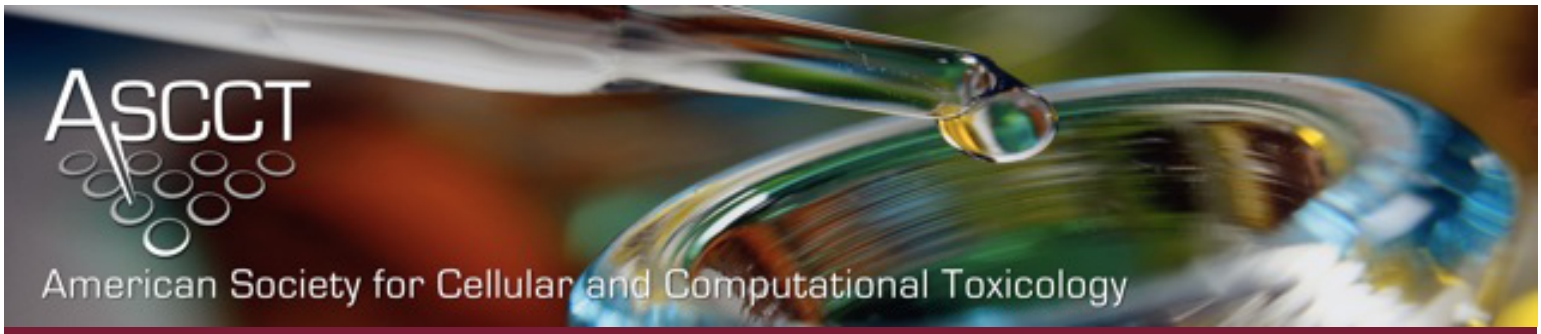

5th Annual Meeting
of the
American Society for Cellular and Computational Toxicology

September 29-30, 2016
US EPA Building
Research Triangle Park, Durham, NC

President's Welcome

Welcome everyone to the 5th Annual Meeting of the American Society for Cellular and Computational Toxicology. It's hard to believe a year has passed since we last met together, but there certainly has been a tremendous amount of activity in the area of alternative methods over the past 12 months. I'll hope you'll enjoy hearing about many of those activities over the next two days. Once again, a big thank you to EPA-NCCT for hosting us in their Research Triangle Park, North Carolina facilities. Thank you for being a part of this meeting!

The organizing committee has worked diligently to bring together an excellent program that highlights two prominent areas that are increasingly emphasized in the field of predictive toxicology. Both days will be filled with platform presentations from ASCCT members that will be bookended by plenary presentations by internationally renowned experts in their respective fields. Our very own Dr. Thomas Hartung will set the stage on Day 1 by highlighting the utility of applying read across techniques as a "non-testing" approach to fill data gaps necessary for effective chemical hazard identification. Day 2 will feature Dr. Mahendra Rao, who will highlight the power of induced pluripotent stem cell-based assays to toxicology research and testing. We'll close the meeting on Day 2 with a Panel discussion focused on applying the predictive models to the pharmaceutical sector. Once again, I'm confident that you'll enjoy hearing presentations throughout the meeting from your fellow members chosen by the Organizing Committee for a platform presentation based on their outstanding abstract submissions.

I want to encourage all attendees to take advantage of the excellent poster discussion session on Thursday afternoon that is a great time to network with your colleagues and discuss cutting edge science. We'll also be recognizing the recipient of the Edward Carney Predictive Toxicology Award, an annual award we started in 2015 to recognize an outstanding poster presentation in memory of our friend and colleague. I'm also excited to see the future of our Society in attendance today – we've invited a large contingent of students to this year's meeting and have set aside a networking luncheon to allow young scientists to interact with many of our more experienced members. I'm sure you'll agree that this is a great opportunity for everyone involved!

Of course, there is a long list of people that have contributed to making this meeting happen, but let me make sure to give all the credit to the annual meeting organizing committee who have put together this fine program. Please thank Kristie Sullivan, Erin Hill, Ray Tice, Carolina Garcia-Canton, Emilia Costin, Elizabeth Baker, Shaun McCullough, Gaddamanugu Prasad, Grace Patlewicz, Agnes Karmaus, Natalia Vinas and Jack Fowle for putting forward their time and effort in designing the program and contacting the speakers. And please make sure to once again commend Kristie for her continued organization and oversight of the outstanding webinars that we've all enjoyed over the past year.

Finally, I want to thank each of you for becoming ASCCT members and for your continued contributions to our Society, which now stands at well over 200 members! And of course a special thank you to the

President's Welcome

those organizations whose contributions have made much of this annual meeting possible – Alternatives Research and Development Foundation, CAAT, Human Toxicology Project Consortium, IIVS, Integrated Laboratory Systems, Inc., PETA International Science Consortium, Ltd, and Physician's Committee for Responsible Medicine.

And don't forget, the ASCCT was originally envisioned as a platform where regulatory and research scientists from both the computational and cellular sides of toxicology could freely exchange ideas. I'm hopeful that we'll successfully meet several of our Society's overall goals while we here in RTP: fostering open dialog between industry, academic, advocacy, and regulatory scientists; include the participation of young scientists to promote their contributions to the field; and strengthen cooperation between cosmetic, pharmaceutical, and chemical industry scientists and professionals.

Have a great meeting!
Dave

The American Society for Cellular and Computational Toxicology (ASCCT)

Mission:

The ASCCT is a scientific society which provides an organized forum for discussion of cellular and computational toxicology approaches, especially as replacements for animal-based toxicology methods. Through its meetings and activities, the Society facilitates the development, acceptance, and routine use of cellular and computational methods through open dialog between industry, academic, advocacy, and regulatory scientists. The Society strives to include the participation of young scientists to promote their contributions to the field.

Goals:

- Facilitate the development, acceptance, and routine use of cellular and computational methods
- Increase the routine application and use of computational and *in vitro* methods for prioritization, classification, and risk assessment purposes
- Foster open dialog between industry, academic, advocacy, and regulatory scientists throughout North America
- Include the participation of young scientists to promote their contributions to the field
- Strengthen cooperation between stakeholders

All Members will receive:

- Quarterly e-newsletter
- Access to a growing library of educational webinars from field leaders
- Discounted subscription rates to the journals ALTEX and Toxicology In Vitro
- Discounted registration for ASCCT events
- News and event updates in the *in vitro* and computational toxicology fields
- The chance to network with regulators, scientists, and policymakers on the cutting edge of non-animal toxicology

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2016 ASCCT

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9:50-10:10: An open-source workflow for *in vitro* to *in vivo* extrapolation
Xiaoqing Chang, Integrated Laboratory Systems, Inc.

10:10-10:30: PyChemSim - comparing chemical metrics across diverse health endpoints
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11:40-12:00: Development and validation of a computational model for androgen receptor activity
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10:05-10:25: Kinetic hepatotoxicity testing using iPSC derived hepatocytes HC2.0 cells
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10:25-10:45: Role of mitochondrial dynamics in assessment of developmental neural toxicity using human iPS cells
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10:45-11:05: Good Cell Culture Practice (GCCP 2.0): Developments towards the 21st Century
David Pamies, CAAT, Johns Hopkins University

11:05-11:20 Break

11:20-12:30 Closing Panel Discussion:

- How can iPS cell models be used to improve the safety assessment ofpharmaceuticals?
- What can interested stakeholders do to help facilitate increased use and acceptance of such models?
- Are there specific barriers to identify or overcome?

Moderator:
Rodger Curren, IIVS

Panelists:
Mahendra Rao, Les Recio, ILS, Inc.
Ilyas Singeç, National Center for Advancing Translational Science

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Plenary Abstracts

Making big sense of big data by read-across

Thomas Luechtefeld[1], and Thomas Hartung[1,2]

[1]Center for Alternatives to Animal Testing (CAAT), Johns Hopkins Bloomberg School of Public Health, Environmental Health Sciences, Baltimore, USA; [2]CAAT-Europe, University of Konstanz, Germany

Modern information technologies have made big data available in safety sciences, i.e., extremely large data sets that may be analyzed only computationally to reveal patterns, trends and associations. Over the last few years, especially the use of omics technologies and systematic robotized testing in a high-throughput manner have created such large dataset. The compilation of large sets of existing data, e.g., as a result of the European REACH regulation, also leave us with big data – the challenge is now to make big sense of these data. The European Chemical Agency (ECHA) warehouses the largest dataset of *in vivo* and *in vitro* toxicology tests. This data was extracted using linguistic search engines into a structured, machine readable and searchable database. The constructed database, downloaded in December 2014, contains data for 9,801 unique substances (identified by EC-Number) – including 3,609 unique study descriptions (i.e. 'Exp Key Skin irritation corrosion') and a total of 816,048 study documents for substances). This data can be used to explore toxicological data on a scale not previously seen.

The biological data available in these datasets combined with *in vivo* endpoints from REACH represent an enormous modeling potential. A case is made, that REACH should systematically open regulatory data for research purposes. Read-across, i.e., the local similarity-based intrapolation of properties, is gaining momentum with increasing data availability and consensus on how to process and report it. It is predominantly applied to *in vivo* test data as a gap-filling approach, but can similarly complement other incomplete datasets. Big data are first of all repositories for finding similar substances and ensure that the available data is fully exploited. Substance similarity analysis was used to determine clustering of substances with similar hazard labels. Here, a new web-based tool under development called REACH-across, which aims to support and automate structure-based read-across, is presented among others.

High-content and high-throughput approaches similarly require focusing on clusters, in this case formed by underlying mechanisms such as pathways of toxicity. The closely connected properties, i.e., structural and biological similarity, create the confidence needed for predictions of toxic properties.
Together with the currently developed Good Read-Across Practice guidance (Ball et al., ALTEX 2016) and the ongoing development of a web-based tool, this will facilitate the application of high quality read-across for the REACH 2018 deadline and beyond.

Using ipSC derived products for toxicology

Mahendra Rao

Mahendra Rao LLC

iPSC based technology has allowed us to obtain human cells of a defined phenotype from any individual. As the cost of the production of cells has come down it has enabled one to consider screens for panels of cells that are prospectively identified and for which clinical and adverse event information is known. Further as we have learnt to direct differentiation of the cells we can now make cells from the same allelic background to obtain more quantitative assessments of cell type toxicity. Finally as we have learnt to make tissues and growth of cells in 3-D cultures models that are more reflective of the in-vivo responses can be developed. These advances in cell culture technology have already begun to yield results and I will describe examples of our efforts in this area. I will also describe how we have been able to couple reporter lines and genomic analysis to better understand the mechanism of action of drugs. I will end with a brief overview of how clinical trials may be performed incorporating these new technologies.

ASCCT 2016

Oral Abstracts

High-throughput literature mining to support read-across predictions of toxicity

Nancy C. Baker[1], Thomas Knudsen[2], Kevin M. Crofton[2], Grace Patlewicz[2]

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Building scientific confidence in the development and evaluation of read-across remains an ongoing challenge. Approaches include establishing systematic frameworks to identify sources of uncertainty and ways to address them. One source of uncertainty is related to characterizing biological similarity. Many research efforts are underway such as structuring mechanistic data in adverse outcome pathways and investigating the utility of high throughput (HT)/high content (HC) screening data. A largely untapped resource for read-across to date is the biomedical literature. This information has the potential to support read-across by facilitating the identification of valid source analogues with similar biological and toxicological profiles as well as providing the mechanistic understanding for any prediction made. A key challenge in using such information is to convert and translate its unstructured form into a computable format that can be linked to chemical structure. We developed a novel text-mining strategy to represent literature information as keyword features (toxicity signatures) at the chemical level. These signatures were integrated with *in vitro* HT data \from ToxCast and curated chemical structures from DSSTox. The signature elements were weighted using a rule-based algorithm that assessed the strength of the literature relationship, thereby providing a mechanism to rank and visualize the signature as literature ToxPIs (LitToxPIs). We applied this methodology to rank and visualize over 6,000 chemicals described in the biomedical literature for a variety of toxicity types such as developmental toxicity, genotoxicity, reproductive toxicity, and thyroid toxicity. We then developed a user interface that facilitates exploration of the literature evidence behind the records of biological activity. This tool allows researchers to substantiate structure based read-across predictions with literature reports of *in vitro* and *in vivo* toxicity and thereby achieve a higher level of confidence in those predictions. *This abstract does not necessarily represent U.S. EPA policy.*

An open-source workflow for *in vitro* to *in vivo* extrapolation

X Chang[1], J Pirone1, SM Bell[1], D Allen[1], W Casey[2], N Kleinstreuer[2]

