abstract

The liver is the main organ responsible for the modification, clearance, and transformational toxicity of most xenobiotics owing to its abundance in cytochrome P450 (CYP450) enzymes. However, the scarcity and variability of primary hepatocytes currently limits their utility. Human pluripotent stem cells (hPSCs) represent an excellent source of differentiated hepatocytes; however, current protocols still produce fetal-like hepatocytes with limited mature function. Interestingly, fetal hepatocytes acquire mature CYP450 expression only postpartum, suggesting that nutritional cues may drive hepatic maturation. We show that vitamin K2 and lithocholic acid, a by-product of intestinal flora, activate pregnane X receptor (PXR) and subsequent CYP3A4 and CYP2C9 expression in hPSC-derived and isolated fetal hepatocytes. Differentiated cells produce albumin and apolipoprotein B100 at levels equivalent to primary human hepatocytes,

while demonstrating an 8-fold induction of CYP450 activity in response to aryl hydrocarbon receptor (AhR) agonist omeprazole and a 10-fold induction in response to PXR agonist rifampicin. Flow cytometry showed that over 83% of cells were albumin and hepatocyte nuclear factor 4 alpha (HNF4α) positive, permitting high-content screening in a 96-well plate format. Analysis of 12 compounds showed an R(2) correlation of 0.94 between TC50 values obtained in stem cell-derived hepatocytes and primary cells, compared to 0.62 for HepG2 cells. Finally, stem cell-derived hepatocytes demonstrate all toxicological endpoints examined, including steatosis, apoptosis, and cholestasis, when exposed to nine known hepatotoxins.

Conclusion: Our work provides fresh insights into liver development, suggesting that microbial-derived cues may drive the maturation of CYP450 enzymes postpartum. Addition of these cues results in the first functional, inducible, hPSC-derived hepatocyte for predictive toxicology.

Genes specifically modulated in sensitized skins allow the detection of sensitizers in a reconstructed human skin model. Development of the SENS-IS assay.

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Score 2 + 1 (Assay/biomarker + predictivity)

Abstract

Analysis of genes modulated during the sensitization process either on mice (LLNA) or human (blisters) combined with data mining has allowed the definition of a comprehensive panel of sensitization biomarkers. This set of genes includes already identified markers such as the ARE family and others not yet associated with the sensitization process (the so-called SENS-IS gene subset). The expression of this set of genes has been measured on reconstituted human epidermis models (Episkin) exposed to various sensitizers and non-sensitizers. Fine analysis of their expression pattern indicates that it is the number of modulated genes rather than the intensity of up-regulation that correlates best with the sensitization potential of a chemical. Moreover, sensitizers that are weak inductors of ARE genes tend to be relevant modulators of the SENS-IS subset. By combining the expression data obtained with both gene subsets, it is now possible to identify a wide variety of sensitizers on a test system (in vitro reconstructed human epidermis) that is very similar to the in vivo situation and compatible with a large variety of test substance characteristics.

Keywords: Alternatives to animal tests; Reconstituted epidermis; SENS-IS; Skin sensitization; Toxicogenomics

Successful validation of genomic biomarkers for human immunotoxicity in Jurkat T cells in vitro.

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Score 3 (Assay)

Abstract

Previously, we identified 25 classifier genes that were able to assess immunotoxicity using human Jurkat T cells. The present study aimed to validate these classifiers. For that purpose, Jurkat cells were exposed for 6 h to subcytotoxic doses of nine immunotoxicants, five non-immunotoxicants and four compounds for which human immunotoxicity has not yet been fully established. RNA was isolated and subjected to Fluidigm quantitative real time (qRT)-PCR analysis. The sensitivity, specificity and accuracy of the screening assay as based on the nine immunotoxicants and five non-immunotoxicants used in this study were 100%, 80% and 93%, respectively, which is better than the performance in our previous study. Only one compound was classified as false positive (benzo-e-pyrene). Of the four potential (non-)immunotoxicants, chlorantraniliprole and Hidrasec were classified immunotoxic and Sunset yellow and imidacloprid as non-immunotoxic. ToxPi analysis of the PCR data provided insight in the molecular pathways that were affected by the compounds. The immunotoxicants 2,3-dichloro-propanol and cypermethrin, although structurally different, affected protein metabolism and cholesterol biosynthesis and transport. In addition, four compounds, i.e. chlorpyrifos, aldicarb, benzo-e-pyrene and anti-CD3, affected genes in cholesterol metabolism and transport, protein metabolism and transcription regulation. qRT-PCR on eight additional genes coding for similar processes as defined in ToxPi analyzes, supported these results. In conclusion, the 25 immunotoxic classifiers performed very well in a screening with new non-immunotoxic and immunotoxic compounds. Therefore, the Jurkat screening assay has great promise to be applied within a tiered approach for animal free testing of human immunotoxicity.

