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Abstracts

In Vitro Toxicology Society Annual Meeting

November 10–11, 2015

Birmingham, United Kingdom

These abstracts were presented at the 2015 annual meeting of the UK *In Vitro* Toxicology Society (IVTS). The meeting was hosted at Birmingham, UK on November 10–11, 2015. The main session topics included hepatoxicology, inhalation toxicology, developmental and reproductive toxicology, and neurotoxicology.

The IVTS (www.ivts.org.uk) was founded in 1988 to support scientists active in the study, practice or development of *in vitro* toxicology.

Speaker Abstracts

S-1.
Evaluation of human liver microtissues in hepatotoxicity screening

Dominic Williams

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Drug-induced liver injury (DILI) is a major cause of attrition during drug development and drug withdrawal. Hence the need for predictive toxicology screening assays which enable early identification and deselection of compounds that have the potential to cause DILI. A novel and promising in vitro model is the 3D InSightTM primary human hepatocyte and non-parenchymal coculture model (hLiMT). In this study we assessed ATP depletion as an indicator of cytotoxicity towards the hLiMTs for marketed drugs reported to cause DILI, liver enzyme elevations or no reports of DILI in man. Test compounds were administered to the hLiMT on days 0, 5 and 9 and cytotoxicity was determined on days 5 and/or 14 using the ATP endpoint assay. The top concentration tested was ≥ 100 fold plasma Cmax or the limit of solubility. Biomarker release was assessed (alpha-Glutathione S Transferase, HMGB1 & miR122) in selected incubations.

S-2.
Complex cell models to bridge in vitro data to human liver injury

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New tools are necessary for studying drug induced hepatotoxicity, liver regeneration, differentiation of induced pluripotent stem cells (iPSC) to hepatocytes and for understanding interindividual differences in drug induced liver injury and liver diseases. Previous in vitro systems where the human liver can be studied for long periods of time, retaining its phenotype allowing to study liver function, regulation of hepatic gene expression, drug and/or viral induced hepatic damage relevant in vitro systems, have been lacking. We are establishing conditions for formation of hepatic spheroids in normal and diseased states which are phenotypically stable for more than 5 weeks in vitro. The diseases include viral hepatitis, cholestasis, steatosis, fibrosis and drug induced hepatitis. These systems are anticipated to be of importance for increased possibilities a. to avoid the development of hepatotoxic drugs, b. to develop novel protocols for formation of hepatocytes from stem cells, c. for understanding the bases for interindividual differences in drug metabolism and drug hepatotoxicity, d. for development of systems for hepatocyte expansion, e.g., for transplantational purposes and for large scale in vitro liver systems, and e. for understanding the genome alterations in different liver diseases with novel possibilities for intervention. The lecture will give a state of the art view of the current stage and results in the development of these new systems.

Underline indicates presenting author.

S-3.
Mechanistic biomarkers and their application to the study of drug induced liver injury in vitro

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The potential of mechanistic biomarkers to improve the prediction of drug-induced liver injury (DILI), a significant cause of patient morbidity, mortality and attrition within drug development, is widely acknowledged. Prediction of clinical DILI remains difficult, particularly in cases characterized by marked inter-individual variation. A lack of sensitivity, specificity and an indirect mechanistic basis of currently used biomarkers of hepatic injury remains a factor for the delayed identification of DILI. Currently, Hy's law represents the regulatory endorsed model to predict serious DILI and is the standard for novel DILI biomarkers to surpass. Clinical and preclinical studies of paracetamol (APAP) overdose have shown the identification and development of circulating biomarkers that provide enhanced hepatic specificity (miR-122) and can inform on mechanistic events such as necrosis (keratin-18, HMGB1), apoptosis (caspase-cleaved keratin-18), mitochondrial dysfunction (glutamate dehydrogenase) and inflammation (acetylated HMGB1). A number of these biomarkers also provide enhanced prognostic utility and the sensitive identification of liver injury following APAP overdose prospective studies. However, significant challenges remain such as their ultily in in vitro models of DILI. These challenges will be discussed alongside the presentation of studies to address these issues and are the current focus of the Predictive Safety Testing Consortium (PSTC) and the IMI Safer and Faster Evidence based Translation (SAFE-T) and MIP-DILI (Mechanism Based Integrated Systems for the Prediction of Drug Induced Liver Injury) consortia. The integrated use of these biomarkers from clinical-preclinical-in vitro translational studies will be discussed in the context of understanding fundamental in vitro mechanistic and predictive drug safety science.

S-4.
Application of hepatocyte-like cells to predictive toxicology

David Hay

University of Edinburgh, UK

The development of renewable human liver models, from genetically defined origins, is likely to be a game-changing addition to the field. However, for these models to have significant impact they must be stable in character and capable of manu-

facture at scale. For this reason, we have chosen to use pluripotent stem cells (PSCs). We have developed highly efficient, scalable, and reliable differentiation procedures for use with PSCs, which demonstrate very promising qualities. In collaboration with industry and academic laboratories, we provide proof of concept that stem cell derived hepatocytes metabolise drugs, possess an intact innate immune system and are capable of predicting human drug-induced liver injury, in line with current gold standard human models. Our most recent advances will be discussed at the meeting.

S-5.
Inhalation toxicology – Regulatory aspects

Kristina Ulrich

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New chemical entities being developed by the inhalation route provide unique technical challenges to the inhalation toxicologist. The non-clinical data requirements to support clinical trials and marketing authorisation applications (MAA), with regards to pharmacology, toxicokinetics and toxicology, are generally the same as those using routine routes of administration. The speaker will discuss Organisation for Economic Co-operation and Development (OECD) test guidelines and International Conference on Harmonisation (ICH) guidance in relation to non-clinical development of inhaled compounds for pharmaceutical development as well as Good Laboratory Practice (GLP). The use of available in vitro methodologies to support clinical trials and MAA applications will also be considered.

S-6.
A model next-level *in vitro* strategy to assess the inhalatory hazard of nanofibres

Martin J. D. Clift, Carola Endes, Savvina Chortarea, Alke Petri-Fink and Barbara Rothen-Rutishauser

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In the field of inhalation toxicology there is a significant lack of representative models that embody the notion of reproducible and effective alternative *in vitro* test systems that adequately mimic invasive *in vivo* testing strategies[1,2]. This bottleneck is an increasing problem within the discipline of nanotoxicology, where the objective is to determine the hazard (and eventual risk) that nano-sized materials (1–100 nm)[3] pose towards human health. Either routinely manufactured for a wide-array of human-based applications (*e.g.* medicine, cosmetics), or accidently released into the atmosphere (*e.g.* bonfires, car exhaust), determining the impact of an inhalatory exposure to nano-sized materials is of a fundamental research impetus towards understanding their human health effects.

Through combining a 3D multi-cellular system, representing the alveolar epithelial barrier[4], with an air-liquid interface cell exposure system[5] it has been possible to conduct realistic exposures, as well as subsequently assess the inhalatory hazard of nanomaterials, with a focus upon nanofibers[6,7]. In addition to using this sophisticated *in vitro* method, state-of-the-art microscopy techniques combined with both gene- and protein-based biochemical methods[8] have enabled a holistic determination of the biological impact of nanomaterials. Based upon this approach, it has further been possible to correlate the exposure levels with those towards humans (*i.e.* life-time exposures)[9] within an occupational scenario, suggestive of the applicability of this system towards mimicking the *in vivo* scenario. In summary, despite improvements towards closing the gap between *in vitro* and *in vivo* still required, a significant foundation has been built towards an advantageous alternative *in vitro* test system.