[1]ILS, RTP, NC, USA; [2]NIH/NIEHS/DNTP/NICEATM, RTP, NC, USA

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In vitro to *in vivo* extrapolation (IVIVE) facilitates the comparison of effects observed in *in vitro* assays to effects observed in *in vivo* animal tests by correlating an *in vitro* effective concentration to *in vivo* plasma or tissue concentrations. We previously used a population-based pharmacokinetic (PK) model to estimate the daily equivalent administered dose of a test chemical that would result in a steady-state blood concentration equivalent to the concentration of that chemical that induces a response in an *in vitro* estrogen receptor transactivation assay. To make the model publicly accessible and broadly applicable, we have constructed model workflows using two open-source platforms, Konstanz Information Miner (KNIME) and Jupyter Notebook. KNIME uses a modular data pipeline concept with a graphical user interface that allows assembly of modules for data preprocessing, modeling, analysis and visualization. To run our IVIVE workflow, the only inputs required are the *in vitro* assay active concentrations (e.g., half-maximal activity concentrations, AC50s) and relevant PK parameters that affect ADME (absorption, distribution, metabolism, and excretion), which are fraction of chemical unbound to protein, intrinsic clearance, and renal clearance. Although experimental PK parameter values are preferred, and are currently available for 448 chemicals, predicted values can be used if necessary. Our workflow provides predicted PK parameters derived from structure-based models for more than 7600 chemicals. The Jupyter Notebook IVIVE workflow uses the same variable inputs, but provides additional user flexibility via a web browser and dashboard. Users can access human-readable notebook documents containing the analysis description and the results (e.g. tables, figures), as well as executable documents which can be run to perform the IVIVE analysis. This presentation provides two examples of using the workflows, one focusing on assays measuring estrogenic activity and the other focusing on developmental toxicity, to demonstrate how they provide a fast and easy approach to IVIVE analysis. *This project was funded in whole or in part with Federal funds from the NIEHS, NIH under Contract No.HHSN273201500010C.*

PyChemSim – Comparing chemical similarity metrics across diverse health endpoints

Tom Luechtefeld[1], Daniel P. Russo[2], Ignacio Tripodi[4], Hao Zhu[2,3], Thomas Hartung[1]

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Data created to serve the European Union's REACH legislation (Registration Evaluation Authorisation and Restriction of Chemicals) provides an unprecedented opportunity to build computational models for diverse human health hazards. This new data source allows for many chemical models to be compared across 75 UN Global Harmonized System Hazards. Comparison of models on different endpoints allows for a characterization of model efficacy.

Models for chemical similarity have two components: a fingerprinting method (which derives vector representations of chemicals) and a similarity metric (which quantitatively compares two chemical fingerprints.) We perform evaluations of combinations of 4 fingerprinting methods with 11 heuristic similarity metrics and then rank fingerprinting + similarity metric pairs for their ability to accurately predict chemical similarities with respect to distinct UN GHS hazards.

In this work we use McNemar's paired statistical test to compare similarity strategies. Our research indicates large differences in chemical similarity strategy performance. This discovery shows that there is no dominant chemical similarity strategy among those tested.

We propose and demonstrate preliminary results for a supervised learning approach to chemical similarity. Supervised models can be trained on chemical pairs where chemicals that share a class are classified as 'similar' and otherwise 'dissimilar'. Heuristic similarity measures, such as those evaluated in this work, are sensitivity-biased, meaning that they tend to accurately predict when two chemicals share a class but fail when chemicals do not share a class. Supervised learning metrics should overcome this disadvantage. The python package PyChemSim was written for this project and is available as an open source python package. All datasets are shared on Datapub.io to enable easy sharing and use of data in R, python, and java.

Developing a fit-for-purpose *in vitro* assay to measure uterine estrogenic activity and guide risk assessment

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Global initiatives are redefining the paradigm of toxicology testing to reduce the use of animals and improve human relevance through the implementation of *in vitro*-only risk assessment strategies. Current methods for *in vitro* screening of potential endocrine disruptors include multiple assays to identify estrogen activity. Yet, while the uterus is a critical target tissue for estrogenic compounds, there is no *in vitro* assay available which uses human uterine cells. We identified an appropriate uterine cell line, Ishikawa (IKA), to use as an *in vitro* model for examining dose-response for uterine effects of potential endocrine disrupting chemicals (EDCs). This cell model recapitulates protein, gene and proliferative responses of uterine tissue to estrogens. We compared the concentration response of these cells with human *in vivo* data to native ligand (estradiol) and the human drugs tamoxifen and ethinyl estradiol. The IKA proliferation model was highly sensitive and responded to these compounds at similar doses to normal adult women. We identified a network of estrogen-specific response pathways which include proliferation, changes in cell survival, transcriptional responses, and altered cell-cell interactions and designed a multiplex high content imaging assay to screen for activation of each of these endpoints in a single analysis. To support screening activities, we scaled the assay to a higher throughput format. We performed preliminary validation studies with a set of 6 EDSP21 compounds to compare the accuracy of our methods to the current ToxCastTM assay suite and have begun screening a larger set of 30 compounds. These results are compared to ToxCastTM assays and, where possible, *in vivo* human response. Comparison to serum concentrations of estrogen, ethinyl estradiol and tamoxifen demonstrate that the Ishikawa assay is able to predict uterine response at doses with effects in the adult female *in vivo*. Our goal in this project is development of a validated fit-for-purpose functional assay which is representative of *in vivo* uterine biology and capable of predicting points of departure for an *in vitro*-based chemical safety assessment.

Single-cell analysis reveals that silver nanoparticle exposure leads to multi-nucleation through defective cell division

Ellen Garcia[1], Cindy Alms[2], Conor Kelly[1], Adam Smith[1], Linsey Marr[3], and Daniela Cimini[1]

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Multiple agencies, including the U.S. Environmental Protection Agency and the National Academy of Science, are urging for a radical pardigm shift from standard, whole animal testing to alternative and novel technologies. To meet this urgent need, we aimed to develop new, cell division-focused, single-cell toxicity assays based on the idea that (i) defective cell division may be a better predictor of risk than measurements of cell death or persistent cell cycle arrest and (ii) single-cell microscopic analysis provides far deeper insight into the underlying toxicity mechanism(s) than bulk cell population measurements. To develop such approaches, we are currently investigating the mechanism of toxicity from silver nanoparticles (AgNPs) on hTERT-immortalized human retinal pigment epithelial (hTERT-RPE-1) cells. AgNPs are a major nanomaterial employed in product manufacturing due to desirable antimicrobial properties, yet toxicity reports are still confounding. Cultured RPE-1 cells were treated with 5, 15, 25, 50, and 75 μg/mL of polyvinylpyrrolidone (PVP)-coated AgNPs and time-lapse, phase-contrast microscopy was used to analyze the behavior of cells undergoing cell division over a 24 hour time period. A physical interaction between cells and particles was visually observed and 100% of treated cells appeared to engulf particles. We found that higher concentrations of AgNPs resulted in large numbers of cells stalling in mitosis, becoming arrested in mitosis, dying, or dividing abnormally. In contrast, untreated cells displayed normal mitotic behavior. High-resolution fluorescence microscopy performed in chronically treated cell populations identified an increased percentage of binucleated and multi-nucleated cells. Further live-cell analysis indicated that two cell division defects could explain the binucleated and multi-nucleated cell phenotypes. Indeed, treated cells failed cytokinesis (cytoplasmic division following mitotic chromosome segregation) and slipped out of mitosis (i.e., exited mitosis without stereotypical chromosome behavior) more often than control cells. Overall, our results indicate that AgNPs specifically impair cell division, not only further confirming toxicity to human cells, but also revealing specific, previously unreported toxicity mechanisms and highlighting the propagation of adverse phenotypes within the cell population after exposure. Furthermore, this work illustrates that cell division-based assays and single-cell analysis could greatly benefit chemical safety experimentation in the future.

Development and validation of a computational model for androgen receptor activity

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Screening thousands of chemicals to identify androgen receptor (AR) agonists or antagonists would require millions of dollars, thousands of animals, and decades of time using current OECD or U.S. EPA validated methods. Alternative methods developed in the Tox21 and ToxCast research programs incorporate High-Throughput Screening (HTS) assays and computational pathway models to rapidly and inexpensively identify potential endocrine-active chemicals. Eleven Tox21/ToxCast HTS AR assays were integrated in a computational model of the AR pathway to distinguish true activity from technology-specific assay interference. The HTS assays assess potential activity at multiple points of the AR pathway (receptor binding, cofactor recruitment, gene transcription and protein production) in multiple cell types. The Tox21 antagonist transactivation assay in MDA-kb2 cells was run with different concentrations of the synthetic AR ligand R-1881 to confirm activity specific to AR antagonism. These confirmation data were combined with cytotoxicity data from multiple assays to provide further insight into potential non-specific activity and provide a confidence score for true AR pathway activity. Validation of results from such alternative screening methods requires a robust set of reference chemicals; therefore we compiled data on 158 putative androgen-active or inactive reference chemicals from international AR test method validation efforts. We conducted semi-automated literature reviews for *in vitro* AR binding and transactivation assays on these chemicals, and extracted detailed assay information and results from identified references into a single database using a standardized ontology. Based on quantitative data such as activating (or inhibiting) concentrations, we identified reference chemicals with consistent results and assigned potency ranges. The AR pathway model based on Tox21/ToxCast data predicted AR activity with 93% (27/29) and 97% (27/28) accuracy for agonist and antagonist reference chemicals, respectively. The model was used to screen 1853 chemicals, identifying 173 as AR agonists or antagonists. An additional 274 chemicals were predicted to have very weak AR pathway activity. The Tox21/ToxCast HTS data support a biologically based computational model distinguishing assay interference from true AR pathway activity and rapidly screening large numbers of environmental chemicals for androgenic or anti-androgenic activity. *This work does not reflect the official policy of any federal agency.*

Development of direct double strand break labeling assay

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The development of fit-for-purpose *in vitro* toxicity assays to better predict and define DNA damaging chemicals has become necessary to predict adverse outcomes via *in vitro* rather than *in vivo* methods. Current genotoxicity assays rely on indirect measurements of DNA damage through assessment of DNA repair foci or micronuclei formation. While these provide valuable information, they rely on high chemical concentrations and lack sufficient resolution at human-relevant concentrations. We modified a published method (direct in situ labeling of double strand breaks (DSBs) for high-throughput compatibility. Effectiveness of this method to detect DNA double strand breaks was tested against known DNA damaging compounds. Initial studies were conducted utilizing aphidicolin, an inhibitor of DNA polymerase α and δ, as a test compound in HT1080 fibrosarcoma cells. Preliminary data indicate the altered method detects DSBs that closely model data from in-house genotoxicity assays (micronucleus and DNA repair centers). Sensitivity studies using prototype chemicals, etoposide (ETP) and methyl methanesulfonate (MMS) showed the direct DSB labeling method (DDL) to be 10 times more sensitive than traditional micronucleus or DNA repair center assays (detected changes in DSBs compared to control cells at 0.001 vs. 0.02 and 0.01μM ETP and 10 vs. 100 and 60μM MMS, respectively). Based on preliminary data, the DDL method provides a novel tool to determine genotoxic potential at human-relevant chemical concentrations compared to traditional *in vitro* models. Future studies are focused on determining accuracy of the assay using positive, false-positive, and negative controls for genotoxicity, and miniaturizing the assay for high-throughput screening.