High-content screening of drug-induced mitochondrial impairment in hepatic cells: effects of statins.

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Score 3 (Assay)

Abstract

A frequent mechanism for drug-induced liver injury (DILI) is mitochondrial impairment, and early evaluation of new drugs for their potential to cause mitochondrial dysfunction is becoming an important task for drug development. To this end, we designed a high-content screening assay to study mitochondrial-induced hepatotoxicity in HepG2 cells in detail. Simultaneous assessment of mitochondrial mass and cell viability in cells exposed for 24 h to compounds provides preliminary information on the mitochondrial- or nonmitochondrial-related hepatotoxic potential of compounds. To fully address the mechanisms implicated in mitochondrial impairment, prelethal changes in mitochondrial superoxide production, mitochondrial membrane potential, mitochondrial permeability transition, intracellular calcium concentration and apoptotic cell death were studied in cells incubated for 1 h with compounds. The assay correctly classified a set of well-known mitochondrial toxicants and negative controls and revealed high sensitivity for the detection of mitochondrial DILI and the establishment of different mitochondrial toxicity risks (low to high). This procedure was used for analysing the potential mitochondrial impairment of six statins to determine their clinical risk. All the tested statins produced mitochondrial impairment, although they showed different levels of toxicity (low-medium toxicity risk). The results suggest that this cell-based assay is a promising in vitro approach to predict the potential of drug candidates to induce mitochondrial-associated hepatotoxicity.

Keywords: Hepatotoxicity; High-content screening; Mitochondrial injury; Statins

Assessment of the eye irritation potential of chemicals: A comparison study between two test methods based on human 3D hemi-cornea models

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Score 3 (Assay)

Abstract

We have recently developed two hemi-cornea models (Bartok et al., Toxicol in Vitro 29, 72, 2015; Zorn-Kruppa et al. PLoS One 9, e114181, 2014), which allow the correct prediction of eye irritation potential of chemicals according to the United Nations globally harmonized system of classification and labeling of chemicals (UN GHS). Both models comprise a multilayered epithelium and a stroma with embedded keratocytes in a collagenous matrix. These two models were compared, using a set of fourteen test chemicals. Their effects after 10 and 60 minutes (min) exposure were assessed from the quantification of cell viability using the MTT reduction assay. The first approach separately quantifies the damage inflicted to the epithelium and the stroma. The second approach quantifies the depth of injury by recording cell death as a function of depth. The classification obtained by the two models was compared to the Draize rabbit eye test and an ex vivo model using rabbit cornea (Jester et al. Toxicol in Vitro. 24, 597–604, 2010). With a 60 min exposure, both of our models are able to clearly differentiate UN GHS Category 1 and UN GHS Category 2 test chemicals.

Keywords: Human 3D hemi-cornea model; Relative MTT-depth of injury; Globally harmonized system of classification and labelling of chemicals; The Draize eye irritation test

Stem cell-derived human dorsal root ganglia-like cells to identify peripheral neurotoxicants

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EUSAAT 2015 Abstract 136

Score 3 (Assay)

Safety sciences and the identification chemical hazard have been seen as one of the most immediate practical applications of human pluripotent stem cell technology. Protocols for the generation of many desirable human cell types have been developed, but optimization of neuronal models for toxicological use has been astonishingly slow, and the wide, clinically-important field of peripheral neurotoxicity is still largely unexplored. Here, a 2-step protocol to generate large lots of identical peripheral human neuronal precursors was characterized and adapted to the measurement of peripheral neurotoxicity. High content imaging allowed an unbiased assessment of cell morphology and viability. The computational quantification of neurite growth as functional parameter highly sensitive to disturbances by toxicants was used as endpoint reflecting specific neurotoxicity. The differentiation of cells towards dorsal root ganglia-like neurons was tracked in relation to a large background data set based on gene expression microarrays. On this basis, a peripheral neurotoxicity (PeriTox) test was developed as first toxicological assay that harnesses the potential of human pluripotent stem cells to generate cell types/tissues that are not otherwise available for prediction of human systemic organ toxicity. Testing of more than 30 chemicals showed that human neurotoxicants, as well as neurite growth enhancers, were correctly identified. Various classes of chemotherapeutics causing human peripheral neuropathies were identified, while they were missed when tested on human central neurons. The PeriTox-test established here shows the potential of human stem cells for clinically-relevant safety testing of drugs in use and of new emerging candidates.