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S-7.
Assessment of pulmonary toxicity of nanomaterials using in vitro test methods and the DF4nanoGrouping decision-making framework

Ursula G. Sauer

Consultant to European Centre for the Ecotoxicology and Toxicology of Chemicals (ECETOC; Brussels, Belgium) Task Force Nanomaterials (Chair: Dr. Robert Landsiedel, BASF SE; Ludwigshafen, Germany) presenting results of BASF SE studies and the ECETOC Task Force Nanomaterials

The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) 'Nano Task Force' proposes a decision-making framework for the grouping and testing of nanomaterials (DF4nanoGrouping) consisting of 3 tiers to assign nanomaterials to 4 main groups (with possible further sub-grouping) and to refine specific information needs. The DF4nanoGrouping covers all relevant aspects of a nanomaterial's life cycle and biological pathways: intrinsic material and system-dependent properties, biopersistence, uptake and biodistribution, and cellular and apical toxic effects. Use, release and exposure route may be applied as 'qualifiers' to determine if, e.g., nanomaterials cannot be released from products, which may justify waiving of testing. The four main groups encompass (1) soluble, (2) biopersistent high aspect ratio, (3) passive, and (4) active nanomaterials. The DF4nanoGrouping foresees a stepwise evaluation of nanomaterial properties and effects with increasing biological complexity: as necessary, intrinsic material properties, system-dependent properties (e.g. surface reactivity and dispersibility that are dependent upon the nanomaterial's respective surroundings, such as culture media or lung lining fluid) and *in vitro* effects are assessed. *In vitro* cytotoxicity testing (preferably using alveolar macrophages as relevant *in vitro* test system for inhalation exposure) plays a central role in determining nanomaterial functionalities. The DF4nanoGrouping facilitates grouping and targeted testing of nanomaterials. It ensures that sufficient data for grouping

and ultimately risk assessment of a nanomaterial are available, and it fosters the use of non-animal methods. At the same time, no studies are performed that do not provide crucial data. Thereby, the DF4nanoGrouping serves to save both animals and resources.

S-8.
Precision cut lung slices for inhalation toxicology

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Advances in translational medicine need predictive human test systems. Precision cut lung slices (PCLS) display such a suitable *ex vivo* tissue model that maintains microanatomy and functionality of the respiratory tract. The model allows the investigation of effects of compounds and drugs directly on cytokine release and functional responses such as bronchoconstriction under similar experimental conditions in different species including man. The tissue can be stimulated with e.g. chemicals, lipopolysaccharides, bronchoconstricting agents and disease-related proteins as well as gases and aerosols such as cigarette smoke. By this, different features of diseases such as asthma, COPD, and lung injury can be investigated. Depending on the underlying immunology, lipopolysaccharides and proteins such as IL-13 induce an acute increase of pro-inflammatory cytokines and/or airway hyperresponsiveness. Effects of chemicals were shown to correlate with *in vivo* inhalation toxicity studies. In addition, the method shows comparable results in different labs as recently shown in a prevalidation study. In summary, different tissue responses can be used for the prediction of toxicological endpoints and adverse health outcomes such as organ injury, respiratory sensitization and inflammation. The presentation will give an overview about the current use of lung tissue in inhalation toxicity.

S-9.
Developmental and reproductive toxicology – the regulatory system

Mary Moxon

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Currently, chemicals are tested for potential effects on developmental and reproductive toxicity with in vivo studies conducted according to regulatory guidelines. The tests provide general information concerning the effects on the integrity and performance of the male and female reproductive systems, including gonadal function, the oestrous cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning, and on the viability, growth and development of the offspring. More recently, the list of endpoints has been extended to include development of the nervous system, immune system and the potential for endocrine disruption. The studies are expensive to run and despite the comprehensive list of endpoints, interpretation of the results is not always straightforward. Observation of parental toxicity is generally required by the guideline to demonstrate the adequacy of the test system. The challenge can then be to determine if the effects seen in the offspring are consequential or a direct effect of the test chemical. *In vitro* tests do not have a role in the current regulatory testing strategy although they may be used to aid initial chemical selection or to provide mechanistic information. What can they offer to help clarify the *in vivo* data bearing in mind that DART is a complex area which attracts strong opinion and emotion that sometimes clouds the science?

S-10.
In vitro techniques for the prediction of developmental toxicology

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In vitro systems, as pre-screens or validated alternatives, appear to be useful tools to reduce the number of whole animals used or refine procedures and hence decrease the cost for the chemical and drug industry. Three validated *in vitro* systems exist for developmental toxicity/embryotoxicity testing: whole embryo culture (WEC), rat limb bud micromass (MM) and the embryonic stem cell test (EST). Results will be reported for such systems with various well-known human teratogens, both in line with the validated method, and as variations. Also in our laboratory, a relatively novel chick heart micromass (MM) culture system has been tested and compared to the validated D3 ESC test. With an avian species, sacrifice of the mother is unnecessary, the eggs are easy to store and handle, and it is easy to control the development and growth of the embryo. A modified protocol based on Atterwill et al., (1992) (mid brain MM culture) and Wiger et al., (1988) (limb bud MM culture) allows the investigation of cytotoxicity and the effects on cellular differentiation of test chemicals. The chick heart MM culture system correctly classified several model human teratogenic molecules and pairs of test chemical analogues. The heart MM culture was also used to evaluate antiepileptic and antidepressant drugs as molecules which have the potential to inhibit embryonic heart cell differentiation, without inducing cytotoxicity. The results of the heart MM culture system were found to be comparable with the D3 mouse ESC system.

S-11.
Towards the *in silico* prediction of teratogenicity: A novel semi-automated mechanistic expert call approach to creating purposeful datasets

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Oestrogen receptor (ER) binding, as a molecular initiating event (MIE), is of relevance to teratogenicity. Using ChEMBL data, we applied a semi-automated process to generate a mechanistic expert call dataset. As proprietary or publically available datasets are often inconsistently collated in various formats, data mining to define potential toxicophores can be quite challenging. This novel semi-automated approach circumvents this problem.

The ChEMBL ER data was first analysed by a human expert and the *in vitro*/*in vivo* data was categorised as binding, agonist, antagonist and mixed agonist. An expert call for activity was then assigned using threshold rules to classify a compound as active, weakly active, equivocal or inactive. This outcome was

then compared to a semi-automated process using KNIME, with analogous threshold rules applied, followed by expert intervention for data points for which the automated system failed to generate an expert call.

The semi-automated expert calls, which were completed in 5 days, compared to 3 weeks for the manual approach, agreed with the human expert calls for 93% of the 6956 chemicals based on a conservative approach for all combined classified calls.

As a proof of concept, the complex nuclear receptor binding/activation data was successfully transformed into a purposeful dataset. This methodology can be extended to other MIEs, including enzyme inhibition. MIE datasets created this way have useful applications, including faster structural alerts development in a transparent *in silico* system like Derek Nexus, for insertion in a purposeful database, to support adverse outcome pathway frameworks and for model building.

S-12.
The neurosphere assay for developmental neurotoxicity testing

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The neurosphere assay is a model for developmental neurotoxicity screening based on human and rat neural progenitor cells. It enables the detection of disturbances in basic processes of brain development, such as proliferation, migration, differentiation and apoptosis, and the distinction of these specific disturbances from general cytotoxicity. Furthermore, the comparison of human and rat data provides useful insights into species differences for toxicodynamics of compounds contributing to human risk assessment of developmental neurotoxicants. A concrete example of application of the neurosphere assay on the risk assessment of epigallocatechin gallate (EGCG) will be presented within the Adverse Outcome Pathways (AOP) framework. By studying the effects of EGCG, we found a relevant molecular initiating event: binding of EGCG to laminin causing the key event of disturbance of β1-integrin function, leading to decreased adhesion and migration of neural progenitor cells. We propose that developmental neurotoxicity can easily be tested in human neural progenitor cells for recognizing potential human developmental neurotoxic compounds, which thus helps to improve regulatory assessment.