International validation study on the Hand1-Luc Embryonic Stem Cell Test (Hand1-Luc EST): A reporter gene assay using engineered mouse ES cells to evaluate embryotoxicity *in vitro*

Hajime Kojima[1], Koichi Saito[2], Hirohisa Nagahori[2], Noriyuki Suzuki[2], Florian Le Coz[2], Takashi Omori[3], Mayumi Kobayashi[3], Azusa Mori[3], Yuichi ito[4], Joshou Ryuu[4], Kazunori Yanagi[5], Takeshi Izukawa[5], Mika Watanabe[6], Mayu Ikezumi[6], Makiko Kuwagata[6], Andrea Seiler[7], Michael-Wilhelm Schaeffer[8], Warren Casey[9], David Allen[9], Eui-Bae Jeung[10], Yoshihiro Ohmiya[11], Kazuhiko Matsumoto[12], Shojiro Yamazaki[13], Noriho Tanaka[14]

[1]JaCVAM, NIHS, [2]Sumitomo Chemical, [3]Kobe University, [4]Kao Corporation, [5]Sumika Chemical Analysis Service, Ltd.), [6]HRI, FDSC, [7]ZEBET, [8]ECVAM, [9]NICEATM, [10]KoCVAM, [11]AIST, [12]Nagoya City University, [13]OTIP, [14]HRI & OTIP

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The embryonic stem cell test (EST) is a promising alternative method for evaluating developmental toxicity. It has been developed to respond to the needs of the 3Rs (replace, refine, reduce) in 1997 by the team of Dr Horst Spielmann. In this test, embryotoxicity of chemicals was evaluated by measuring cytotoxicity and differentiation toxicity using mouse ES cells. Differentiation toxicity was analyzed by microscopically counting the beating of embryoid bodies after 10 days of culture. However, improvements were necessary to reduce the laborious manipulations involved and the time required to obtain results. We thus developed the Hand1-Luc EST. This test uses stable transformant of ES cells with promoter of Hand1 (heart and neural crest derivatives expressed transcript 1) gene upstream of luciferase reporter gene previously reported to be an essential transcriptional factor for mammalian heart development and a quantitative and objective molecular endpoint for predicting embryotoxicity. Three endpoints were measured and used in the prediction model: IC50 (concentration of chemical that reduces the viability of cells to 50% of the control level against the vehicle control), ID50 (concentration of the test chemical that reduces the luminescence by 50%) and maximum dose (concentration for the one the chemical dissolves in the assay medium).

From February 2013 to February 2016, the protocol undertook the validation process. With discussions with members of International Cooperation on Alternative Test Methods (ICATM) along with experts in the domain, we significantly improved the protocol. Three participant laboratories took part in the validation. After protocol modifications, determination of criteria, analysis adjustment (curve fitting) and the revision of the prediction model, we managed to obtain a high reproducibility. Both within- and between-laboratory reproducibilities were higher than 75%. Concerning the predictivity of the test, although we only got an accuracy of 60.6%, the positive predicted value was very high (80.8%) (results based on 71 chemical's data). All those results lead us to think that Hand1-Luc EST is a powerful screening test to detect strong embryotoxicants.

The study was supported in part by Ministry of Economy, Trade and Industry in Japan and Japan Chemical Industry Association (JCIA) Long-range Research Initiative (LRI).

Optimization of the validated *in vitro* Skin Irritation Test (OECD TG 439) to address the assignment of EPA hazard categories

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The dermal safety assessment of chemicals by the Draize rabbit test is part of United States Environmental Protection Agency (US EPA) battery of acute toxicity tests known as the "six-pack". The advancement of the *in vitro* toxicology field has made available testing platforms using reconstructed tissue models that are now validated to address the skin corrosion and irritation endpoints. While these assays can be used for the hazard identification of chemicals irritant to skin in accordance with the United Nations (UN) Globally Harmonized System (GHS) for Classification and Labeling of Chemicals, they are not calibrated to address the classification system used by the US EPA. The validated *in vitro* Skin Irritation Test (SIT – OECD TG 439) can be used to discriminate between skin irritants (GHS Category 2) and non-irritants (No Category) based on a single exposure time (60 minutes, using the EpiDerm™ model from MatTek Corporation, Ashland, MA, USA) followed by a 42 hours post-exposure period. A single cut-off value of 50% tissue viability separates GHS Category 2 from the GHS No Category prediction. To support the current efforts of the US EPA to modernize the battery of acute toxicity tests, we investigated whether the validated SIT could be used to obtain EPA labeling information. A retrospective analysis of paired *in vivo-in vitro* data for 41 chemicals used for the validation of SIT revealed an over-prediction of some EPA Category III and Category IV chemicals. We also conducted preliminary testing of a sub-set of chemicals from the group of 41 using an optimized protocol based on a 15 minute exposure followed by a 24- or 42 hours post-exposure period in addition to the validated method. The results were analyzed using a new prediction model: 15- and 60 minutes exposure, 24- and 42 hours post-exposure, and a revised cut-off value of the 20% viability endpoint which improved the prediction of the EPA Category III and IV chemicals. We are currently investigating a larger set of chemicals with already assigned EPA skin hazard categories to assess the validity of this new prediction model for EPA labeling.

Computational fluid dynamic modeling to support the development of flow-based hepatocyte culture systems for metabolism studies

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Accurate estimation of metabolic parameters is a significant outstanding challenge in toxicology. The best predictions are based on experimental data from *in vitro* systems using primary hepatocytes. The predictivity of the primary hepatocyte-based culture systems, however, is still limited due to well-known phenotypic instability and rapid decline of metabolic competence within a few hours. Dynamic flow bioreactors for three-dimensional cell cultures are thought to be better at recapitulating tissue microenvironments and show potential to improve *in vivo* extrapolations of chemical or drug toxicity based on *in vitro* test results. These more physiologically relevant culture systems hold potential for extending metabolic competence of primary hepatocyte cultures as well. In this investigation, we used computational fluid dynamics (CFD) to determine the optimal design of a flow-based hepatocyte culture system for evaluating chemical metabolism *in vitro*. The main design goals were 1) minimization of shear stress experienced by the cells to maximize viability, 2) rapid establishment of a uniform distribution of test compound in the chamber, and 3) delivery of sufficient oxygen to cells to support aerobic respiration. Two commercially available flow devices—RealBio® (RB) and QuasiVivo® (QV)—and a custom developed fluidized-bed bioreactor (FB) were simulated, and turbulence, flow characteristics, test compound distribution, oxygen distribution, and cellular oxygen consumption were analyzed. Experimental results from the bioreactors were used to validate the simulation results.

Our results indicate that maintaining adequate oxygen supply is the most important factor to the long-term viability of liver bioreactor cultures. Cell density and system flow patterns were the major determinants of local oxygen concentrations. The experimental results closely corresponded to the *in silico* predictions. Of the three bioreactors examined in this study, we were able to optimize the experimental conditions for long-term hepatocyte cell culture using the QV bioreactor. This system facilitated the use of low system volumes coupled with higher flow rates. This supports both cellular respiration by increasing oxygen concentrations in the vicinity of the cells, and facilitates long-term kinetic studies of low clearance test compounds. These two goals were achieved while simultaneously keeping the shear stress experienced by the cells within acceptable limits.

Implications of the recent 2016 amendment of the toxic substances control act (TSCA) on the development and implementation of non-animal methods

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The Frank R. Lautenberg Chemical Safety for the 21st Century Act was signed into law by President Obama and came into force on June 22, 2016. This amendment to TSCA increases the authority of the Environmental Protection Agency to obtain information on both new and existing industrial chemicals and includes the following: mandatory requirement for the Environmental Protection Agency (EPA) to evaluate existing chemicals with clear and enforceable deadlines; new risk-based safety standard; increased public transparency for chemical information; and consistent source of funding for EPA to carry out the responsibilities under the new law. In addition to requiring the use of the "best available science," the amendment also includes a section requiring both EPA and any person developing information under the Act to reduce and replace vertebrate testing "to the extent practicable, scientifically justified, and consistent with the policies of this title." This section of the bill also requires EPA to "promote the development and timely incorporation" of non-vertebrate animal methods by developing a strategic plan to do so and to publish and update regularly a list of acceptable methods and approaches. Under amended TSCA, existing chemicals will be subject to prioritization via a risk-based screening process into high and low priority. High priority chemicals ("those that may present an unreasonable risk" or for which there is insufficient information to make a determination) must undergo a risk evaluation. New chemicals will all be subject to review, and if EPA cannot make a determination regarding safe use, may ask for additional information. The amended Act is therefore likely to require the generation of massive amounts of new information on both existing and new industrial chemicals; at the same time there is pressure to reduce vertebrate testing, creating an immediate need for increased implementation of non-vertebrate evaluation tools. Such rapid development and implementation will require strongly coordinated efforts between industry, agency scientists and regulators, and other stakeholders to leverage exiting approaches form other sectors and expand available methods and approaches. This presentation will offer some suggestions for immediate action.

Impact of autism-associated CHD8 mutation on iPSC-derived mini-brains

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Autism spectrum disorders (ASD) are a major public health concern, affecting 1 in 68 children in the US. The increase of ASD prevalence cannot be explained solely by genetics and/or changes in diagnostic criteria. Increasing evidence suggests that exposure to drugs and environmental chemicals may have a substantial impact on ASD risk. Idiopathic autism is a multifactorial disease that makes it challenging to find the disease causation. Mutation in the chromodomain helicase DNA binding protein 8 (*CHD8*) gene is one of the top genetic risk factors in autism and was linked to ASD by regulating a number of ASD related genes. The main goal of our research is to identify and mechanistically understand environmental contributions to risk and rise of ASD. In particular, to pursue the hypothesis that individuals with genetic predispositions to autism (such as *CHD8* mutation) are more sensitive to environmental exposure and that the interplay of genetics and environment triggers the manifestation of disease. To address this question, we used our human induced Pluripotent Stem Cells (iPSC)-derived 3D brain model (mini-brains). We used iPSC derived from healthy donor (*CHD8*+/+), where *CHD8* mutation was introduced to generate isogenic heterozygous *CHD8*+/- and homozygous *CHD8*-/- knockouts. We were not able to generate mini-brains from homozygous knockout. Mini-brains from healthy and heterozygous mutant cell lines were characterized as to their cellular composition. Then we analyzed the susceptibility of *CHD8*+/- isogenic mini-brains vs. healthy mini-brains to organophosphate chlorpyrifos and its metabolite chlorpyrifos-oxon. First, we observed that mini-brains were more sensitive to chlorpyrifos than to oxon, suggesting that the mechanism of toxicity is not through acetylcholinesterase inhibition. Second, we could not detect any differences in susceptibility between different cell lines at early stages of differentiation (NPC and 2 week mini-brains). However, *CHD8* knockout was more sensitive to chlorpyrifos than control cell line later in development (4 and 8 week mini-brains), as shown by viability and mitochondria membrane potential assays. We will use this preliminary data to further investigate potential metabolic biomarkers of the mutation and treatment. Expression of ASD-risk gene and miRNA sets will be quantified under all conditions.

Kinetic hepatotoxicity testing using iPSC derived hepatocytes HC2.0 cells

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Hepatotoxicants act through different kinetics and mechanisms. An ideal *in vitro* hepatotoxicity analysis system would combine convenient, kinetic monitoring with metabolically competent, long lasting human hepatic cells. We report such a system with iPSC derived hepatocyte HC2.0 monitored on Real Time Cell Analysis (RTCA) xCELLigence instrument. Differentiated HC2.0 cells were seeded, maturated and treated with hepatotoxic chemicals in 96 well EPlates which were engaged in xCELLigence instruments, and the whole process were monitored and corresponding Cell Index data generated from impedance signal of cell grown on electrodes at the bottom of EPlates were displayed. HC2.0 cells in this system are viable for 3 weeks with 2 weeks window for toxicity testing. Distinctive kinetic response profiles were observed from hepatotoxic compounds such as amiodarone, chlorpromazine and troglitazone, reflecting different toxicity pathways. P450 inhibitors such as ketoconazole and 1-aminobenzotriazole decreased toxicity from known bioactivated compounds such as aflatoxin b1 and cyclophosphamide. Cellular redox modulators DL-buthionine-(S,R)-sulfoximine and N-acetylcysteine altered acetaminophen toxicity by changing viability or alter kinetic response profiles. These suggest HC2.0 cells are metabolically active and they preserve the regulation networks. Altogether, compared to other *in vitro* system, this novel system present the following advantages: 1) extended hepatocyte life which enabled chronic and repeated dosing toxicity testing; 2) non-invasive, kinetic response profile would help to predict not only testing compound potency, but also potential signaling pathways under physiological conditions; 3) ease of growth, dosing and monitoring make the system ideal candidate to study multiple different regulatory pathways. With almost unlimited supply of iPSC cells, metabolically active HC2.0 combined with RTCA technology present unique advantages over cell lines-lack of metabolic activity and primary hepatocytes-limited supply/batch differences/ limited life span for hepatotoxicity testing.