BoNT activity determination by stimulus-dependent release of neuro-secretory vesicle-targeted luciferase from neuronal cell lines

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EUSAAT 2015 Abstract 16

Score 3 + 1 (Assay + potential high impact on reducing animal tests)

Botulinum toxin is a bacterial toxin that inhibits neurotransmitter release from neurons and thereby causes a flaccid paralysis. It is used as drug to treat a number of serious ailments and,more frequently, for cosmetic medical interventions. Botulinum toxin for pharmacological applications is isolated from bacterial cultures. Due to partial denaturation of the protein, the specific activity of these preparations shows large variations. Because of its extreme potential toxicity, pharmacological preparations must be carefully tested for their activity. For the current gold standard, the mouse lethality assay, several hundred thousand mice are killed per year.

Alternative methods have been developed that suffer from one or more of the following deficits: In vitro enzyme assays test only the activity of the catalytic subunit of the toxin. Enzymatic and cell based immunological assays are specific for just one of the different serotypes. The current study takes a completely different approach that overcomes these limitations: Neuronal cell lines were stably transfected with plasmids coding for luciferases of different species, which were N-terminally tagged with leader sequences that redirect the luciferase into neuro-secretory vesicles. From these vesicles, luciferases were released upon depolarization of the cells. The depolarization-dependent release was efficiently inhibited by of botulinum toxin in a concentration range (1 to 100 pM) that is used in pharmacological preparations. The new assay might thus be an alternative to the mouse lethality assay and the immunological assays already in use.

Note: This work has now been published as a full paper - DOI: http://dx.doi.org/10.14573/altex.1503061

Micellar liquid chromatography as an alternative for in vivo and expensive in vitro tests commonly used in prediction of human intestinal absorption

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EUSAAT Abstract 54

Score 3 *(Assay)*

Oral administration is one of the most important and abundant routes of drug administration, therefore estimation of intestinal absorption is crucial for pharmaceutical compounds, especially for drugs with poor solubility [1]. That is why it is important to determine the extent of drug absorption for new drug entities (NDE) in preformulation studies. This study proposes a novel, simple, economic, accurate and precise way to predict intestinal absorption using a modified chromatographic method.

Introduction: Animal testing is the standard method currently used to predict the extent and rate of intestinal absorption [2,3], i.e. through in vivo testing, for pharmaceutical compounds. In this study a form of chromatography, known as MLC, has been applied to predict passive intestinal absorption with a selection of model compounds through measurement and calculation of the partition coefficient, Pmw. In this method bile salts were used as a mobile phase (instead of the more standard head and tail surfactants) to provide an environment more closely simulating the human intestinal environment.

Methods and Reagents: Two bile salts were considered separately, namely sodium deoxycholate (NaDC) and sodium taurodeoxycholate (NaTDC) no buffers or organic modifiers were added during the study to simulate intestinal conditions as closely as possible. Low concentrations of 13 model drugs were prepared in the corresponding mobile phase concentration to avoid saturation of the bile salt mobile phase. The samples were injected into the chromatographic system with capacity factors obtained by analysis of the retention data recorded for the model drugs used. Modelling of Human intestinal absorption (% HIA), intestinal permeability of Caco-2 and PAMPA was performed by multiple linear regression. The models obtained confirmed the ability of MLC to predict human intestinal absorption (% HIA), Caco-2 and PAMPA intestinal permeability coefficients. Conclusion: The use of NaTDC in MLC was found to be a better method in the prediction of Human intestinal absorption while NaDC was

found to be better for prediction of PAMPA and Caco-2 intestinal permeability coefficients although both bile salts provided a superior prediction for both the in vivo (% HIA) and in vitro (Caco-2 and PAMPA) methods for intestinal absorption determination compared with current methods. Therefore, it was concluded that the MLC method was found to be a successful tool in prediction of human intestinal absorption (% HIA), Caco-2 and PAMPA intestinal permeability. Since MLC is a more economic [4], time saving [5], eco-friendly [6] method that does not include the use of animals, it can be considered to be a superior method over all available alternative options for prediction of intestinal absorption.