S-13.
The concept of an adverse outcome pathway (AOP) applied to regulatory neurotoxicity evaluation

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To support a paradigm shift in regulatory toxicology testing and risk assessment, the Adverse Outcome Pathway (AOP) concept has recently been applied to different organ toxicity evaluations, including neurotoxicity. AOPs provide a description of causal rationales for qualitative and quantitative predictive modeling of the human adverse outcome that result from chemical triggering of a molecular initiating event, followed by key events at the cellular and organ level for which testing methods, including high-throughput methods, can be developed.

There are a large number of cellular and molecular processes known to be critical to proper brain development, maturation and function. However, comprehensive understanding of pathways leading from chemical exposure to an adverse outcome in the central nervous system is sparse. This has hampered both the judgment of the predictive ability, as well as the regulatory use of in vitro data. Lately, there has been an attempt to organize the available scientific knowledge in the field of toxicity (including neurotoxicity) by creating AOPs and making them publicly available in a knowledge base (https://aopkb.org).

During this talk, an introduction to the AOP concept and the challenges to apply this framework for developmental neurotoxicity (DNT) testing, followed by an example of an AOP specific for DNT entitled *''Binding of antagonist to NMDA receptors during brain development (synaptogenesis) induces impairment of learning and memory abilities''* will be presented.

Moreover, across the range of potential DNT AOPs common key events will be presented, which could help towards the development and/or interpretation of strategies for Integrated Approaches to Testing and Assessment (IATA) in the field of DNT. This potential application of AOPs related to DNT will increase the use of data derived from in vitro assays and the possibility of correctly identifying potential developmental neurotoxicants, even if toxicity is mediated by various pathways.

S-14.
Studying the blood-brain barrier on a microfluidic chip

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A realistic model of the blood-brain barrier (BBB) is valuable to perform drug screening experiments and to improve the understanding of the barrier's physiology at normal and pathological conditions. Although the conventional in vitro systems (e.g. Transwell systems) have been used for this, they lack reproducibility and have a static environment. To overcome these disadvantages so called ''organs-on-chips'' have been developed, which use microfluidics and (human) cells to mimic organ function.

An example of the BBB chip is shown in the work of Griep et al., where human cerebral endothelial cells (hCMEC/D3) were cultured in a microfluidic device made of polydimethyl siloxane (PDMS). Recently we improved this model. Two PDMS parts

with microchannels are placed on top of each other, with a porous membrane in between at the intersection serving as scaffold for the cells. hCMEC/D3 cells (kindly provided by INSERM, Paris, France) were cultured in the chip for up to 15 days. With the four integrated electrodes, which did not block view on the intersection, reliable transendothelial electrical resistance measurements were carried out. Additionally, using immunohistochemistry it was shown that the endothelium expressed tight junction proteins, which is an essential characteristic of the BBB.

To further improve the physiological relevance of this promising platform, the cells inside the channels will be cultured under fluid flow. As application, this platform will be used to study the transport of nanocarriers with Alzheimer medication through the BBB. In addition, the clearance of Alzheimer-associated proteins (amyloid β) by the BBB can be examined.

S-15.
It's all about transport - prediction of blood-brain barrier permeation and *in silico* safety assessment

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With an increasing understanding of the function and physiological role of ABC-transporters, their major contribution to bioavailability, brain permeation, and clearance of drug candidates became evident. P-glycoprotein (P-gp), the paradigm transporter in the field, has been discovered more than 30 years ago as being responsible for multiple drug resistance in tumor cells and for low blood-brain barrier permeability of a large range of compounds. Thus, designing in and designing out substrate properties comprises a hot topic and – due to the polyspecificity of the transporter – also a major challenge for medicinal chemists. Recent progress in the structural biology of ABC-transporter paved the way for the application of structure-based design methods. The combination with ligand-based *in silico* methods derived from the Open PHACTS Discovery Platform allowed to identify molecular features driving drug-transporter interaction. This experimental data guided docking approach was also successfully applied to selected neurotransmitter transporters, such as SERT, DAT, and GAT1.

Successful case studies of this integrated modeling approach comprise ligand- and structure-based design studies on inhibitors of P-glycoprotein as well as ligands of neurotransmitter transporters. Furthermore, mining public data bases allowed the creation of predictive classification models for transporters, as well as the prediction of neuro- and hepatotoxicity based on ligand-receptor interaction profiles.

Acknowledgments

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S-16.
Microscopic evaluation of in vitro neuronal networks to assess synaptic connectivity and synaptotoxicity

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During brain development, orchestrated activation of genetic programs, as well as spontaneous electrical activity guide the correct wiring of neuronal networks. Mature neuronal networks are characterized by the expression of synaptic markers, synchronized electrical activity and the presence of dendritic spines, tiny protrusions from the dendritic shaft that compartmentalize single synapses to ensure optimal regulation of synaptic strength.

We established an *in vitro* model based on primary hippocampal neurons that recapitulates features of mature neuronal networks. We optimized a set of microscopy workflows to quantitatively characterize both morphological (neurite outgrowth, synapse density, dendritic spine density) and functional (spontaneous electrical activity) aspects of the established neuronal networks.

Using calcium imaging, we pinpointed a critical period in which stochastic activity of individual neurons turned into robust, synchronized network activity, indicative of the formation of functional synapses. This synchronization coincided with an increase in neurite outgrowth and synapse density, while dendritic spine density increased mainly after synchronization of the activity, suggesting that the latter enables fine-tuning of synaptic connections. We further showed that synchronized network activity is mediated by the NMDA receptor, and that interference with microtubule stability alters the bursting pattern.

In brief, we have established a robust platform for pharmacological and/or genetic interrogation of synaptic connectivity in *in vitro* neuronal networks. Our approach is easily amenable to upscaling, which makes it an attractive model for high content synaptotoxicity screening.

Poster Abstracts

P-1.
Antioxidant and cytoprotective activities of phytochemicals against free radicals

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Accumulation of reactive oxygen species (ROS) induces oxidative stress, which plays a causative role in disease conditions such as non-alcoholic steatohepatitis (NASH). Even though most cells possess cytoprotective mechanisms, persistent exposure to high ROS levels depletes cellular antioxidant mechanisms, augmenting cell damage. Hence therapeutic focus has shifted to exogenous antioxidants, with keen interest on those of plant origin.

Antiradical properties of quercetin, curcumin, sulforaphane and rosmarinic acid (RA), and key RA metabolites - caffeic acid (CA), ferulic acid (FA), m-coumaric acid (m-CoA) and 3,4-dihydroxyphenyllactic acid (DPLA) were investigated against free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in a non-cellular assay. Hepatoprotection against 0.5 mM tert-butyl hydroperoxide (tBHP) was evaluated in human HepG2 cells via neutral red uptake, after 20 hours pre-exposure and 5 hours co-exposure with phytochemicals. DMSO and methanol were used as negative controls.

Caffeic acid, 3,4-dihydroxyphenyllactic acid and rosmarinic acid were equi-potent with positive control, quercetin (mean EC50: 0.21 ± 0.01 mM), but were stronger DPPH radical scavengers than curcumin (mean EC50: 0.39 ± 0.03 mM) and ferulic acid (mean EC50: 0.78 ± 0.13 mM) in decreasing order. Sulforaphane and m-coumaric acid were ineffective against DPPH and tBHP (5 hr co-exposure).

In HepG2 cells, cytoprotection against tBHP (5 hr co-exposure) indicated quercetin > curcumin > caffeic acid > rosmarinic acid in order of decreasing potencies, while curcumin was more potent than quercetin and sulforaphane in 20 hr pre-exposure experiments. With the exception of caffeic acid, all RA metabolites used in this study were ineffective against 0.5 mM tBHP. However, antioxidant activities of curcumin and sulforaphane could be hindered by their toxicities at high concentrations, which could be modulated via apoptotic mechanisms. Although physicochemical properties of radical scavengers hinder their cytoprotective activities, quercetin, curcumin, CA and RA could act as direct and indirect phyto-antioxidants against oxidative damage. Quercetin proved to be an effective positive control for evaluating antioxidant activities of phytochemicals in both cellular and non-cellular models. Hence this study demonstrates the efficiency of in vitro models in assessing toxicities and antioxidant properties of plant-derived chemicals.