Role of mitochondrial dynamics in assessment of developmental neural toxicity using human iPS cells

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Human iPS cells (iPSCs) are expected to provide a platform to assess various toxicities as human cell model. Many lines of evidence suggest that mitochondria changes its morphology in response to environmental conditions and is regulated by fusion proteins (Mfn1, Mfn2 and Opa1) and fission proteins (Drp1 and Fis1). Here we report a role of mitochondrial dynamics in developmental neural toxicity (DNT) of chemicals using human induced pluripotent stem cells (iPSCs). We first selected several positive compounds for DNT, such as tributyltin (TBT), chlorpyrifos and nicotine. We found that exposure to TBT at nM levels decreased cell viability in iPSCs. TBT also reduced intracellular ATP levels. We next assessed the effects of TBT on mitochondrial dynamics. Staining with MitoTracker revealed that TBT induced mitochondrial fragmentation. Immunoblot analysis revealed that levels of Mfn1 were significantly reduced by TBT. Moreover, Mfn1 degradation was abolished by knockdown of the E3 ubiquitin ligase MARCH5, suggesting that TBT at nM levels induces mitochondrial dysfunction via MARCH5-mediated Mfn1 degradation in iPSCs. In addition to TBT, other chemicals, including chlorpyrifos and nicotine, also induced both mitochondrial fragmentation and Mfn1 degradation in iPSCs. Thus, mitochondrial dynamics might be a good endpoint for evaluation of chemical-induced DNT.

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Good cell culture practice (GCCP 2.0): Developments towards the 21st century

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The techniques available for cell culture have developed dramatically in the last decade due to demands for cheaper, faster, humanized and more mechanistic methods. However, there is too often a lack of quality control. Good Cell Culture Practice (GCCP) defines fundamental standards to maximize the reproducibility, reliability, credibility, acceptance and proper application of cell culture. The first guidance on Good Cell Culture Practice dates back to 2005. This document expands this to aspects of quality assurance for *in vitro* cell culture to the increasingly diverse cell types and culture formats used in research, product development, testing and manufacture of biotechnology products and cell-based medicines. It provides a set of basic principles of best practice which can be used in training new personnel, reviewing and improving local procedures and helping to assure standard practices and conditions for the comparison of data between laboratories and experimentation performed at different times. Since then, limited implementation of GCCP and fast-paced developments of new technologies have been observed, especially with regard to human stem-cell-derived models and organo-typic cell cultures, including organoids, organ-on-chip and even human-on-chip approaches. A new and updated version of GCCP called “GCCP 2.0” is under development through collaboration between many countries, providing the secretary at the Center for Alternatives to Animal Testing (CAAT) of Johns Hopkins University. With the goal to develop a revised GCCP 2.0, two workshops have been convened in 2015 in the US and Europe to map the challenge and organize the process. This presentation summarizes some of the results of the GCCP 2.0 collaboration.

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Poster Abstracts

A review of single-dose acute toxicity tests in new drug applications from 2011-2015

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The Food and Drug Administration (FDA) informally states that single-dose acute toxicity testing is not required for pharmaceutical testing. The purpose of this study was to determine if single-dose acute toxicity testing is still conducted and submitted to FDA, despite FDA's informal statements. New Drug Applications (NDAs) for approved drugs from 2011-2015 were reviewed to gather the data. While final numbers are being compiled, the data show single-dose studies are still submitted to FDA and multiple animal species are used for testing. This presentation will include the purpose for conducting the study, the methods employed to gather data, results and next steps, including suggestions for FDA regulatory action.

Effects of non-specific binding to different organic polymeric components in a bioreactor system

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Primary hepatocytes rapidly lose their metabolic competence in culture. In order to maintain metabolic activity in these primary cultures, more physiologically relevant culture methods have been in development. Specifically, 3D hepatocyte cultures in flow-based bioreactor systems have shown considerable improvement in maintaining basal metabolic activity and viability of primary hepatocytes in long term cultures. However, unaddressed concerns remain regarding secondary aspects of the system that affect performance such as non-specific binding of compounds to system components (e.g., tubing).

We have evaluated the degree of non-specific binding of chemicals to different bioreactor components under various conditions in closed-circuit and open-circuit systems, using 7-ethoxycoumarin (7EC) as a test compound. Single- and multiple-component systems were examined, and the time course of 7EC concentrations was used to extract kinetic parameters describing adsorption and desorption from several materials.

In single material studies, Teflon tubing showed the lowest non-specific binding, with increasing levels of binding to PharMed and Tygon. When a mixture of different polymers was used in the system, Tygon was found to be responsible for the majority of the non-specific binding.

Overall, while Teflon performed best by minimizing non-specific binding to 7EC, the stiffness of this material makes it difficult to use with peristaltic pump systems and also increases the total length of tubing needed since it cannot be used for sharp bends. PharMed is more malleable and is compatible with peristaltic pumps. Our results suggest an optimal system using primarily Teflon with short lengths of PharMed tubing incorporated as necessary. In conjunction with a computational model that captures the kinetics of adsorption and desorption, this system is suitable for accurate estimation of metabolic activity in 3D hepatocyte cultures in flow-based bioreactors.

Drug metabolizing enzyme expression in long term 3D culture of human hepatocytes in alginate-hydrogels

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To increase the accuracy and physiological relevance of *in vitro*-to-*in vivo* extrapolations of chemical toxicity, we have designed and characterized a 3D alginate-encapsulated hepatocyte cell culture system to be used as an alternative to traditional short-term primary hepatocyte culture in suspension or 2D systems. We have shown that our alginate-encapsulated system better recapitulates the hepatic microenvironment, as reflected in the extension of cell viability to >28 days, which compares favorably to ~6 hours for suspension culture, the current gold standard for metabolism studies. The extension of cell viability can facilitate the investigation of metabolism, particularly for low clearance compounds that require longer incubation times. In order to evaluate that metabolic competence is retained over a similar time frame, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to explore the time dependence of expression of drug metabolizing enzymes (DMEs). Using fresh cell suspensions as a benchmark comparison, we measured levels of CYP2E1, CYP1A2, CYP3A4, CYP2D6, and CYP2C9 mRNA as markers for phase I metabolism, and levels of UGT2B7, SULT1A1, and CES1 mRNA as markers for phase II metabolism. Our results indicate only a slight drop in expression for the selected enzymes (retaining >50% baseline activity on average) within a few days post-encapsulation (PE), followed by a rise to near reference levels by day 7 PE for most enzymes, with further evidence for continued recovery over the following two weeks. Moving forward, our efforts will be targeted at replicating our results using hepatocytes from additional human donors. The data we collect will be used to establish our alginate-encapsulated culture system as a useful tool for studying metabolism over longer time frames than are possible using current methods.

Modeling a complex *in vivo* response *in vitro*: Exploring heterogeneity and mechanisms associated with ozone adaptation

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Predicting the health effects of exposure to ozone, a ubiquitous air pollutant, is challenging because acute and repeated exposures produce different outcomes. Acute ozone exposure produces pulmonary inflammation and lung function impairment, but repeated exposures result in the attenuation of these effects. "Ozone adaptation" has been observed for nearly fifty years in human and animal studies, but it is poorly understood due to inter-individual variability and a lack of exploratory models. Importantly, the health implications of ozone adaptation remain unclear. To facilitate the efficient exploration of inflammatory ozone adaptation, we utilized differentiated primary human bronchial epithelial cells cultured at air-liquid interface from a panel of human donors. Using this *in vitro* system, we compared responses between single and repeated ozone exposures in pro-inflammatory (IL-8, COX-2, IL-6) and oxidative stress-responsive genes (HMOX-1). We found that cells exhibited broad range of donor-specific inductions after a single ozone exposure. However, after repeated exposure responses became homogeneous and were globally reduced, mimicking *in vivo* ozone adaptation. This global reduction was driven by donors which were initially highly-ozone responsive, but then exhibited robust adaptive responses (e.g. IL-8; 6/13 donors). By correlating initial induction and the magnitude of reduction, we found that adaptation is a feature of ozone responsiveness in IL-8, HMOX-1, and perhaps COX-2. *In vitro* adaptation was often apparent by two exposures and persisted for at least three days. Contrary to previous hypotheses, adaptation was not associated with the upregulation of oxidative stress-responsive genes. These results suggest that inflammatory ozone adaptation is mediated on a cellular level by the reprogramming of inflammatory gene expression and may be exclusive to a certain subpopulation. Moreover, it may be an important yet unappreciated susceptibility factor in pollutant-related cardiopulmonary mortality and multi-pollutant exposure effects. The *in vitro* model of ozone adaptation presented here can be used to further investigate ozone response heterogeneity, mechanisms underlying adaptation, and implications for air pollutant susceptibility.

miRNAs as common regulators of the transforming growth factor (TGF)-β pathway in the preeclamptic placenta and cadmium-treated trophoblasts: Links between the environment, the epigenome and preeclampsia

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Preeclampsia (PE), a condition traditionally categorized by high blood pressure and increased protein in the urine of pregnant women, accounts for 40-60% of maternal deaths in the U.S. PE can also be detrimental to the unborn baby, resulting in premature births, birth deformities, and death. While the etiology is unknown, poor placentation of the placenta due to aberrant signaling of growth and angiogenic factors has been postulated as causal factors of PE. Environmental contaminants, such as the metal cadmium (Cd), have also been linked to placental toxicity and increased risk of developing this disease. Previously, we have observed the transforming growth factor beta (TGF-β) pathway was upregulated in women with preeclampsia compared to those without. The TGF-β pathway is important for regulating several cellular processes in the placenta, such as apoptosis and placental endometrial invasion. In addition, genes within the TGF-β pathway displayed increased expression in both preeclamptic placentas and Cd-treated trophoblasts, with miRNAs that target the TGF-β pathway significantly altered as well. Integrative analysis resulted in the identification of a subset of Cd-responsive miRNAs common to preeclamptic placentas and Cd-treated trophoblasts that have previously been linked to PE and are predicted to regulate members of the TGF-β pathway. Furthermore, trophoblasts treated with Cd exhibited impaired migratory capabilities and these miRNAs may be used in the future for epigenetic reprogramming to repair cellular mobility. Investigating the underlying biology of preeclampsia and ptential environmental influences can provide insight into the specific effects of toxicants on placental molecular phenotypes that increase risk of PE.

The h-CLAT for assessment of dermal sensitization potency of commercially available mixtures and the OECD proficiency chemicals

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In order to explore the applicability domain for more pure chemicals and mixtures (including commercial products), we performed a series of studies using the Human Cell Line Activation Test (h-CLAT) protocol under the latest OECD Test Guideline. THP-1 cell expression of CD86 and CD54 from a new, expanded set of validation chemicals and several complex mixtures were measured. A variety of products from the petroleum, agrochemical, food, beauty and chemical industries, were obtained via retail outlets and evaluated. Known positive (via safety data sheets) mixtures were assessed along with similar non-sensitizing mixtures. These included: non-PPD containing hair dye, propolis extract, diesel fuel additive, a pesticide, and commercial acrylate-based sealants. In addition to mixtures, we evaluated the OECD proficiency test chemicals, which includes DNCB, Phenylenediamine, Nickel Sulfate, 2-Mercaptobenzothiazole, R(+)Limonene, Imidazolidinyl Urea, and the non-sensitizers Lactic Acid, Isopropanol, Glycerol, and 4-Aminobenzoic Acid. All chemicals were able to be exposed at a low or non-irritating concentration, yielding a CV75 or higher viability, as determined by Flow Cytometry. Sensitizer potency was measured by the concentration of test chemical that induced a Relative Fluorescence Intensity (RFI) that was a threshold positive response (CD86 = 200%, CD54 = 150%) of control. Two sets of draft OECD guidelines proficiency chemicals were tested for a total of 16 pure chemicals (6 non-sensitizers and 9 sensitizers). The h-CLAT correctly predicted 9 of 9 sensitizing and 5 of 6 non-sensitizing chemicals, for an overall Accuracy of 93.7%.

Further Evaluation of Chemicals and Mixtures for Skin Sensitization Potential and Potency Using a Reconstructed Human Epithelium (3D) Tissue Model and the IVSA

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This research project combined a 3D reconstructed human epidermal (RHE) tissue that is co-cultured with human plasmacytoid Dendritic Cells (pDCs) for use as an *in vitro* CoCulture dermal sensitization assay. In this assay, RHE tissues were placed at the air-liquid interface above a media suspension of pDC. The tissues are then exposed to test materials, and after 4 hours of incubation together, the RHE tissues and pDC were separately cultured for an additional 20 hours. The RHE media was analyzed for IL-18 release by ELISA, and the pDC were analyzed for changes in CD86 surface expression by flow cytometry. Two non-sensitizing irritants (Lactic Acid and Phenol), along with two weak/moderate sensitizers: Eugenol and Hexylcinnamaldehyde, and two strong sensitizers: 1-Chloro-2,4-Dinitrobenzene and 4-Nitrobenzyl Bromide were assayed. A positive response from the RHE tissues was determined to be a 2-fold increase in IL-18 secretion, and a 1.5 fold increase in CD86 expression on pDC. Tissue viability was measured using the MTT assay. The responses we obtained in both the RHE tissue versus pDC were very consistent. Increases in both secretion of IL-18 and expression of CD86 were detected after exposure to dermal sensitizers. A prediction model was developed in which a sensitizer result for a chemical is defined as either a positive result in the RHE tissue (IL-18) or a positive result in pDCs (CD86). From three individual experiments, and using a 2x2 contingency table to determine Cooper statistics, we obtained an Accuracy of 100%, 83%, and 83% (89% mean Accuracy). All four of four sensitizers were positively predicted in each experiment (100% Sensitivity). This research was funded by the Society of Toxicology Grant for Alternatives Research (sponsored by Colgate-Palmolive).