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In vitro assays for the potency determination of botulinum neurotoxin serotypes A and B

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EUSAAT Abstract 17

Score 3 + 1 (Assay + potential high impact on reducing animal tests)

Botulinum neurotoxins (BoNTs) have a paralytic effect, which is broadly applied in the fields of medicine and cosmetics. The toxins produced by the bacterium Clostridium botulinum have to be tested for their specific activity and potency, what is currently mostly done with LD50 tests in mice. As these tests cause a high distress for the animals, it is desirable to find alternatives to the LD50 test, for which at least 600,000 mice are used annually [1]. The combined assay presented here could be a suitable candidate for this task [2]. BoNTs use an elaborate intoxication process leading to a high toxicity with a human lethal dose of approximately 1 ng per kilogram body weight. During intoxication, BoNTs specifically recognize receptors on efferent nerves preceding neuromuscular junctions. In consequence of the binding, the toxin is taken up via endocytosis. Conformational changes caused by alterations of the pH and redox milieu in the endosome allow the light toxin subunit containing the protease domain to traverse the endosomal membrane and to simultaneously separate from the receptor bound toxin subunit. This leads to an activation of the protease, which then starts to cleave specific proteins involved in the release of the neurotransmitter acetylcholine. The blocked neurotransmitter release from the efferent nerve causes a flaccid paralysis of the respective muscle. The combined assay for potency determination of BoNTs which has been developed in our laboratory is based on the binding capacity as well as the proteolytic activity of the toxins. As the medically relevant BoNT serotypes A and B differ in their receptor proteins as well as in their substrates, two separate combined assays were developed. In these assays, BoNTs are bound to receptor molecules coated on a microtiter plate. As receptor molecules, the ganglioside GT1b as well as a receptor peptide (derived from synaptic vesicle protein 2c for BoNT/A, and synaptotagmin for BoNT/B) are applied. After binding, the protease domain is separated from the residual molecule by reduction and transferred to a second microtiter plate

coated with the substrate for the protease (synaptosomal-associated protein 25 for BoNT/A, and synaptobrevin for BoNT/B). Finally the cleaved substrate is detected with antibodies directed against the cleavage site. The development of the assay for the activity determination of BoNT/B has already been completed. In-house validation studies have shown that the assay is highly sensitive, with a detection limit below 1 pg/ml. For BoNT/A, assay optimization is ongoing. In addition to being preferable from an ethical point of view, the combined assay is also easier to perform, cheaper and faster than the LD50 test.

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Migration of Human Neural Crest Cells as Functional Endpoint to Screen for Developmental Neurotoxicity

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Society of Toxicology 2016 Abstract 1171

Score 3 (Assay)

Introduction: Neural crest cells (NCCs) are a transient stem cell population arising at the time of neurulation. NCCs migrate to various body parts and differentiate into cells of the peripheral nervous system, melanocytes as well as craniofacial bones and other structures. Therefore, failure of NCC to migrate can lead to severe developmental defects, so called neurocristopathies. Aim: We developed an in vitro test system using human NCCs and screened an 80-compound library of potential environmental toxicants for compounds interfering with NCC migration.

Methods: NCCs were differentiated from human pluripotent stem cells and cryopreserved until further use. For the cMINC migration assay, cells were seeded in plates containing silicon stoppers to create a cell-free circular area. Removal of the stoppers allowed the start of migration. After 48 h, the number of viable cells in the circular zone was quantified by high-content image analysis. The whole library, originally assembled and published by the national toxicology program of the US (NTP), was tested in three independent biological experiments at the highest non-cytotoxic concentration (≤ 20 µM). Compounds causing > 20% migration inhibition were re-ordered for confirmation and concentration-response curves were obtained. Results: Internal quality controls lead to consistent results and all negative controls were correctly classified. The screen identified 26 compounds as potentially migration-inhibiting, of which 22 were confirmed upon retesting. Among these migration-inhibiting compounds, seven were organophosphates, five were drug-like compounds, three were organochlorines and three were polybrominated flame retardants. Moreover, there were three other pesticides, and an industrial chemical. Conclusions: The cMINC assay yielded stable and reproducible results. Comparison to data already available on the library compounds from in vivo testing and other screens suggested a high sensitivity of our assay. We expect that a battery of related developmental toxicity tests may be used to generate alerts on potential toxicants, and to prioritize them for further testing.

Human Relevant Intra-Laboratory Validated In Vitro Vasculo/Angiogenesis Test

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Society of Toxicology 2016 Abstract 2176