P-2.
Elucidating the mechanism of Aag-dependent cell death

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DNA damage results from exposure to endogenous and environmental genotoxic agents such as alkylating agents. Base excision repair (BER), initiated by an enzyme called alkyladenine DNA glycosylase (AAG), is a DNA repair process acting on alkylation base damage.

AAG activity on alkylated DNA bases generates abasic sites (AP sites), which are further processed to form single strand breaks (SSBs). These BER intermediates are very toxic to the cell if not repaired. Since BER is a multistep repair process, any imbalance in the pathway can lead to accumulation of these toxic intermediates and potentially trigger cell death via a mechanism involving hyperactivation of poly (ADP-ribose) polymerase 1 (PARP1), an enzyme that uses energy in the form of NAD+ to coordinate BER.

In our study, we seek to elucidate the mechanism of Aag-dependent cell death by using Aag proficient and deficient cells and analyzing their response after treatment with an alkylating agent. The temporal changes in BER intermediate incidence were characterised. In addition, intracellular levels of NAD+ and ATP were measured and the temporal changes in levels of NAD+ and ATP were characterised.

The results show that the kinetics and amount of BER intermediate formation is different in Aag proficient versus Aag deficient cells. Also, we found that while intracellular levels of NAD+ and ATP are depleted both in alkylation treated Aag proficient and deficient cells, the kinetics and magnitude of this depletion varies with genotype. These results will be used to build a mathematical model to explain AAG-mediated alkylation-induced cell death.

P-3.
Human neural stem cells as a model to predict the neurotoxicity of methyl mercury

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The neurotoxicity of methyl mercury (MeHg) is well documented both in human and experimental animals. In the adult, MeHg poisoning causes damage to the visual cortex, cerebel-

lum, and myelin disruption to the peripheral nerves. While in the foetus, the immature nervous system is extremely sensitive to it and causes a diffuse and wide damage which result in cerebral palsy, deafness, and blindness. Human neural stem cells have been proposed to detect the neurotoxicity of MeHg in vitro, since these cells have the ability to divide, migrate, and differentiate into other cells. Therefore, this might to some extent recapitulate the processes involved in the development of the human nervous system. These cells were in passage number 20–25; they were seeded in 48 well plates which were coated with Poly-D-Lysine and Laminin. MeHg was added in different concentrations to the media. After 6 days, the resazurin test and kenacid blue assay were performed to study the effect of it on cell viability and total cellular proteins respectively. For Immunocytochemistry, the cells were seeded into 8 well chamber slides which have the same double coating. They were stained with Tubulin and GFAP antibodies to measure the length of neuronal processes by using Volocity software version 6.3.1. 1*104 cells were seeded in 2 ultra-low attachment 96 well plates, one for measuring the neurosphere sizes and the neurospheres in the other plate were transferred into coated 48 well plate to study the migration distance. It appears that MeHg affects the cell viability at the doses $>5\,\mu$M while its effect on total protein amount was clear at the doses $>2.5\,\mu$M. Regarding the other end points, cell migration, neurite length, and neurosphere sizes were significantly reduced at $>2.5\,\mu$M. These results agree with the previous finding of the effect of this chemical on rat cerebellar granular cells.

P-4.
Death of an epithelium: ketamine-induced apoptosis in human bladder epithelium in vitro

Simon C. Baker and Jennifer Southgate

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Since 2007, the recreational abuse of ketamine has been increasingly associated with an emergent bladder pain syndrome, characterised by chronic inflammation and ulceration of the bladder epithelial (urothelial) lining. This study investigated the mechanism of ketamine-induced bladder damage using normal (non-transformed) human urothelium maintained in organ culture or as finite cell lines in vitro.

Cultured urothelial cells exposed to ≤ 1 mM ketamine (non-cytotoxic) showed a rapid release of ATP, that activated purinergic P2Y receptors and stimulated the inositol trisphosphate receptor to provoke transient release of calcium from the endoplasmic reticulum into the cytosol. Cytotoxic ketamine concentrations (>1 mM) provoked a larger-amplitude and unresolving increase in cytosolic $[Ca^{2+}]$. This continued elevation of cytosolic $[Ca^{2+}]$ was associated with decreased mitochondrial oxygen consumption and a consequent reduction in cellular ATP which may contribute to the initiation of apoptosis.

Exposure of urothelial cells and organ cultures to ketamine resulted in apoptosis, characterised by mitochondrial cytochrome c release and caspase activation. Damage to the urinary barrier is thought to initiate bladder pain and this study suggests urothelial loss through apoptosis might explain the loss of urothelium observed histologically in ketamine-induced cystitis patients. This study is the first to provide evidence for the mechanism by which ketamine drives the intrinsic apoptotic pathway in urothelial cells.

P-5.
Manipulation of in vitro models to assess differential mitochondrial toxicity between structurally-related compounds

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Drug-induced mitochondrial toxicity is implicated in the aetiology of idiosyncratic adverse drug reactions, yet current models lack clinical applicability. In particular, the lack of reliance of carcinoma-derived cell models on oxidative phosphorylation when cultured in glucose media confers resistance to mitotoxicants. It was therefore hypothesised that the galactose-conditioning of cells facilitates the identification of direct mitochondrial toxicants.

A panel of compounds associated with mitochondrial toxicity, alongside structurally-related negative compounds; flutamide, 2-hydroxyflutamide, bicalutamide, tolcapone and entacapone were applied to HeLa cells in glucose or galactose media (2 h). Cell viability (MTT assay) and intracellular ATP content assessment were used to identify mitotoxicants by comparison of IC50 values. Subsequently, the mechanism of mitochondrial toxicity was investigated using Seahorse XFe96 extracellular flux analysis in intact and permeabilised cells.

Significant differences in intracellular ATP content between glucose and galactose-conditioned cells were produced by tolcapone (glucose; $106.6 \pm 3.7\%$, galactose; $86.7 \pm 4.1\%$), flutamide (glucose; $97.3 \pm 10.1\%$, galactose; $41.10 \pm 7.6\%$) and its primary metabolite, 2-hydroxyflutamide (glucose; $87.3 \pm 7.5\%$, galactose; $64.60 \pm 4.5\%$) at $100\,\mu$M, suggesting direct mitochondrial toxicity. This observation was supported by Seahorse analysis which demonstrated significant fold changes in basal respiration following $100\,\mu$M compound exposure (tolcapone; -0.75 ± 0.07, flutamide; -0.65 ± 0.07, 2-hydroxyflutamide; -0.78 ± 0.14). Further to this, differences were also noted in parameters including proton leak, spare respiratory capacity and ATP-linked respiration. In permeabilised cells, 2-hydroxyflutamide, as well as flutamide was found to be a potent inhibitor of respiratory complex I. Overall, this study has demonstrated the utility of the galactose-conditioning of cells in combination with Seahorse analysis in investigating mechanisms of mitochondrial toxicity.