Update on the society of toxicology – Colgate palmolive grant for alternative research: *In Vitro* Co-Culture Assay for Identification of Dermal Sensitizers

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This research project combined a 3D reconstructed human epidermal (RHE) tissue that is co-cultured with human plasmacytoid Dendritic Cells (pDCs) for use as an *in vitro* CoCulture dermal sensitization assay. In this assay, RHE tissues were placed at the air-liquid interface above a media suspension of pDC. The tissues are then exposed to test materials, and after 4 hours of incubation together, the RHE tissues and pDC were separately cultured for an additional 20 hours. The RHE media was analyzed for IL-18 release by ELISA, and the pDC were analyzed for changes in CD86 surface expression by flow cytometry. Two non-sensitizing irritants (Lactic Acid and Phenol), along with two weak/moderate sensitizers: Eugenol and Hexylcinnamaldehyde, and two strong sensitizers: 1-Chloro-2,4-Dinitrobenzene and 4-Nitrobenzyl Bromide were assayed. A positive response from the RHE tissues was determined to be a 2-fold increase in IL-18 secretion, and a 1.5 fold increase in CD86 expression on pDC. Tissue viability was measured using the MTT assay. The responses we obtained in both the RHE tissue versus pDC were very consistent. Increases in both secretion of IL-18 and expression of CD86 were detected after exposure to dermal sensitizers. A prediction model was developed in which a sensitizer result for a chemical is defined as either a positive result in the RHE tissue (IL-18) or a positive result in pDCs (CD86). From three individual experiments, and using a 2x2 contingency table to determine Cooper statistics, we obtained an Accuracy of 100%, 83%, and 83% (89% mean Accuracy). All four of four sensitizers were positively predicted in each experiment (100% Sensitivity). This research was funded by the Society of Toxicology Grant for Alternatives Research (sponsored by Colgate-Palmolive).

A large dataset of acute oral toxicity data created for cesting *in silico* models

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Acute toxicity data is a common requirement for substance registration in the US. Currently only data derived from animal tests are accepted by regulatory agencies, and the standard *in vivo* tests use lethality as the endpoint. Non-animal alternatives such as *in silico* models are being developed due to animal welfare and resource considerations. We compiled a large dataset of oral rat LD50 values to assess the predictive performance currently available *in silico* models. Our dataset combines LD50 values from five different sources: literature data provided by The Dow Chemical Company, REACH data from eChemportal, HSDB (Hazardous Substances Data Bank), RTECS data from Leadscope, and the training set underpinning TEST (Toxicity Estimation Software Tool). Combined these data sources yield 33848 chemical-LD50 pairs (data points), with 23475 unique data points covering 16439 compounds. The entire dataset was loaded into a chemical properties database. All of the compounds were registered in DSSTox and 59.5% have publically available structures. Compounds without a structure in DSSTox are currently having their structures registered. The structural data will be used to evaluate the predictive performance and applicable chemical domains of three QSAR models (TIMES, PROTOX, and TEST). Future work will combine the dataset with information from ToxCast assays, and using random forest modeling, assess whether ToxCast assays are useful in predicting acute oral toxicity. *This abstract does not necessarily represent U.S. EPA policy.*

Defining toxicological tipping points using microelectrode array recordings of developing neural networks

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Current guideline studies for developmental neurotoxicology (DNT) hazard are resource intensive and time-consuming, and therefore have only been conducted for a limited set of compounds. The EPA is developing more efficient methods to screen and prioritize the thousands of chemicals with undefined DNT hazard. *In vitro* microelectrode array recording of cellular electrical activity is a promising assay for development of functional neuronal networks that is amenable to high-throughput screening. We have used this approach on developing cultures of primary rat cortical neurons to derive 16 network parameters that quantify aspects of network activity and coordination during exposure to a library of 70 compounds. Assessing these 16 parameters at four times across 7 concentrations for each compound produced a rich dataset of 31,360 total endpoints. To prioritize compounds for further investigation, we have developed two complimentary methods to quantify the potency of each compound in producing network effects. An area-under-the-curve metric applied to network parameter values over time simplifies concentration-response modeling without loss of developmental delay effects, and allows for estimation of concentration where 50% of network activity is lost (EC50) for each network parameter. To integrate data across network parameters and model network adaptive response to exposure, we also calculated the total scalar perturbation at each timepoint. This allows for estimation of system velocities indicating whether a given concentration causes network failure or allows for recovery by adaptive response. Critical concentrations (i.e, tipping points) that mark the point at which toxicity begins to overwhelm the system were defined from the system velocities of 35 compounds. Comparison of these DNT tipping points to network and cell viability EC50 estimates for the same compounds suggests tipping points are often more sensitive than individual network parameters, capture selective effects, and may be useful to prioritize compounds for DNT hazard. (This abstract does not represent EPA Policy).

A novel cell panel based real time cellular assay to detect and differentiate endocrine disruptors

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Endocrine disruptors mimic endogenous hormones, alter the homeostasis of the endocrine systems, leading to health problems including development and cancer. To date, due to the complexity of endocrine pathways, there is an unmet need to identify these chemicals, especially those affecting estrogen receptor (ER), androgen receptor (AR) and thyroid hormone receptor (TR) (EAT receptors). Here, we describe a cell panel-based kinetic profiling approach using real-time impedance monitoring to detect and differentiate modulators of these hormone systems. A panel of three mammalian cell lines responsive to modulators of the EAT receptors were selected. In a specific cell line, compounds with similar activity produced similar response profiles that were receptor-specific. In addition, because of their different tissues and species origins, each cell line generated a unique response profile to modulators of each hormone system, reflecting their physiological and genetic differences. Quantitative analysis of the profile revealed differences in level of detection, potency, and specificity among different cell lines in the cell panel. Our results reveal that the rat pituitary tumor cell line, GH3, exhibit the most sensitive response to TR hormones, while the human prostate cancer cell line, LNCaP, is most sensitive to AR hormones and the human breast cancer cell line, T-47D, is most sensitive to ER hormones. In addition, our data suggests that T-47D cells can be utilized to detect and differentiate between ER- and AR- target pathways by the varying kinetic profiles and antagonist-specific responses. Our findings indicate that the cell panel-based tests can be used as a screening approach to identify and differentiate endocrine disruptors targeting EAT receptors.

A biologically relevant *in vitro* culture system for DMPK analysis

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Biologically relevant DMPK analysis of drugs and environmental compounds is of critical importance. The classical, static 96-well plate has been a mainstay of medium/high through *in vitro* cellular safety and efficacy testing. The biological relevance of these static cultures is improved through the use of primary cells, 3D cells (formed through scaffolds, gels, and spheroids), and co-cultures of multiple cell types. Recently, fluidic culture systems have even more dramatically increased the biological relevance of *in vitro* DMPK studies. In this study we compare the newly released SciFlowTM 1000 Fluidic Culture System to a traditional static 96-well culture plate for drug toxicity testing. The SciFlow 1000 is a unidirectional, 96-well, gravity driven fluidic system manufactured entirely from tissue culture treated polystyrene. This system sequentially links 10 cellular chambers forming a cascading flow of fluids (media, drugs, toxicants, metabolites, growth factors,..) without pumps or tubing. This study utilized high-content imaging, biochemical assays, and LC/MS for real-time, endpoint, and mechanistic assessments of cell health, viability, metabolic competency, and metabolite quantification.

Using a fluorescein tracer, we demonstrate non-linear exposures across the fluidic system with greater similarly to an *in vivo* plasma drug concentration curve. We tested the cytotoxic effects of Aflatoxin B and acetaminophen (APAP) on a metabolically competent human hepatocyte cell line (HepaRG) using Hoechst stain, CellTox Green, CellTiter-Glo, GSH-Glo Glutathione assay, and LC/MS. The fluidics and gradients of the SciFlow 1000 allowed us to differentiate between the direct (parent compound) effects of Aflatoxin B and the metabolite mediated effects of acetaminophen; this mechanistic information was lost in the static plates. Additionally, we constructed an *in vitro* oral absorption and metabolism system by combining engineered intestinal cells with liver cells. We used fluorescent CYP450 substrates for monitoring CYP activity, in real-time. We were able to measure intestinal CYP activity, permeation of the fluorescent CYP substrates, followed by additional metabolism of the CYP substrates by the HepaRG liver cells. This *in vitro* oral absorption, metabolism, and first pass liver metabolism system brings a new level of biological relevance to *in vitro* DMPK analysis.

Compilation, curation, and prioritization of food-use chemicals

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Thousands of chemicals, many with little or no toxicological evaluation, are directly added or come in contact with food. Diversity among food-use chemicals and a lack of hazard and exposure data create challenges in curating and prioritizing chemicals for evaluation. To address this challenge, we compiled an inventory of food-use chemicals in the U.S. and demonstrate approaches for testing prioritization. A list of 8,659 unique food-use chemicals was compiled from 12 public sources and mined against the ToxCast inventory to identify 1,530 food-use chemicals with ToxCast assay data. Each chemical was then manually evaluated for current registration status and categorized based on exposure likelihood as a direct food additive, indirect additive, pesticide/residue, or deemed non-food. Ultimately, 319 chemicals were categorized as non-food and removed from the list, leaving 556 direct additives, 339 indirect additives, and 406 pesticides/residues. Comparing cytotoxicity in ToxCast across categories revealed that only 10% of direct additives elicited cytotoxicity, while 24% of indirect additives and 41% of pesticides/residues were cytotoxic. Finally, to address the need to prioritize chemical mixtures, we used frequent itemset mining (FIM) to identify which individual chemicals or combination of chemicals appear with the greatest frequency in food contact substances. More specifically, we applied FIM to the registrations from the U.S. Food and Drug Administration's Effective Food Contact Substances (EFCS) database as these substances are often registered as defined mixtures. The FIM approach identified acetic acid, hydrogen peroxide, and peracetic acid as the most abundant chemicals in EFCS registrations, each occurring in 42 (or 4.3%) of 978 registered mixtures. The combination of peracetic acid and hydrogen peroxide occurred most frequently, appearing in 40 (or 4.1%) of the 978 registered mixtures. The current inventory and analysis of ToxCast cytotoxicity and EFCS mixture prioritization represent the first evaluation of food-use chemicals on this scale, providing insight into this overlooked but critical chemical inventory. This work does not reflect EPA policy.

Perinatal endothelial progenitor cells for epidemiological studies

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Human variability in response to chemical exposures, including drugs and environmental toxicants, cannot be addressed by animal models. Because testing human responses to toxicants is unethical, addressing individual variability in human populations is the principal limitation of current approaches in toxicological testing. However, at a cellular level human variability in response to toxicants can be assessed via using donor-specific cells.

We hypothesized that utilizing progenitor cells isolated from blood or other body fluids can serve for population-based toxicological testing. Specifically, we proposed that cord blood-derived endothelial progenitor cells can be used as a population-based platform for screening drugs and environmental toxicants for developmental cardiovascular toxicity. Formed during fetal development, endothelial progenitor cells can be harvested at birth in an entirely non-invasive manner, providing an opportunity for establishing a population-based collection of perinatal cells.

Currently, we are establishing a collection of cord blood-derived late outgrowth endothelial colony-forming (progenitor) cells (ECFCs). We present the results demonstrating that ECFCs is a versatile model amenable for high-throughput and high content screening approach. Here we demonstrate that ECFCs proliferation is a sensitive marker of exposure to a wide variety of environmental hazards, including ionizing radiation, cadmium, and endocrine disruptors. Using several donor-specific cell lines, we show the variability in response to these agents. An assessment of donor-specificity of cell growth inhibition is our immediate next step. In the future, proving that donor-specific characteristics can be assessed at the cellular level is a task of paramount importance as it will allow to bring toxicological and epidemiological studies to a new level.