Score 3 *(Assay)*

Formation of vascular network is a crucial process during embryonic development, and it also contributes to the pathogenesis of numerous disorders such as cancer and diabetes mellitus. One of the principal teratogenic mechanisms suspected to be associated with medication is vascular disruption (van Gelder et al.,Therapie 69(1), 13-24. 2014). Vascular network is formed either by vasculogenesis (in situ differentiation and growth of blood vessels) and angiogenesis (growth of new blood vessels from the preexisting ones). The vasculogenesis/angiogenesis test developed in FICAM utilizes primary human adipose stem cells and primary human umbilical cord vein endothelial cells. The 3D coculture formed contains 3D endothelial tubule structure (vWF) with lumen, younger and more mature vessels, basement membrane (colIV), adherens junctions (ve-cadherin, occludin) between endothelia, pericytes (PDGFRb, SMMHc, SMA) and endothelial cells characterized by immunostaining and electron microscopy. Further, the maturation process covering vasculogenesis and angiogenesis was confirmed by measuring the expression of the key marker genes (e.g. ANGPT1/2, FGF2, VEGF, PDGF, VEGFR1/2, TIE-2, PDGFRb). The test passed successfully intra-laboratory validation according to the OECD 34 guideline: reproducibility, repeatability and relevance were proven. There was no statistical difference between the results of different technicians (P=0.44, ANOVA) or different repeats of the same technician (CV<15%). The relevance to inhibit tubule formation was shown with drugs with published human data e.g. Erlotinib, Levamisole, 2-methoxyestradiol, acetylsalicylic acid, anti-VEGF, Cladribine, and Doxorubicin resulting in a good concordance (Pearson R=0.6921). This indicates that this human cell based test has potential be used as predictive in vitro test in assessing vascular disrupting potential of chemical substances in man. The applicability covers development of vascular-disrupting drugs as well as testing of chemicals for their potential to disrupt vasculature formation during embryogenesis.

High Density Human Stem Cell Micromass to Model Teratogen-Induced Limb Malformations

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Society of Toxicology 2016 Abstract 2178

Score 3 *(Assay)*

Limb malformations are a major feature of exposure to many developmental teratogens. Embryonic limb mesenchyme high density micromass culture is an excellent system for studying skeletal tissue development in vitro. We have developed micromass cultures with human adult bone marrow-derived mesenchymal stem cells (hBMSC), a more homogenous cell population, that can undergo chondrogenesis and chondrocyte hypertrophy in a predictable and physiological manner. In this study, we challenge hBMSC-based micromass with three toxicants, thalidomide, warfarin and mifepristone, to test the possibility that this hBMSC-based

culture may be used to study the effects of teratogenic compounds on limb development. hBMSCs micromass were induced to undergo chondrogenesis in TGF-β1 containing medium. On day 10, cultures were either analyzed for chondrogenic differentiation or induced to undergo hypertrophy by TGF- β1 withdrawal and T3 supplementation. Candidate teratogens were added throughout the culture period and the effect on chondrogenic and hypertophic markers were assayed histologically and by qRT-PCR as a function of dose. Control micromasses demonstrated robust chondrogenesis and hypertrophy, indicated by increased Alcian Blue staining and alkaline phosphatase (ALP), activity, respectively, and stage-specific gene expression. Thalidomide did not show any detectable impact on chondrogenesis and hypertrophy at any dose. Warfarin had minimal impact on chondrogenic gene expression but a significant effect on hypertrophic gene expression, matrix elaboration and ALP activity. Mifepristone significantly reduced chondrogenic and subsequent hypertrophic gene expression. The effects of both warfarin and mifepristone occurred at doses near reported maternal serum levels. The 3 toxicants tested affected hBMSC micromasses in ways related to their mechanism of action. Thalidomide, an inhibitor of angiogenesis, did not show any detectable impact on chondrogenesis or hypertrophy at any dose in this avascular model Warfarin, an inhibitor of post-translational γ-carboxylation critical for cartilage matrix production, affected mainly hypertrophy. Mifepristone, a glucocorticoid antagonist, strongly inhibited both chondrogenesis and hypertrophy. We conclude that high density, 3-dimensional hBMSC-based micromass cultures represent a reproducible and controlled model to screen for potential limb teratogens.

7. Conclusions

The review of the most recent EUSAAT, SoT, and SEURAT-1 conference proceedings, and an extensive literature search, yielded 29 abstracts describing projects which we believe the Judges should consider as potential candidates for the Lush Science Prize shortlist. These are given in Section 6.

Once again we have reviewed many more papers and abstracts than in the previous year (over 3,100 this year compared with around 2,200 in 2015). We also scored more abstracts this year than last (85 vs 50 in 2015). It was pleasing to find a larger proportion of high scoring (3 and 4) abstracts this year – 34% - compared with 2015 (10%).

The nominated abstracts are very diverse, and cover developing models for new toxicity assays, improvements in the phenotypic status of human iPSC-hepatocytes, high-throughput- and high content- screening, *in silico* analyses, and demonstrations of the utility of modern tt21d approaches. We believe that they are all worthy candidates for the 2016 Science Prize.