P-6.
Study of mitochondrial and glycolytic activity in vitro may aid differentiation between genotoxic and non-genotoxic carcinogens

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Perturbations of cellular metabolism are increasingly being recognized as a hallmark of cancer. Such associations with

carcinogenicity suggest that altered cell metabolism could be a powerful indicator of neoplastic transformation. The present study's objective was to establish how carcinogens influence bioenergetic endpoints and whether these data correlated with genotoxicity (DNA damage) induction. The human lymphoblastoid cell lines TK6 and MCL5 were treated with test chemicals for 4 h or 23 h. The In Vitro Micronucleus Assay coupled to the semi-automated MetaSystems Metafer Slide Scanning Platform was used to obtain DNA damage dose-responses. The Seahorse XFe Analyzer was then used to measure the effects of carcinogens on two major metabolic pathways: glycolysis and mitochondrial metabolism. Oxygen consumption rate (OCR) represented mitochondrial function, whereas extracellular acidification rate (ECAR) indicated levels of glycolytic activity. Positive micronucleus dose-responses were obtained for acetaldehyde, benzo[a]pyrene, cadmium chloride, hydrogen peroxide and methyl methanesulfonate. Negative responses for micronucleus induction were observed for bis(2-ethylhexyl) pthalate, nickel (II) chloride, quinacrine, methyl carbamate and urethane. Bioenergetics analysis revealed some chemical-dependent changes in cell metabolism. Interestingly, some non-genotoxic agents appeared to reduce mitochondrial activity specifically. In conclusion, bioenergetic analysis could prove important for distinguishing between genotoxic and non-genotoxic carcinogens in in vitro carcinogenicity testing.

P-7.
Prototype risk assessment for biopersistents using AOP approaches

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The traditional safety risk assessment of inhalable biopersistent materials relies on estimation of the consumer lung exposure coupled with generation of in vivo inhalation toxicity data. Our aim is to replace the need for in vivo studies using a combination of exposure-based waiving and an understanding of the Adverse Outcome Pathways (AOP) for key events in lung responses to fibrosis. The AOP framework includes exposure, transport to the alveoli, and initiation of gene activation/protein production/altered signalling, inflammation/cell proliferation/ tissue remodelling and lung fibrosis and cancer. We are exploring the use of this AOP framework for risk assessment using specific examples of inhalable biopersistent materials. By understanding the multistage pathogenesis of well-studied human lung disorders induced by model biopersistents, early markers of pre-disease in in vitro systems can be determined for use in consumer safety risk assessments for the use of novel biopersistent materials.

P-8.
Development and characterization of a functional 3D liver spheroid model

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More predictive in vitro liver models are a critical requirement for preclinical screening of compounds demonstrating hepatotoxic liability. 3D liver spheroids have been shown to have an enhanced functional lifespan compared to 2D monocultures, however the analyses of spatiotemporal function and structure of spheroids has received little attention. We have developed and characterized the structure and function of a 3D liver spheroid model formed from C3A hepatoma cells. Spheroids maintained a compact in vivo-like structure and steadily proliferated, with no necrotic core, for up to 32 days. MRP2 and Pgp transporters had polarised expression on the canalicular membrane of cells in the spheroids, with MRP2 able to functionally transport CMFDA substrate into these canalicular structures. Spheroids were able to stably synthesise and secrete albumin and urea. Penetration of doxorubicin throughout the spheroid core was also demonstrated. Dose-dependent toxicity occurred in response to model hepatotoxins, with spheroids showing increased susceptibility to diclofenac than 2D monolayer cultures, with an IC50 of 270 μM and 370 μM respectively. We have successfully optimised a method for creating C3A liver spheroids and demonstrated cell polarisation, liver-specific functionality and toxicological response, confirming a more in vivo-like liver model when compared to standard 2D liver models.

P-9.
Spheroids for the detection of hepatotoxicity

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Hepatotoxicity is the second leading cause of drug attrition during drug discovery at AstraZeneca (AZ). Multiple mechanisms lead to drug induced liver injury (DILI), which are often unable to be recapitulated in simple in vitro models. Therefore, the development of complex and biologically relevant surrogate liver microtissue screens is under much research focus. Deployment early in the drug discovery process may better identify potential hepatotoxic compounds enabling selection of lead compounds with fewer hepatotoxicity 'flags' and enabling intervention to ameliorate this undesirable property. Spheroids (3D cell colonies), consisting of the liver derived cell line HepG2 (clone C3a) and genetically modified primary hepatocytes (Upcytes®) were formed in ultra-low attachment plates and characterized for their potential to detect hepatotoxic and non-hepatotoxic compounds. Cell seeding densities, volumes and incubation times were optimized to derive spheroids using both cell systems. These spheroids were characterized for their size (imaging), basic histology (H&E and pHH-3 mitotic staining) and cytochrome P450 (CYP) functional activity over 5–11 days in culture. In addition, ATP depletion as an indicator of cytotoxicity was determined following repeat dosing of 16 marketed drugs with known hepatotoxicity profiles. The rank order of hepatotoxic compounds in HepG2 (C3a) and Upcyte® spheroids was derived and compared to results obtained using the hanging drop primary hepatocyte system (InSphero®). Upcyte hepatocyte spheroids did not give any significant advantage over spheroids consisting of HepG2 (C3a) cells under the experimental conditions in this study. Classification of the compounds ranked by IC50 values for the HepG2 (C3a) cells (IC50 < 35; 35–100 and >100 uM) showed a close alignment to

the primary human liver microtissues (InSphero®). Preliminary analysis of CYP expression, assessed by detecting metabolite formation following incubation with probe substrates for CYP 3A4, 2D6, 2C9, 1A2 and 2C19, showed a progression away from baseline to an increase in activity from day 7 to 11 in both the HepG2 (C3a) and Upcyte® spheroids for 4 out of the 5 CYPs. Overall, this data suggests the 3D structural arrangement of the HepG2 (C3a) and Upcyte® cells as spheroids may be important to improve their functional hepatic competence and repertoire. This will enable cost effective and predictive hepatoliability screening to meet the demand from AZ drug discovery projects.

P-10.
The effect of the first- and second-generation of antipsychotic drugs on SH-SY5Y brain cells and their toxicity

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Antipsychotic drugs are primarily used to manage several psychiatric disorders, including schizophrenia, bipolar mania, and related mental illnesses. The present study examined the effect of the first and second generation of antipsychotic drugs on neuronal and non-neuronal cells. The toxicity of both-generation of antipsychotics was tested in both the SH-SY5Y brain cell line and the COS7 kidney cell line. According to the LC50 values for chlorpromazine (1st generation), trifluoperazine (1st generation) and olanzapine (2nd generation), the neurotoxicity of the two classes in SH-SY5Y exceeded their common cytotoxicity in COS7 cells, indicating that neuronal cells are at greater risk of cell death with low concentrations of antipsychotics at micro molar comparing to non-neuronal cells. Detailed studies looking at the mechanisms of cell death induced by these antipsychotic drugs indicate that both apoptosis and necrosis play a role, while autophagy does not.

P-11.
Biogelx Ltd: Designer gels for cell culture

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Biogelx Limited is a biomaterials company that designs tuneable peptide hydrogels, offering artificial tissue environments to cell biologists for a range of cell culture applications.

The hydrogels are highly tuneable, cell-matched biomaterials, capable of revolutionising the way cell biologists control and manipulate cell behaviour in the laboratory. This is of direct relevance to fundamental cell research, including the study of stem cells and disease models within academic and medical labs. However, the major commercial significance this has is the dramatic impact on the development of cell-based assays and drug discovery/toxicology platforms within large pharmaceutical companies, representing a rapidly growing global market with revenues of over $5 billion per annum.

Biogelx offers a range of hydrogel platforms that are three dimensional (3D), 99% water and have the same nanoscale matrix structure as human tissue (see figure below). This gives control back to the cell biologist, as the gels can be tuned to meet the needs of any given cell type.

This poster will discuss the underlying chemistry of Biogelx's peptide hydrogels, highlighting the range of chemical and mechanical modifications that can be implemented within the gels, in order to address a wide range of cell based applications. Some examples of academic and industrial collaborative work shall also be presented. Finally, the company's progress from research-based technology through to commercial entry into high value markets will be discussed, highlighting Biogelx's current successes and future aspirations.

