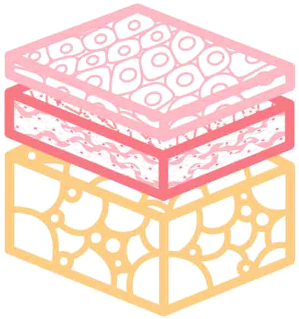

Building a Better Epithelium

Breaking the Barrier to the Next Generation of Toxicity Testing

Abstract Submissions

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March 10, 2018

San Antonio, Texas

PDF on Demand

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A Cellular Game of Telephone: Trans-Cellular Reprogramming in Responses to Toxic Stimuli

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Exposure to air pollution is a leading cause of cardiopulmonary morbidity and mortality; however, while these effects outside the lung have been associated with aberrant oxidative stress and inflammation, the underlying molecular mechanisms are poorly understood. We hypothesized that air pollutant exposure of human bronchial epithelial cells (HBEC) could alter the expression of pro-inflammatory and oxidative stress genes in adjacent, but physically separated cells through alterations to the epigenome. To test this hypothesis, we exposed confluent monolayers of HBEC, grown on the apical surface of Transwell permeable membranes, to diesel exhaust particles (DEP) while monocytic THP-1 cells were present in the basolateral compartment. A variety of pro-inflammatory and oxidative stress genes were induced in THP-1 cells cultured in the presence of DEP-exposed HBEC. Using IL-8 and HMOX1 as representative genes in the pro-inflammatory and oxidative stress responses, respectively, we then determined whether these changes in gene expression were associated with alterations to the epigenome. We assessed the abundance of four histone modifications with established roles as key regulators of gene expression – trimethyl histone H3 lysine 4 (H3K4me3), pan-acetyl H4 (H4ac), di/trimethyl histone H3K27 (H3K27me2/3), and unmodified histone H3 (H3). The abundance of H3K4me3 and H3K27me2/3 increased within the promoters of both IL-8 and HMOX1 in THP-1 cells cultured in the presence of DEP-exposed HBEC. These indirectly exposed THP-1 cells also exhibited increased H4ac within IL-8 and HMOX1 enhancer regions. In addition, a reduction in H3 occupancy accompanied the above changes in histone modifications. This trans-cellular epigenetic reprogramming within the THP-1 cells was also associated with an enhanced pro-inflammatory and oxidative stress response to a secondary challenge with lipopolysaccharide. Our findings demonstrate that pollutant-exposed epithelial cells reprogram the expression and responsiveness of pro-inflammatory and oxidative stress genes in adjacent cells. These findings ultimately provide a potential mechanism by which exposure to air pollution can cause adverse health effects in tissues that are not directly exposed, and further heighten the importance of understanding the effects of air pollution on tissues beyond the airway.

Attendee Presenter

Context Matters: Using an Organotypic Airway Model to Assess the Response to Inhaled Toxicants

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Exposure to air pollution is associated with increased morbidity and mortality from pulmonary and cardiovascular diseases worldwide. As a significant contributor to air pollution, diesel exhaust particles (DEP) are known to induce pulmonary oxidative stress and pro-inflammatory responses in both in vivo studies and in vitro models. Many in vitro studies of DEP exposure have focused only on bronchial epithelial cells grown in two-dimensional cultures on plastic substrata; however, the cellular microenvironment in vivo is more complex involving additional cell types such as fibroblasts in the stroma. Intercellular communication is an important aspect of response to environmental stimulants, and it has been shown that fibroblasts in the lung influence epithelial cell growth, differentiation, and gene expression. We hypothesized that incorporating fibroblasts into an organotypic model of the airway epithelium would alter epithelial cell response to DEP and provide an opportunity to examine effects of DEP exposure on cell types beyond the epithelium. To test this, we developed a transwell-based organotypic airway model where bronchial epithelial cells are grown on a collagen matrix on the apical side of a porous membrane, and lung fibroblasts are grown in the basolateral compartment. We conducted exposures by adding DEP to the confluent epithelial cell layer and assayed changes in the expression of interleukin-8 (IL-8) and heme oxygenase-1 (HMOX1), markers of pro-inflammatory and oxidative stress, respectively, in both cell types. Exposure of epithelial cells in the organotypic model resulted in a 25- and 5-fold induction of HMOX1 and IL-8, respectively, which was greater than the 15- and 4-fold inductions observed in matched exposures lacking fibroblasts. Further, despite a lack of direct exposure, HMOX1 and IL-8 were induced by 3,300- and 3-fold, respectively, in fibroblasts in the basolateral compartment. Our findings indicate that toxic exposures can have effects outside of the directly exposed cells, suggesting this organotypic airway model can elucidate aspects of exposure response not well-addressed by traditional monoculture models. Utilizing this model in further studies could facilitate understanding the role that fibroblasts play in local and systemic adverse health effects of toxic exposures. This abstract does not reflect EPA policy.