Improving the accuracy of *in vitro* sensitization integrated testing strategies by incorporating a metabolic source

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Skin sensitization is an important consideration for ingredients and formulations and much of the necessary testing can now be done using non-animal methods. Two *in vitro* assays for sensitization, KeratinoSens™ and the Direct Peptide Reactivity Assay (DPRA) have published OECD test guidelines. Given the complexity of the sensitization pathway, there are limitations associated with each assay. One limitation is the inability of these assays to provide a sufficient source of metabolism. Pro-haptens require enzymatic oxidation to act as sensitizers and often lead to false negative results in these assays. There are limited metabolic capabilities with KeratinoSens™ and no metabolic capabilities with the DPRA. In this study we sought to assess the potential of incorporating an *in silico* method (OECD Toolbox) to make initial sensitization predictions. Along with these predictions we designed an incubation step using human liver microsomes as a source of metabolism for the test articles. We used initial predictions to decide if the traditional assay steps would be sufficient or if the addition of the microsomes was needed in order to correctly predict the test articles in our set of 13 chemicals. The results indicated that OECD Toolbox was a useful tool at distinguishing sensitizers, non-sensitizers, and pro-haptens. Also, with the traditional assays, only 3 out of 5 pro-haptens were correctly predicted in KeratinoSens™ and only 1 out of 5 pro-haptens were correctly predicted in the DPRA. After the addition of the microsomes, 4 out of 5 pro-haptens were correctly predicted in KeratinoSens™ and 4 out of 5 pro-haptens were correctly predicted in the DPRA. With the incorporation of OECD Toolbox we were able to avoid false positive results with the non-sensitizers in our chemical set. Taking a cautious approach with a positive result in either assay leading to a classification as a sensitizer, we correctly predicted all of the chemicals in our chemical set and have shown that the addition of a metabolic source can improve the accuracy of these assays.

Environmental quinones impair mitochondrial function in human airway epithelial cells using seahorse extracellular flux technology

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Air pollution is a global public health concern linked to cardiopulmonary morbidity and mortality. Quinones are components of ambient particulate matter (PM) air pollution and may contribute to health effects through oxidative mechanisms. Mitochondrial dysfunction has been proposed to underlie PM-induced oxidative stress. 1,2-naphthoquinone (1,2-NQ) is an electrophile that can adduct macromolecules and participate in single electron redox reactions to produce reactive oxygen species. We have previously shown that exposure to 1,2-NQ increases mitochondrial H2O2 production, leading to activation of inflammatory signaling. In the present study, we utilized extracellular flux analyses to characterize 1,2-NQ-induced disruption of mitochondrial function in human airway epithelial cells. 1,2-NQ caused a marked increase in the oxygen consumption rate (OCR) that was shown to be largely attributable to redox cycling in the cytosol using permeabilized-cell and isolated mitochondria preparations. In these preparations, 1,2-NQ impaired Complex I-linked substrate utilization, specifically the oxidation of pyruvate but not glutamate. Complex I activity of NADH oxidation was not blunted by 1,2-NQ exposure, suggesting inhibition of pyruvate-specific uptake and/or metabolism. In addition, 1,2-NQ exposure did not affect Complex II-linked substrate oxidation. These findings show that exposure to redox-active compounds like 1,2-NQ can elicit H2O2 production through redox cycling and mitochondrial dysfunction simultaneously. Though Seahorse Extracellular Flux Technology is a powerful method of assessing mitochondrial function *in vitro*, accurate interpretation of OCR effects induced by toxicologic compounds requires consideration of alternative sources of oxygen consumption.

Forwarding xeno-free stem cell techniques to advance human-relevant models for drug testing

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Induced pluripotent stem cells (iPSCs) approaches are rapidly becoming powerful *in vitro* human-relevant models for toxicological testing. With the advent of clustered regularly interspaced short palindromic repeats (CRISPR) technology, iPSCs can be readily manipulated to develop cellular models of many human diseases to replace animal models for testing drug safety and efficacy. However, the predictive power of stem cell-derived models of human diseases is limited in part by contamination with animal-derived products during the process of iPSC generation and differentiation into specialized cells. This contamination issue is not only a challenge for regenerative medicine, but may reduce the predictive power of iPSC-related assays for toxicity screening. In recent years, methods and reagents have been developed to replace animal-derived products for iPSC generation and differentiation. To assist scientists and toxicologists to adopt these techniques to generate xeno-free human models for drug testing, we are creating an online database of xeno-free protocols for iPSC research. To date, we have catalogued peer-reviewed publications on such methods and are working towards standardization to improve usability and reproducibility of protocols. Next, we will prototype a searchable, interactive online resource that will allow for user experience sharing. This xenofree stem cell toolkit, a first of its kind, will serve as a framework to not only advance the development of alternatives to animals for toxicology testing but also aid in reproducibility, increase transparency of methods, reduce cost, and assist in the clinical translatability of iPSC research.

PPARα activation drives zone-specific transcriptional programs in the rat liver

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The liver is comprised of millions of parallel functional units (lobules) each containing several hundred hepatocytes. The physiology and metabolic specialization of hepatocytes differ greatly between these zones: periportal hepatocytes are smaller and primarily engage in oxidative energy metabolism, whereas centrilobular hepatocytes are large, polynucleated cells specializing in glycolysis and xenobiotic metabolism. Zone-specific expression of proteins in liver tissues has been previously observed upon chemical perturbation of other ligand-activated receptor systems (e.g., AhR). To better understand zonal biology and its relationship to chemical sensitivity, we used laser capture microdissection (LCM) to evaluate zonal differences in transcriptomic response to PPARα activation. Male Sprague-Dawley rats were treated with a selective agonist of PPARα (GW7647) at 0, 0.1, 0.25, 1.0, 2.5, 10, or 20 mg/kg/d via oral gavage for 4 days. Livers from these animals were evaluated by immunohistochemistry or LCM of periportal, midzonal, and centrilobular hepatocytes. We observed zone-specific activation of PPARα-regulated marker proteins in the periportal region of the liver using immunohistochemistry. Gene expression microarray analysis of these subpopulations revealed that more genes were differentially expressed in periportal cells than in centrilobular (2837 vs 432 at 20 mg/kg/d). Additionally, the magnitude of the transcriptional changes was larger in the periportal subpopulation and the dose at which cells responded was lower (AC50 = 0.38 vs 1.94 mg/kg/d). Analysis of canonical PPARα transcriptional targets, as well as pathway analysis using BMDExpress, revealed that all three zones respond with alterations in fatty acid metabolic machinery. However, there are a number of processes—including cell cycle, apoptosis, and cell-cell adhesion Gene Ontology categories—that are altered exclusively in periportal hepatocytes. Differential periportal responses may be a key step in tumorigenesis driven by PPARα agonists in rodent livers. We discuss these results in the context of our *in vitro* culture experiments and development of a hepatocyte-based proliferation assay.

Utilizing the adverse outcome pathway framework in the development of organotypic screening platforms: a breast cancer case study

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The adverse outcome pathway (AOP) framework has become popular in regulatory toxicology as the AOP helps identify early molecular or cellular endpoints to incorporate into chemical screening platforms. In addition to its traditional use, we hypothesize that the AOP can be used in the development of more advanced organotypic screening platforms by identifying critical cellular components necessary to recapitulate the molecular, cellular and tissue level readouts that are predictive of *in vivo* response. To test this hypothesis, we used the estrogen receptor (ER) mediated breast cancer AOP to identify the cellular components and readouts needed to recapitulate ER+ breast cancer development, then evaluated each in an organotypic mammary duct model. We generated a collagen-embedded mammary duct structure lined with MCF7 human breast cancer cells for our base platform, and tested the response to ER agonists and pro-agonists after addition of embedded stromal fibroblasts and adipocytes. Upon exposure to the ER agonist 17-β estradiol (E2), we found that exposing our base platform to E2 initiates the molecular initiating event, ER activation, in a concentration-dependent manner (EC50 = 1.85 nM). On a cellular level, we found that ER activation leads to a 5-fold increase in the expression of ER regulated genes TFF1 and PGR and a 2-fold increase in the percentage of Ki67+ cells. To evaluate a tissue level response, we cross sectioned the ductal structures and found that estradiol exposure increased luminal filling of the epithelium. We found the presence of fibroblasts significantly increased the potency of ER agonists, E2, bisphenol-A and diethylstilbestrol, as indicated by a 10-fold leftward shift in EC50, but not the E2 pro-agonist testosterone. Only after the addition of pre-adipocytes in the stroma did we find that exposure to testosterone initiated molecular, cellular and tissue level responses in our organotypic platform. These findings highlight the potential importance of incorporating additional cell types into cancer models, rather than traditional *in vitro* cancer models that evaluate the cancer cell alone. Taken together, we demonstrate the AOP framework can be used in the selection of readouts and cellular components of organotypic cancer models.

IATA for skin sensitization potential – 1 out of 2 or 2 out of 3?

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To meet EU regulatory requirements and to avoid or minimize animal testing, there is a need for non-animal methods to assess skin sensitization potential. Given the complexity of the skin sensitization endpoint, there is an expectation that integrated testing and assessment approaches (IATA) will need to be developed which rely on assays representing key events in the pathway. Three non-animal assays have been formally validated: the direct peptide reactivity assay (DPRA), the KeratinoSensTM assay and the h-CLAT assay. At the same time, there have been many efforts to develop IATA with the "2 out of 3" approach attracting much attention whereby a chemical is classified on the basis of the majority outcome. A set of 271 chemicals with mouse, human and non-animal sensitization test data was evaluated to compare the predictive performances of the 3 individual non-animal assays, their binary combinations and the '2 out of 3' approach. The analysis revealed that the most predictive approach was to use both the DPRA and h-CLAT: 1. Perform DPRA – if positive, classify as a sensitizer; 2. If negative, perform h-CLAT – a positive outcome denotes a sensitizer, a negative, a non-sensitizer. With this approach, 85% (LLNA) and 93% (human) of the non-sensitizer predictions were correct, in contrast to the '2 out of 3' approach which had 69% (LLNA) and 79% (human) of non-sensitizer predictions correct. *The views expressed are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.*

Information dependent enrichment analysis – A novel algorithm for enrichment analysis

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Over-representation analysis is widely used to calculate if an ontological category is statistically associated with a given set of genes. Traditionally, statistical and/or fold-change thresholds are used to select the gene set. These arbitrary cutoffs are sensitive to statistical power and do not take into account the larger biological pattern of gene expression. We have developed a novel method - Information Dependent Enrichment Analysis (IDEA) - that quantifies the information contained within the pattern of gene expression by using the entire transcriptomic response for over representation analysis. IDEA examines how the pattern of expression changes in a cohort of genes drives enrichment. We applied IDEA to two distinct gene expression datasets where standard over-representation analysis was not feasible or problematic due to few significant genes at lower exposures conditions. In the first dataset, we applied IDEA to calculate transcription factor enrichment from gene expression profile of MCF7 cells treated to 1nM E2 at 2h,4h,8h and 24h post treatment. Using IDEA instead of standard over-representation analysis uncovered the contribution of different transcription factors to overall gene expression and how the pattern evolves over time. Using this information, we were able to establish a putative transcription factor network for estrogen activity in MCF7 cells. In the second case, we investigated effects in C57BL/6 Wild Type mice lung and liver after Styrene exposure for 1 day at 5, 10, 20, 40 and 120ppm. IDEA was able to identify more enriched categories from Liver samples than were detected by traditional enrichment analysis, but these did include all of the pathways enriched by traditional analysis. Lung results were similar, including the finding that most significant enrichment was a result of changes at exposures less than the maximum dose despite the large numbers of differentially expressed genes at 120ppm. While IDEA results included those from traditional enrichment analysis in both cases, IDEA was able to capture more of the dynamics of the functional response at the lower exposures, where traditional methods were not applicable.

Variability within systemic *in vivo* toxicity studies

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In vivo studies have long been considered the gold standard for toxicology screening. Often time models developed *in silico* and/or using *in vitro* data to estimate points of departures (POD) are compared to the *in vivo* data to benchmark and evaluate quality and goodness-of-fit. However, recent work has illustrated that currently available *in vivo* data are not without flaws and inherent variance presents a challenge in predictive modeling. The goal of the current work was to characterize the amount of variance that exists within systemic *in vivo* data. The present study was done using the US EPA's Toxicity Reference Database (ToxRefDB) which contains around 5,000 *in vivo* toxicity studies from the Office of Pesticide Programs (registrant-submitted studies), National Toxicology Program, pharmaceutical industries, and publically available literature covering over 1,000 chemicals. Using multilinear regression to calculate the residual sum or squares, we accounted for known variability in study conditions to quantify the unexplained variance of the log10 (POD) to be about 0.35. The leave-one-out method was used to assess the amount of variance explained by various study conditions (e.g., species, purity of test material) and chemicals were found to be the biggest contributor. Stratifying the dataset by species and administration methods showed similar results, indicating stability of the unexplained variance. Considering and quantifying the unexplained variance will provide a benchmark and lower bounds on the mean-square-error for predictive toxicity model development. This work provides an upper bound on the level of precision predictive models can attain when trained on conventional PODs.