P-12.
A pH dependent in silico model of human liver glycolysis

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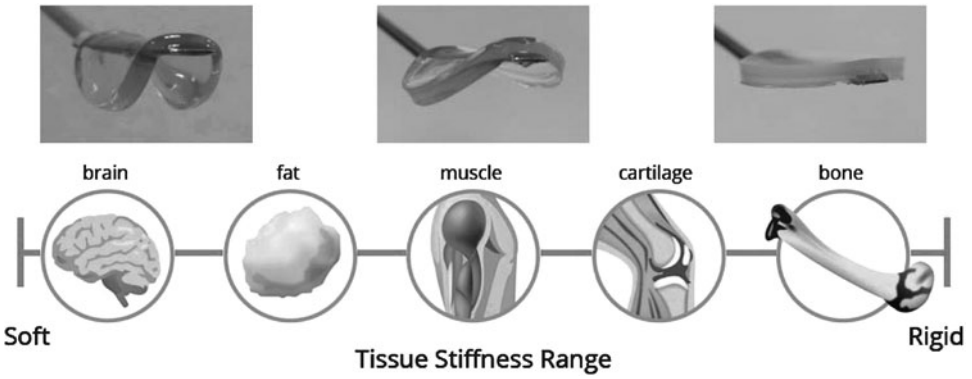
Drug induced mitochondrial dysfunction is an understated mechanism of toxicity responsible for the withdrawal of several drugs from the market. Recently, off-target toxicities of this nature have become more widely acknowledged, with more effort being assigned to better understanding the underlying mechanisms of mitochondrial toxicity. Unfortunately, current methods have poor predicative capabilities due to 1) outdated tools, 2) discordance between animal models.

The mitochondria are an extremely complex system and some insight into these complexities has been gained recently by in silico models of mitochondrial bioenergetics. However, a comprehensive model that incorporates oxidative phosphorylation,



Tricarboxylic acid cycle, ion transport as well as glycolysis has yet to be constructed for the purpose of assessing mitochondrial toxicity. Furthermore, the inclusion of dynamic buffering of protons and metal cation binding followed by resulting rapid conversion of biochemical species has also yet to be modelled.

In this study, we have constructed an in silico model of human liver glycolysis, incorporating pH-dependent enzyme kinetics and reaction equilibria to compute the time course of pH changes. The model can be coupled with extracellular acidification (ECAR) data from extracellular bioenergetic flux analysis.

This model is the first step to dynamically modelling cellular bioenergetics that is able to quantify proton uptake and release by all biochemical reactions in the metabolic pathway and the effects of the resultant pH change on thermodynamics and reaction kinetics; a necessary feature to assess mitochondrial toxicity.

P-13.
Assessing the vacuolated (foamy) macrophage phenotype using a high content screening approach

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Introduction: The vacuolated 'foamy' macrophage (FM) appearance is common to cells undergoing different drug-induced processes, including phospholipidosis, apoptosis, and autophagy. We developed an in vitro screen to identify FM induction and differentiate between these phenotypes using pharmaceutically-relevant inducers: amiodarone (phospholipidosis), staurosporine (apoptosis) and polyvinyl acetate nanoparticles (PVA-NP; induces vacuolation by an unknown mechanism).

Methods: A mouse macrophage cell line, J774.1, was exposed to 3 compounds – amiodarone, staurosporine and PVA NP for varying duration (24, 48 and 72 h). Post exposure the cells were imaged in 96-well plates using an IN Cell Analyzer (GE Healthcare) to measure cell size, nuclear size, number of vacuoles, vacuolar area and intensity of incorporated dye. To validate this assay a similar morphometric analysis was performed using transmission electron microscopy (TEM).

Results: Comparison of % vacuolar area per cell versus fluorescence intensity of LipidTox Red (phospholipid stain) distinguished between vacuolated cells with phospholipid accumulation (amiodarone treatment; 0.0003 – 20 μM; t = 24–72 h; n = 3 independent experiments) and vacuolated cells with no phospholipid accumulation (PVA NP treatment; 0.001 – 5 mg/ml; t = 24–72 h; n = 3 independent experiments). Treatment with staurosporine did not result in any accumulation of LipidTox Red, but induced a progressive increase in nuclear area with increasing dose and duration of treatment (staurosporine treatment; 0.00003 – 0.004 μM; t = 24–72 h; n = 3 independent experiments). These results were in agreement with the results obtained from TEM imaging.

Discussion/Conclusion: Although electron microscopy has been the standard methodology for morphometric analysis of the foamy macrophage phenotype, it is time consuming, costly, labour intensive, low throughput and unsuitable for testing libraries of compounds for hazard ranking. We have developed a 96-well plate in vitro high throughput screening (HTS) method for analysis of FM phenotype. This technique is a significant methodological advance which can screen for FM induction in vitro and enable differential phenotypes, dose-response and temporal responses to be probed.

P-14.
Establishing species-specific 3D liver microtissues for repeat-dose toxicology and advancing in vitro to in vivo translation through computational modelling

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Liver injury is a rising problem in the UK and in the US, e.g. over 50% of cases of acute liver failure (ALF) in the US are accounted for by adverse drug reactions (ADRs) (39% Acetaminophen).

Such toxicity issues account for $\sim$21% drug attrition during drug development, and liver toxicity is the most common reason for drug failure during the developmental process.

Current 2D in vitro model systems developed to assess ADRs and hepatotoxicity have a number of down falls and the emphasis on producing relevant and representative 3D models has therefore expanded in order to bridge the complexity gap between 2D systems and in vivo.

3D cultures of hepatocytes in vitro have recently emerged as an improved platform to recapitulate the in vivo liver structure and to maintain long-term hepatic functions as compared with conventional 2D cultures. In the present study, we constructed spheroids using the liquid-overlay technique in a multiwall format as an in vitro 3D model using freshly isolated primary rat hepatocytes. This method allows the formation of reproducible spheroids of varying cell seeding densities in 3 days and provides a high-throughput platform for investigation.

We have demonstrated that these primary rat spheroids display in vivo characteristics including direct cell-cell contacts, bile canaliculi formation, cellular polarisation and a 3D morphology.

Immunofluorescent analysis demonstrated formation of secondary structures throughout the hepatosphere.

However, histological analysis has raised questions with regards to potential storage issues within spheroids and whether or not they would be suitable for long-term repeat-dose investigations.

P-15.
Towards a zonated bioartificial liver for bridge treatment of liver failure and drug metabolism and toxicity assessment

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Liver disease is a major cause of death, with incidences increasing yearly. The demand for liver transplants is therefore outstripping supply. Bioartificial livers (BALs) could provide a bridge treatment to support patients on transplant waiting lists, while also having applications within the pharmaceutical industry for more accurate drug metabolism and toxicity testing. This work describes the use of hollow fibre bioreactors (HFB) to create zonated BALs for hepatocyte culture in vitro by mim-

icking the in vivo metabolic zonation of liver lobules. Zonation is caused by the gradient between oxygen-rich blood entering the lobule and depleted blood exiting it, and cell metabolism is affected by location along the gradient. Our aim is to increase the functionality of hepatocytes (typically low in traditional 2D culture) by culturing in a more in vivo-like environment.

The HFB consists of permeable fibres (acting as hepatic sinusoid capillaries) glued into a glass casing, with cells seeded on the outside of the fibres and media delivered through the fibre lumens. Using mathematical modelling the dimensions of the hollow fibre bioreactor and flow rates have been tuned to mimic the oxygen partial pressures seen in the liver lobule. Seeding conditions for HepG2/C3A cells have been optimised and the effect of media permeation flow rates during the growth period have been assessed. HFB input and output oxygen levels are monitored to ensure desired oxygen gradients are established and maintained. Metabolic zonation is assessed using molecular and biochemical assays. Cellular function in the HFB is measured by assessing albumin secretion and paracetamol clearance.

Cell seeding rates of $\sim$15% are routinely achieved without the addition of any attachment factors, and a low permeation flow rate was found to be beneficial to the growth rate. Following a 7 day growth period the cell density achieved in the HFB is equal to that of a 7 day routine culture on 2D tissue culture plastic. Using atmospheric oxygen growth conditions, both albumin secretion and paracetamol clearance were found to be elevated in the HFB compared to 2D cultures.

The HFB system is a versatile system in which cells can be seeded and grown to relevant numbers. Studies under atmospheric oxygen conditions indicate an increased functionality (albumin secretion and paracetamol clearance) of cells grown in the HFB compared to 2D. Oxygen zonation can be achieved in the HFB and analysis of metabolic zonation and cell function is underway.

P-16.
Simulating afterdepolarisations as a predictor of drug-induced arrhythmic risk

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A novel simulated afterdepolarisation-based marker for the prediction of drug-induced arrhythmias was created using mathematical electrophysiological cell models. Interventions to provoke afterdepolarisations were applied to the model, including: increasing L-type calcium current conductance; decreasing rapid delayed rectifier potassium current conductance; and shifting the voltage inactivation curve of the fast sodium current. The threshold levels of these interventions required to produce an afterdepolarisation were detected and recorded. There were significant inter-model differences between the thresholds obtained from different cell models. These interventions were combined with simulated drug block of ion channels and used to classify drugs into risk categories using linear discriminant analysis, based on clinical training data on drug-induced torsades incidence. The L-type calcium current conductance increase protocol combined with the ten Tusscher 2006 M cell model was the best predictor. Most of the protocols were more predictive than hERG-only risk markers, and some protocols were more accurate than the APD90 measure suggested by Mirams et al. (2011). These results indicate that simulating afterdepolarisation tendency has potential for use in the early stages of drug development as an improved marker for drug-induced arrhythmic risk.

P-17.
How many cell death pathways that Doxorubicin can affect HepG2 cells?

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Doxorubicin (DOX) is a potent antibiotic anti-cancer drug that is used either in isolation or in combination, for treating ovary, haematological, breast, stomach, liver, and prostate cancers. This drug has the ability to damage DNA and inhibit macromolecules (DNA and RNA) by producing free-radicals. Several studies have shown that DOX induces P53 activation leading to apoptosis in both normal and tumour cells, by causing cytochrome c release from the mitochondria which ultimately leads to apoptosis via caspase 3. We have investigated the molecular mechanisms of DOX induced hepatic cell death. This study shows that DOX can induce cell death in HepG2 cells through two different mechanisms. The use of caspase substrates and caspase inhibitors confirm that apoptosis through caspase 9 and caspase 3 are involved. Using HepG2 cells transfected with LC3-GFP, it was also noted that a high percentage of LC3-GFP punta were seen using fluorescence microscopy, following DOX treatment, which suggests that autophagy is also involved. However, lactate dehydrogenase release assays and the use of necrostatin, on DOX treated cells indicate that necrosis is unlikely to be involved.

P-18.
Assessment of embryotoxicity for 6-gingerol in chick embryonic cardiomyocytes and mouse embryonic stem cell-derived cardiomyocytes

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Despite the fact that there is lack of safety information for most herbs, there is growing trend of using herbal medicines as 'safe alternatives' for pregnant women, particularly during the organogenesis period of gestation. Morning sickness troubles up to 50% of pregnant women in the early stages of pregnancy. It is believed that ginger can relieve this condition; however, there are limited studies regarding its teratogenicity. 6-gingerol is the most abundant compound in the fresh rhizome of ginger (Zingiber officinale) (Zick et al, 2008). Many studies have examined the antiemetic effect of ginger being taken for morning sickness by pregnant mothers (Dante et al., 2014). In this study, two cardiomyocyte lineages were targeted during development. Firstly, the cardiac lineage which represents early embryonic heart cells but is anatomically underdeveloped (Bellairs et al., 1998), for which the chick embryonic cardiomyocyte micromass system has been used, (Ahir et al., 2011). Secondly, mouse embryonic stem cells were differentiated into cardiomyocytes by using the hanging drop technique. The potential effects of 6-gingerol were assessed by measuring contractile activity using a manual scoring system, resazurin reduction assay to measure cell activity in the cultures, kenacid blue to evaluate total protein content of the cultures and finally the dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay to measure reactive oxygen species for cell populations of both systems. By culturing embryonic cardiomyocytes in the micromass system with a wide range of 6-gingerol concentrations (0.75, 1.5, 3, 6, 12.5, 25, 50 and

100 μM), a significant ($P < 0.05$) decrease in morphological scoring system (contractile activity), cellular activity, protein content at concentrations (12.5–100 μM) and significant increase ($P < 0.05$) of reactive oxygen species (ROS) at (100 μM) was observed. However, the result for 6-gingerol-treated stem cells for the same range of concentrations showed a significant ($P < 0.05$) decline for the same parameters but starting from 1.5 μM up to 100 μM alongside a significant ($P < 0.05$) increase of ROS at both lowest (0.75 μM) and highest (100 μM) concentrations measured. Currently, ginger is already widely accepted for use during pregnancy, therefore it is important to recommend that pregnant mothers use this treatment with caution.

P-19.
Combinatorial approach to use of non-mammalian organisms to predict developmental and reproductive toxicity

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We exploit the combinatorial power of two genetically tractable eukaryotic model organisms, the nematode worm Caenorhabditis elegans and the social amoeba Dictyostelium discoideum, to determine the mode of action of drugs and identify developmental and reproductive toxicity of compounds. Model organisms have proved invaluable for drug mode of action studies and ellucidating pathways associated with disease states. Both C.elegans and Dictyostelium have rapid, well-defined multicellular developmental cycles involving pathways well-conserved with humans. However, no single model organism can act as a perfect surrogate for humans as some pathways will not be conserved and organism-specific effects will complicate analysis. Comparison of the effects of small molecules, or molecular pathway disruption, in two organisms greatly increases the predictive power of results. Robust effects of a small molecule in two organisms consistent with a common pathway target, for example, hugely increases the confidence of target prediction. Disruption of signalling pathways (either by pharmacological intervention or genetic manipulation) leads to scorable developmental aberrations or defined alterations in gene expression patterns. We are developing a library of markers for each organism for mode of action studies and libraries of mutants in both systems can be easily screened for defined phenotypes, or drug resistance. Comparison of genetic defects leading to increased sensitivity or resistance to a compound in two organisms confirms validity of targets and provides rapid mode of action identification. Loss or gain of function mutations can easily be generated in both organisms for genes of interest and comparison of phenotypic consequences. We have expertise in pathways involved in development and of alterations histone modifications and potential epigenetic effects in these organisms. Exploiting the combinatorial power of two non-animal models greatly increases the power of the analysis and has the potential to generate a rapid, effective predictive pipeline for adverse effects in mammals.

P-20.
New possibilities in mathematical toxicology

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Bayesian Networks have recently been advocated to predict hazard and potency class of chemicals in the context of skin sensitization by using only animal-free assays. We have used developments in probability and statistics to both simplify and generalize these initial results. Our new approach provides robust procedures for constructing Bayesian Networks, avoiding earlier pitfalls involving data imputation, loss of information and cross-validation while maintaining (or improving) the accuracy of hazard and potency prediction.

Importantly, our methods use Machine Learning, avoiding need for mechanistic knowledge of the biological processes in question. This gives us hope that Bayesian Networks may be successful in general toxicity prediction using only Machine Learning and animal-free assays. In this spirit preliminary results for liver carcinogenicity, where the underlying biological mechanisms are much less understood, will be discussed.

By extending this probabilistic thinking, we have also developed a rigorous framework to derive optimal integrated testing strategies for toxicity assessment using animal-free testing alone. We combine a population model (accounting for individual-level differences in exposure and in reaction to that exposure) with an explicit cost structure (including both testing and misclassification costs) to derive optimal integrated testing strategies based on the powerful mathematical machinery of Markov Decision Problems. It turns out that, even in the simplest set-ups, optimal policies turn are typically adaptive. In other words, our mathematics demonstrates that one-size-fits-all testing policies cannot possibly be optimal.