This abstract does not necessarily reflect U.S. EPA policy.

Feasibility analysis of incorporating *in vitro* Toxicokinetic Data as a Surrogate for *in vivo* data for read-across predictions

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The underlying principle of read-across is that biological activity is a function of physical and structural properties of chemicals. Analogs are typically identified on the basis of structural similarity and subsequently evaluated for their use in read-across on the basis of their bioavailability, reactivity and metabolic similarity. While the concept of similarity is the major tenet in grouping chemicals for read-across, a critical consideration is to evaluate if structural differences significantly impact toxicological activity. This is a key source of uncertainty in read-across predictions. We hypothesize that inclusion of toxicokinetic (TK) information will reduce the uncertainty in read-across predictions. TK information can help substantiate whether chemicals within a category have similar ADME properties and, hence, increase the likelihood of exhibiting similar toxicological properties. This current case study is part of a larger study aimed at performing a systematic assessment of the extent to which in-vitro TK data can obviate in-vivo TK data, while maintaining or increasing scientific confidence in read-across predictions. The analysis relied on a dataset of ~7k chemicals with predicted exposure data (chemical inventory), of which 819 chemicals had rat and/or human in-vitro TK data (analog inventory), and 33 chemicals had rat in-vivo TK data (target inventory). The set of chemicals with human *in vitro* TK data was investigated to determine whether structurally related chemicals had similar intrinsic clearance. An unsupervised feature selection was performed (using Chemotyper and PubChem fingerprints) on chemicals in the analog inventory to remove the features with low variance. Unsupervised clustering was performed on the reduced feature set using the K-means algorithm. Preliminary results (using both fingerprints) show a correlation between structural fingerprint-based clusters and intrinsic clearance. The chemical inventory was then explored using principal component analysis based on both fingerprints to help select target chemicals that were representative of the inventory, and also associated with in-vivo TK data. 11 target chemicals were found to have at least 1 analog with in-vitro data. Next steps will include an analysis of the correspondence between in-vitro and in-vivo TK data using the target chemicals identified.
This abstract does not necessarily represent U.S. EPA policy.

Integration of different data gap filling techniques to facilitate assessment of polychlorinated biphenyls: A proof of principle case study

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Data gap filling techniques are commonly used to predict hazard in the absence of empirical data. The most established techniques are read-across, trend analysis and quantitative structure-activity relationships (QSARs). Toxic equivalency factors (TEFs) are less frequently used data gap filling techniques which are applied to estimate relative potencies for mixtures of chemicals that contribute to an adverse outcome through a common biological target. For example, The TEF approach has been used for dioxin-like effects comparing individual chemical activity to that of the most toxic dioxin: 2,3,7,8-tetrachlorodibenzo-p-dioxin. The aim of this case study was to determine whether integration of two data gap filling techniques: QSARs and TEFs improved the predictive outcome for the assessment of a set of polychlorinated biphenyl (PCB) congeners and their mixtures. PCBs are associated with many different adverse effects, including their potential for neurotoxicity, which is the endpoint of interest in this study. The dataset comprised 209 PCB congeners, out of which 87 altered *in vitro* Ca(2+) homeostasis from which neurotoxic equivalency values (NEQs) were derived. The preliminary objective of this case study was to develop a QSAR model to predict NEQ values for the 122 untested PCB congeners. A decision tree model was developed using the number of position specific chlorine substitutions on the biphenyl scaffold as a fingerprint descriptor. Three different positional combinations were explored on the basis of equivalence between ortho, meta and para positions. Five different decision trees were developed on the basis of restrictions on tree growth. The training dataset of 87 tested PCBs was evaluated using 5-fold cross validation and leave-one-out (LOO) internal validation to ultimately predict NEQ values for the 122 untested PCBs. The evaluation statistics of the “best” decision tree model resulted in LOOCV RMSE: 0.29, 5-fold CV test RMSE: 0.34, and R2: 0.79. The results demonstrate the utility of using the TEF approach as an alternative data gap filling technique.
This abstract does not necessarily represent U.S. EPA policy.

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The GARD assay – assessment of skin sensitizing chemicals

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Chemicals have improved our welfare in numerous aspects, but severe symptoms can arise in exposed persons. An adverse reaction is Allergic Contact Dermatitis (ACD), that can result after repeated contact. To decrease chemicals inducing ACD (skin sensitizers) in household products and occupational environments, chemicals are tested. Previous test systems included animals, but EU legislation and OECD test guidelines demand the use and development of animal-free assays.

Genomic Allergen Rapid Detection (GARD) is an *in vitro* assay for assessment of sensitizing capacity of a substance of interest. Activation of dendritic cells *in vivo*, is one of the first immunological events in ACD. The GARD assay mimics this process: a human myeloid dendritic cell line is exposed to the chemical to be tested and any activation is assessed by Nanostring analysis comprising the gene expression of roughly 200 genes. The accuracy of GARD is estimated to 89% based on 26 blinded chemicals (Johansson et al. 2014). Potency classifications are requested by chemical regulators to provide correct risk assessments. An ultimate objective is to utilize the GARD assay for this end-point, however further research is needed for that assessment.

The GARD assay, unique with its high informational content readout, can further be used for pathway analysis to understand the underlying mechanisms of ACD. This makes GARD interesting not only for the chemical industry but for academia and the pharma industry as well.

Quantitative imaging analysis of amyloid beta toxicity using cultured neurons

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Synaptic function is modulated by the balance between dynamic and stable actin structures in dendritic spines. Drebrin is an actin-binding protein located at mature dendritic spines and forms a stable actin structure. Therefore the loss of drebrin is a good marker of synaptic dysfunctions. In fact in a number of cognitive disorders, including Alzheimer's disease which suffered from synaptic dysfunction, drebrin disappeared from dendritic spines. We developed high throughput imaging analysis of synaptic function using drebrin as a marker. Frozen hippocampal neurons prepared from E18 rat brain (SKY-neuron) were inoculated in 96-well culture dishes. After 21 days *in vitro*, the cell were treated pharmacologically, and double-immunostained with anti-drebrin and anti-MAP2 antibodies, and analyzed by IN Cell Analyzer 2200 (GE Healthcare Japan, Tokyo). The number of drebrin clusters decreases by acute glutamate treatment (10 min) in a dose-dependent manner. In addition, antagonist of NMDA-type glutamate receptor has shifted the dose-response curve of the glutamate to the right. Interestingly without agonist, overnight treatment of antagonist increased the number of drebrin clusters in a dose-dependent manner. We then examined the toxicity of Amyloid beta (Aβ) to the synapse. The number of drebrin clusters decreases by the soluble oligomers (Aβ-derived diffusible ligands, ADDLs) after 6 hours of the treatment. The decrease of drebrin clusters were induced without reducing drebrin expression, meaning that acute decrease of drebrin clusters is not due to the cell death but due to the exodus of drebrin from dendritic spines. Next we examined whether the toxicity of ADDLs is inhibited by histone deacetylase inhibitor, suberoylanilide hydroxamic acid (SAHA), while SAHA treatment did not affect the density of drebrin clusters in control neurons. This suggests that SAHA attenuates the ADDL toxicity to the neuronal synapse.

Mechanistic modeling: The pathway to precision medicine

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There is a growing and critical need for integrating molecular systems science with computation to model complex disease processes for accelerating drug discovery, drug repurposing, validation of complementary and alternative medicine (CAM) therapies, and identification of efficacious multi-combination therapeutics, while ensuring a personalized and precise medicine. Such needs cannot be advanced without collaborative integration of knowledge across biological disciplines. This talk will share the recent successes, through multiple case studies, in the use of CytoSolve, a computational systems biology collaboratory, developed at M.I.T., that provides an integrative approach to address these critical needs.

Previous approaches, largely based on statistical techniques, have been unscalable and largely useless to scientists who seek to understand complex biological mechanisms. CytoSolve's successes have been published in peer-reviewed journals and have received recognition in Nature for its potential to develop multi-combination therapies. These successes including: FDA allowance for a multi-combination pancreatic cancer therapeutic; the Department of Defense (DoD) and the United States Pharmacopeia (USP) understanding of toxicity and adverse reaction multi-combination nutritional supplements; and, modeling of rare diseases in orphan drug domains such as Neuromyelitis Optica (NMO) and Hereditary Angioedema (HAE) have inspired major nutraceutical researchers, cancer centers such as MD Anderson, National Cancer Institute and others to explore the use of CytoSolve for integrating CytoSolve's collaboratory with modern *in vitro* and *in vivo* methods to accelerate the development of multicombination therapeutics. This talk that will provide an introduction to a disruptive platform that will likely revolutionize development of therapeutics in the 21st century.

Validation of a high content imaging assay for steatosis in a micropatterned human hepatocyte Co-culture model

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Hepatic steatosis, the abnormal accumulation of triglycerides within hepatocytes, is the most prevalent liver disease in the U.S., estimated to affect over 20% of the population. Although well tolerated by itself, steatosis is hypothesized to sensitize the liver to secondary insults that can cause progression to more severe forms of fatty liver disease. Some drugs and environmental chemicals have been linked to fatty liver through diverse modes-of-action, including mitochondrial impairment, enhanced cytokine production, and altered hepatic lipid secretion. To establish a human relevant fit-for-purpose *in vitro* assay that can detect chemical-induced liver steatosis we developed a multiplexed high-content screening strategy using a 96-well micropatterned co-culture system of primary human hepatocytes. Reference compounds were selected for assay development based on their high specificity and well understood mechanisms: cyclosporin A, methotrexate, and amiodarone for steatosis, and caffeine as a negative control. Optimization of dosing parameters and kinetics resulted in a 96 hour workflow with a fixed endpoint, 4-channel assay to quantitatively determine hepatocyte-specific neutral lipid accumulation, cell counts, and viability. High-content imaging parameters for lipid endpoints were restricted to analyzing lipid droplet size, intensity, and frequency. Anticipated concentration-response curves for the lipid endpoints were observed for all of the compounds except for methotrexate. In addition, cytotoxicty parameters were validated and performed as expected. Preliminary results from a validation screen of 29 compounds, including *in vivo* steatosis inducers and non-inducers, show the expected trends with positive hits correlating to known inducers of steatosis *in vivo*. The results support utility of this *in vitro* platform for evaluating chemical effects on human fatty liver disease.

Prediction of skin sensitization potency using machine learning approaches

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Replacing animal tests currently used for regulatory hazard classification of skin sensitizers is one of ICCVAM's top priorities. Accordingly, U.S. federal agency scientists are developing and evaluating computational approaches to classify substances as sensitizers or nonsensitizers. Some regulatory agencies require that sensitizers be further classified into potency categories. We have built machine learning models to classify substances as strong sensitizers, weak sensitizers, or nonsensitizers according to the Globally Harmonized System of Classification and Labeling of Chemicals for both local lymph node assay (LLNA) and human outcomes. The models were based on data from three in chemico or *in vitro* assays (direct peptide reactivity assay, human cell line activation test, and KeratinoSens™ assay) and six physicochemical properties. Combinations of these input variables were modeled with four machine learning approaches: classification and regression tree, linear discriminant analysis, logistic regression, and support vector machine (SVM). Two different strategies were used for modeling: a one-tiered multi-class strategy modeled all three categories of response, while a two-tiered binary strategy modeled sensitizer vs. nonsensitizer responses and then differentiated between strong and weak sensitizers. Models were developed on a training set and evaluated using an external test set. Leave-one-out cross validation (LOOCV) was used to evaluate the models for overfitting. The two-tiered models performed better than the one-tiered models and SVM outperformed the other machine learning approaches. Models using the variable group with all input variables performed better than variable groups with assay data only, or physicochemical properties only, or assay data plus log P. The two-tiered model using SVM and all input variables provided the best performance: accuracy for the LOOCV was 90% for LLNA (120 substances) and 85% for human (87 substances) outcomes. Accuracy for the best one-tiered models was 78% for LLNA outcomes and 75% for human outcomes. This compares to an accuracy of 69% for LLNA prediction of human potency categories. These results suggest that computational approaches may be useful for assessing skin sensitization potency. This project was funded in whole or in part with Federal funds from the NIEHS, NIH under Contract No.HHSN273201500010C. This abstract does not represent EPA policy.