P-21.
Comparing the toxicity of brake abrasion dust and diesel exhaust particles in human macrophages

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Background: Despite brake abrasion dust (BAD) being a major component of urban particulate matter and rich in toxic metal ions, the adverse effects that these particles might cause to pulmonary health have been under-investigated. Instead toxicological studies have focused almost solely on the outcome of exposure to exhaust-related pollutants.

Methods: In order to establish whether BAD exposure may be detrimental to pulmonary health, the adverse effects that BAD (Svensk Maskinprovning, Sweden) have on U937 macrophages were characterised and compared with those of SRM-2975 diesel exhaust particles (DEP). Cellular responses to particle exposure (4–25 μg/ml, 24 h) were established using MTT viability assays, cytokine ELISAs and quantifications of cellular capacity to phagocytose Staphylococcus aureus. After establishing the metallic content of BAD and DEP with ICP-MS, the contributions that metal ions made to the anti-phagocytic activities of both particles were assessed using the non-specific metal ion chelator; desferroxamine.

Results: While neither particle significantly reduced the viability of U937 macrophages at the concentrations tested, BAD induced a dose-dependent inflammatory response; evidenced by two-fold increased secretions of IL-8, IL-10 and TNF-α. This was not significantly different to the inflammatory response induced by DEP and was accompanied by significant dose-dependent inhibition of phagocytosis behaviour for both particles (up to 73% for BAD and 32% for DEP, compared to controls). Although BAD was considerably richer in metals than the DEP, chelation of metal ions restored the ability of U937s to ingest Staphylococcus aureus after exposure to either particle.

Conclusions: The anti-phagocytic activities of BAD and DEP indicate that both exhaust and non-exhaust related particles could contribute to the burden of respiratory infections in urban areas by inhibiting clearance of inhaled pathogens. As no discrepancies were seen between the toxic mechanisms of BAD and DEP, we conclude that exhaust and non-exhaust particles merit equal toxicological investigation.

P-22.
Evaluating E-cigarette cytotoxicity using human bronchial epithelial cell cultures in vitro

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The use of E-cigarettes as 'smoking cessation tools' has rapidly increased in recent years. Although E-cigarettes are marketed as safe alternatives to traditional cigarettes, limited information is available on their acute and chronic health effects.

An E-cigarette typically consists of propylene glycol, glycerol, flavourings and water (with or without nicotine). The main aim of this study was to investigate the cytotoxicity of E-cigarette extract (ECE) on the human bronchial epithelial cell line (BEAS 2B) and the macrophage cell line (J774).

Four different E-cigarette brands, with several different nicotine contents and flavours, were used in this study. E-cigarette extract was obtained by bubbling E-cigarette vapour through 10 mL of sterile culture media at a rate of 35 mL/2s (ISO 3308). The ECE's were then diluted with fresh medium to four different concentrations; undiluted (100%), 50%, 25% and 12.5%. These dilutions were applied to submerged cultures of BEAS 2B and J774. Cell viability was assessed by XTT assay 24 h post ECE exposure.

Results showed that the varying nicotine content in E-cigarettes did not have any significant impact on the cell viability of either cell line at all concentrations tested. However, there was a significant difference in the cytotoxic effects between the different flavours tested, specifically the fruit flavours, which proved to be more cytotoxic. In addition, the BEAS 2B cell line appeared to be more sensitive to the different flavours than J774.

The observed results suggest that toxicity associated with E-cigarettes may not be due to the nicotine present and may in fact be linked to the E-liquid flavourings. It is interesting to note that sensitivity to the different flavourings is cell-type dependent as this has implications for effective defence of the airways. Future works include testing the influence of E-cigarette vapour on bronchial epithelial and fibroblast co-cultures at air-liquid interface (ALI) and subsequent histological analysis. Further investigation of the effects of E-cigarettes is needed and this will require standardisation of protocols and use of relevant human, in vitro models.

P-23.
Differentiating genotoxic and non-genotoxic carcinogens through automated cell phenotypic analysis

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In vitro carcinogenicity tests are often aimed at one type of cell abnormality, such as chromosomal defects or gene mutations. However carcinogenesis is a multistage process meaning identification of different carcinogen groups may be challenging. This project aims to improve in vitro safety assessment, to reduce and replace animal use in research. The procedure includes testing the effects of known carcinogens on DNA damage, cell-cycle and cell phenotype

Human lymphoblastoid TK6 cells were treated for 1.5 cell-cycles with the genotoxic carcinogens Methyl-Methanesulfonate (MMS) and N-Nitroso-N-Methylurea (MNU), the non-genotoxic carcinogens Methyl-Carbamate and Bis(2-ethylhexyl)-phthalate (DEHP), and the misleading-positive 2,4-Dichlorophenol. The Cytokinesis-Block Micronucleus (CBMN) Assay was then used to analyse genotoxicity and changes in cell morphology were detected using high-throughput automated image analysis (In-Cell-Analyzer 2000) and Matlab. Cell-cycle alterations were assessed using the MicroFlow technique and flow cytometry.

Significant genotoxicity was initiated by MMS and MNU at 0.7 and 0.3 μg/mL in the CBMN Assay (statistics assessed with Dunnett's t-test, $p < 0.05$). These genotoxins also caused a dose-dependent increase in nuclear area and a G2/M phase cell-cycle block. A significant dose-dependent increase in cell area was seen in response to genotoxic carcinogens MMS and MNU, whilst non-genotoxic carcinogens Methyl-Carbamate and DEHP caused a significant decrease in cell area. The misleading-positive 2,4-Dichlorophenol had no effect on genotoxicity or cell-cycle, but this chemical caused a lower percentage of elongated cells.

The increase in genotoxicity shown by MMS and MNU abides with their cell-cycle block, since these chemicals are known to cause G2/M cell-cycle arrest in order to initiate DNA repair or cell death. The contrasting effect on cell area for the genotoxic and the non-genotoxic carcinogens provides a potential differentiating feature for these carcinogen groups. The induction of smaller cells in non-genotoxic carcinogens may be mechanistically linked to mTOR signalling, a protein that affects cell size. Also the non-genotoxic carcinogen Methyl-Carbamate has been shown to downregulate P21 and SGK1 gene expression which are modulated by mTOR. Linking these data-sets could help to indicate how effects of specific compounds relate to their mechanisms of action, potentially improving carcinogenic identification in vitro.

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In silico and 3D in vitro models of drug metabolism and toxicity

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Current 2D in vitro test systems are poorly predictive of the toxicity of chemicals entering the systemic circulation. There is therefore an urgent need for models of systemic toxicity with improved predictivity across the pharmaceutical and chemical industry. We are currently developing a 3D hollow fibre bioreactor (HFB) system for hepatotoxicity testing. Previously HFBs have shown promise for use as bioartificial livers and their use in hepatotoxicity testing is a natural extension to this work. To assist with the development of the HFB, the design

has been mathematically modelled to inform its operating set up, interpret data from HFB outputs and aid in optimizing design to mimic certain hepatic physiological conditions. Additionally, the mathematical model has been used to identify the key HFB and compound parameters that will affect xenobiotic clearance. The analysis of this model has produced novel analytical results that allow the operating set up to be calculated and predictions of compound clearance generated efficiently and in a highly accessible form. The mathematical model predicts the inlet oxygen concentration and volumetric flow rate that gives a physiological oxygen gradient in the HFB to mimic a liver sinusoid. It has also been used to predict the concentration gradients and clearance of a test drug and paradigm hepatotoxin, paracetamol (APAP). The effect of altering the HFB dimensions and fibre properties on paracetamol clearance under the condition of a physiological oxygen gradient is analysed. These theoretical predictions can be used to help design the most appropriate 3D in vitro experimental set up and data analysis to quantitatively compare the functionality of cell types that are cultured within the HFB to those in other systems and in vivo. This work has been funded by NC3Rs CRACK IT.