How GLPs Enhance the Quality of Both Regulated and Non-Regulated *In Vitro* Toxicology

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A recent article published in the journal Nature points out that more than half of the researchers surveyed have failed to reproduce their own experiments. The reproducibility of promising new assays is a key factor in the decision to invest further resources into a project to move it from academic discovery to industry use. Lengthy assay optimization phases are often required to standardize materials and procedures to reliably reproduce results. Applying the Good Laboratory Practices (GLPs) as a quality management system provides the framework to ensure methods are conducted reproducibly within a laboratory, and to effectively support transferability of methods to external stakeholders. Additionally, following GLPs plays an important role in the acceptance of multi-laboratory validation trials by regulatory agencies and validation authorities prior to promotion of new methods through industry test guidelines. This poster will highlight some concepts in GLPs designed to promote study reproducibility and data integrity within an *in vitro* toxicology framework. It will reference current US (FDA and EPA) and global (OECD) regulations and briefly point out additional points raised in the FDA's recently proposed draft revisions to the GLPs. Early adoption and implementation of GLP concepts can ensure the execution of robust, repeatable, reliable studies downstream.

Cosmetic safety testing roadmap to regulatory approval in north america, Europe, and Japan

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The cosmetics industry is a robust business, achieving greater than $250 billion in worldwide sales in 2013. Although major recognizable brands comprise the largest fraction of this total, there are thousands of small companies that operate in the same markets. While the industry association International Cooperation on Cosmetic Regulation (ICCR) seeks long-term harmonization of testing requirements, currently unaligned governmental agencies in different countries make navigation of safety requirements daunting for major manufacturers and Kafkaesque for smaller firms. While also laudable, the development, validation, approval and implementation of new non-animal safety assays often make the path to cosmetic approval seem more formidable than ever. To aid in navigation of the kaleidoscopic variables of cosmetic ingredient and final cosmetic formulation safety testing, the authors have developed a roadmap to safety assessment, focusing on requirements of the United States and the European Union.

TCDD represses osteogenic differentiation of human bone-derived mesenchymal stem cells

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Normal bone development requires a strict temporal and spatial coordination of gene regulatory networks that govern commitment of mesenchymal stem cells (MSC) to an osteogenic lineage, and subsequent differentiation to become mature, matrix-secreting osteoblasts. There is increasing concern, however, that exposure to environmental xenobiotic stressors may perturb osteogenic pathways and serve as the basis for developmental and adult skeletal diseases. Aryl hydrocarbon receptor (AHR) ligands including TCDD are one class of chemicals known to disrupt bone and cartilage formation in multiple *in vivo* and *in vitro* models. In this study, we elucidate TCDD-mediated inhibition of osteogenesis *in vitro* using human bone-derived mesenchymal stem cells (hBMSCs) cultured in an osteo-promoting medium. At early stages of hBMSC differentiation treatment with 10 nM TCDD resulted in a significant reduction DLX5, an upstream of master osteogenic regulators RUNX2 and OSX. At intermediate stages of differentiation exposure to 10 nM TCDD attenuated OSX expression along with with a concomitant reduction in alkaline phosphatase activity. Additionally, TCDD-exposed hBMSCs demonstrated diminished expression in apical markers of bone extracellular matrix (ECM) SPP1 and a marked reduction in Alizarin red S-positive staining. Co-exposure to AHR antagonist GNF351 blocked TCDD-mediated inhibition of osteogenic differentiation thus highlighting the AHR as a potential target for adverse skeletal outcomes. Current work is underway to identify additional signaling pathways perturbed by TCDD exposure and to confirm these results in hBMSCs derived from additional donors. This study highlights the potential of using hBMSCs to screening the osteotoxic and osteopromoting potential of xenobiotic compounds and pharmacological mediators.

Uncertainty quantification in high throughput screening models of endocrine disruption

Eric D Watt and Richard S Judson

Using uncertainty quantification, we aim to improve the quality of modeling data from high throughput screening assays for use in risk assessment. ToxCast is a large-scale screening program that analyzes thousands of chemicals using over 800 assay-endpoints representing hundreds of biochemical and cellular processes. Over 2.6 million concentration response curves are fit to models to extract parameters related to potency and efficacy. Models built on ToxCast results are used to rank and prioritize toxicological risk of tested chemicals and predict the toxicity of tens of thousands of chemicals not yet tested *in vivo*. Large amounts of data also present challenges. When fitting data, choice of models, model selection strategy, and hit call criteria must reflect the need for computational efficiency and robustness, requiring hard and somewhat arbitrary cutoffs. When coupled with unavoidable noise in experimental concentration response data, hard cutoffs cause uncertainty in model parameters and the hit call. The uncertainty will then propagate through all models built on the data. Left unquantified, this uncertainty causes difficulty in fully interpreting the data for risk assessment. We used bootstrap resampling methods to quantify uncertainty in model fits of concentration response data. Bootstrap resampling allows calculation of confidence intervals for both potency and efficacy parameters and to shift model selection and hit calling from a binary determination to a probabilistic quantification. These uncertainty estimates were then propagated through mathematical models built to predict bioactivity from ToxCast data. We explored our method's utility by quantifying uncertainty in models of endocrine disruption. Examples include estrogen model agonist scores, androgen antagonism selection, and selectivity scoring of *in vitro* thyroid peroxidase potency relative to cytotoxicity and non-specific enzyme inhibition. By quantifying uncertainty in model predictions, we were able to better identify false positives and negatives in model predictions. Bootstrap resampling allowed us to improve separation of biological activity from assay noise and enhance the quality of model output, and thus increase confidence in model predictions for use in risk assessments.

This abstract does not necessarily reflect U.S. EPA policy.

Three-dimensional paper-based culture platform for screening endocrine disruptors in breast cancer models

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The Environmental Protection Agency (EPA) formed the Endocrine Disruptor Screening Program (EDSP) to screen nearly 10,000 chemicals for endocrine disruption activity. To efficiently screen all 10,000 molecules, high-throughput *in vitro* screens are used to predict for potential endocrine disruption *in vivo*. Current *in vitro* screens commonly rely on competitive ligand binding assays. These assays cannot predict if the test molecule is an agonist or antagonist, making accurate *in vivo* predictions difficult. *In vitro* cell-based assays have been developed as an alternative to ligand binding assays, and are preferred because they provide a cellular response to test molecules leading to more accurate predictions of endocrine disruptors *in vivo*. Current cell-based assays rely on two-dimensional (2D) monolayer cultures, which are not representative of cellular organization found in tissues. Cells cultured in three-dimensional (3D) architectures yield cellular phenotypes and responses that are more representative of *in vivo* tissue, and could potentially improve our ability to accurately identify potential disrupting agents.

It remains unclear how cellular responses to endocrine disruptors differ between 2D monolayer and 3D cell cultures. Here we present a paper-based cell culture platform for screening endocrine disruptors. The paper scaffolds used for this assay are patterned in a 96-well plate format in which hydrophilic zones are separated by hydrophobic wax-patterned borders; these zones support the culture of cells suspended in an extracellular matrix. To investigate endocrine disruption, we utilize a T47D-KBluc cell line engineered with a luciferase reporter gene downstream from three estrogen responsive elements. This cell line detects endocrine disruption activity with a luciferase-based readout. Here, we compare the responsiveness of the T47D-KBluc cells in 2D and 3D culture formats. Specifically, we quantify the maximal response to 17β-estradiol, the EC50 for estradiol, and the IC50 for the antiestrogen ICI 182,780. The 3D paper-based screening platform will be further validated with a library of compounds that are known endocrine disruptors.

ASCCT 2016

Speaker Bios

Thomas Hartung, MD, PhD, Johns Hopkins University

Director, Center for Alternatives to Animal Testing
Professor and Chair for Evidence Based Toxicology

The goal of Dr. Hartung's work is toward a paradigm shift in toxicity testing to improve public health. He was formerly the head of the European Centre for the Validation of Alternative Methods of the European Commission (2002-2008) and is now involved in the implementation of the 2007 NRC vision document "Toxicity Testing in the 21st Century – a vision and a strategy". He has furthered the translation of concepts of evidence-based medicine to toxicology (evidence-based toxicology). This aims for systematic assessment of the quality of all tools for regulatory toxicology and the development of new approaches based on annotated pathways of toxicity (the Human Toxome). Dr. Hartung's background in clinical and experimental pharmacology and toxicology is documented in more than 480 publications. In addition to directing CAAT, he has established a bioinformatics group and a laboratory for developmental neurotoxicity research based on genomics and metabolomics, which were made available by a Thought-Leader Award from Agilent.

Mahendra Rao, MD, PhD

VP Strategic Affairs - Q Therapeutics
VP Regenerative Medicine - New York Stem Cell Foundation - NYSCF

Dr. Rao was the founding Director of the NIH Center of Regenerative Medicine and also the Chief of the Laboratory of Stem Cell Biology at the NIH. Dr. Rao's laboratory is focused on translational work related to Parkinsons disease and glial cell pathology. Dr. Rao is currently the the VP of Strategic Affairs at Q Therapeutics, serves as a consultant on regenerative medicine for the New York Stem Cell Foundation, and continues to provide consultant and advisory services to biotechnology companies such as Cesca, Megakaryon, Stempeutics and Hemacare. Dr. Rao was recently named one of the top ten influential people in the stem cell field and was honored recently by the Federation of Biologists (FABA) India and awarded the NBRI medal (India) for his contributions to neuroscience research.

Awards

Edward Carney Predictive Toxicology Award

Dr. Edward Carney was an active and dedicated member of the American Society for Cellular and Computational Toxicology, and a partner, mentor and friend to many in our fields. His passion and and leadership will continue to inspire investigators in *in vitro* and *in silico* toxicology through the Edward Carney Predictive Toxicology Award. This award will be provided to the first author of a winning poster at each ASCCT annual meeting, starting in 2015. The winner will receive a $500 cash award to assist with travel and/or research expenses.

The 2016 winner will be announced at the member reception on September 29.

The William and Eleanor Cave Award

The William and Eleanor Cave Award is presented to recognize achievements in developing and implementing alternatives to the traditional use of animals in testing, research or education.
It is presented biannually by the Alternatives Research & Development Foundation and this year will carry a $10,000 prize.

Past recipients (and their affiliations at the time of the award) have included:

Ruy Tchao, *University of the Sciences*
George Russell, *Adelphi University*
John Sheasgreen, *MatTek Corporation*
Leon Bruner, *The Gillette Company*
Daniel Smeak, *The Ohio State University*
Rodger Curren, *Institute for In Vitro Sciences*
Mel Andersen, *The Hamner Institute for Health Sciences*
Frank Gerberick, *The Procter & Gamble Company*

A special award was presented in 2010 to the journal ATLA (Alternatives To Laboratory Animals), for its invaluable contributions to advancing the science of alternatives.

William and Eleanor Cave were devoted officers of The American Anti-Vivisection Society for decades. They recognized the opportunities in developing new technologies and alternative methods to address the problems of animal experimentation. They dedicated resources to fund research, eventually resulting in the establishment of the Alternatives Research & Development Foundation.

ARDF's mission is to fund and promote the development, validation and adoption of non-animal methods in biomedical research, product testing and education. ARDF has awarded over 3 million dollars in grants to researchers developing alternative test methods at major universities across the U.S. and sponsors scientific meetings such as the World Congresses on Alternatives and Animal Use in the Life Sciences.

Information is available at www.ardf-online.org.

Notes

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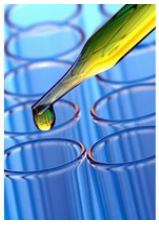
HUMAN TOXICOLOGY PROJECT CONSORTIUM

Advancing a New Paradigm for Assessing Chemical Safety



Alternatives Research & Development FOUNDATION

PCRM PHYSICIANS COMMITTEE FOR RESPONSIBLE MEDICINE

PETA INTERNATIONAL SCIENCE CONSORTIUM, LTD.

ILS

Advancing Science, Improving Lives

IIVS

Institute for In Vitro Sciences

Advancing Science & Animal Welfare Together

CAAT

JOHNS HOPKINS UNIVERSITY

Center for Alternatives to Animal Testing