Attendee Presenter

Cytotoxicity of Applied Nanoclusters for Cellular Imaging

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Recently human microglial cells were either treated with chloroauric acid ($HAuCl_4$) or Au and Fe NCs for up to 72 hrs. Exposure to HAuCl4 or Fe NCs resulted in a dose dependent loss of cell viability within the first 8hrs of exposure, with a continuing loss through 72 hrs. High HAuCl4 exposure lead to significant increased reactive oxygen species (ROS) within 12 hrs following exposure, which continued through the exposure study. Similarly, Fe NCs resulted in ROS within two hours after exposure, signal was found to rise through the exposure time. Interestingly, Au NCs did not produce a notable increase in ROS during the first 24 hrs, however; once induced, ROS signal plateaued after another 24hrs. Fluorescent imaging revealed increased cell fluorescence following exposure to all compounds, with fluorescence seen in the cytoplasm during HAuCl4 and Au NC exposures; and dispersed throughout the cell during Fe NC exposure. Transmission electron microscopy confirmed cellular uptake of all compounds and suggest localization within cellular vesicles. Inductively coupled plasma-mass spec also indicated increased uptake of materials in a temporal and concentration dependent manner. Taken together these results highlight i) the capacity for human neuronal cells to self-synthesize NCs following HAuCl4 treatment, ii) the bioavailability and cytotoxicity of pre-synthesized nanocluster, iii) the ability for human cells to uptake pre-synthesized NCs, and iv) the resulting increased intracellular fluorescence from all these material treatments.

Attendee Presenter

Diesel Exhaust Particles Downregulate PI3K/Akt/mTOR Signaling and Mitochondrial Bioenergetics in a Novel Organotypic Model of the Airway Microenvironment.

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The pathophysiological importance of oxidative stress generation and induction of cytokines in diesel exhaust particle (DEP)-mediated metabolic dysregulation, cardiovascular disease, and pulmonary toxicity is well established. Most in vitro studies have focused on the effects of exposures on epithelial cells; however, stromal cells, such as airway fibroblasts, play a key role in maintaining tissue health and have the potential to play a central role in mediating bioenergetic and inflammatory endpoints following chemical exposure. Utilizing a novel 3D organotypic model of the human airway, we investigated whether DEP disrupts pulmonary and metabolic homeostasis in the airway microenvironment by impacting normal fibroblast functions through generation of oxidative stress and activation of the p38 MAPK pathway, a central mediator of cellular function. Confluent monolayers of bronchial epithelial HBEC grown on a collagen substrate in permeable Transwell inserts were exposed to DEP while IMR90 fibroblasts were present in the adjacent basolateral compartment. Here we show that an in vivo-like DEP exposure of airway epithelial cells strongly enhances phosphorylation of p38 as well as upstream MKK3/6 and downstream effectors MAPKAPK2, HSP27, and p53 in adjacent fibroblasts. Activation of the p38 pathway in fibroblasts was accompanied with the induction of the pro-inflammatory and oxidative stress genes interleukin-8 (IL-8) and heme oxygenase 1 (HMOX-1), respectively, which were attenuated by treatment with LY222820, an inhibitor of p38 kinase activity. We also examined the potential crosstalk between MAP kinases and the PI3K/Akt/mTOR pathway, known for regulating protein synthesis, bioenergetics, and tissue regeneration. DEP exposure decreased phosphorylation of PI3K, Akt, and mTOR in the fibroblasts while increasing phosphorylation of cell cycle regulators, p27kip1 and Rb. Maximal mitochondrial respiratory rate was reduced in fibroblasts; however, these changes were not associated with alterations in gross morphology or cytotoxicity. Altogether our findings demonstrate that indirect exposure of DEP to airway fibroblasts induces mitogenic crosstalk and overall downregulation of nutrient response, protein translation, and cell cycle progression, supporting a prominent functional role for fibroblasts in maintaining cellular homeostasis in the airway microenvironment.

Attendee Presenter

Differentiation State of Airway Epithelium Organotypic Culture Models Is a Potential Mediator of Silver Nanoparticle Toxicity

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Engineered nanomaterials, including silver nanoparticles (AgNP), are one of the largest groups of emerging toxicants. They are used in hundreds of consumer products due to their antimicrobial properties, and have a potential to aerosolize through the manufacturing and usage of these products. AgNP are respiratory toxicants with potential to cause allergic airway inflammation; however, the genetic, environmental, and temporal factors that may mediate their toxicity have yet to be fully elucidated. This study has developed airway epithelium organotypic culture models (AE-OCM) as a platform to identify these potential mediators and use them to inform Adverse Outcome Pathways (AOP) for allergic airway diseases, such as asthma. We are quantifying gene × environment × time interactions (G×E×T) using AE-OCM derived from two inbred founder strains of Collaborative Cross mice (AJ and C57BL/6J), differentiated under two conditions (- IL-13 and + IL-13; 25 ng/mL), and treated with AgNP (12.5, 25, and 50 µg/mL) at two time points—a four-hour repeated exposure over five days (5×4 hours), and 24 hours. Endpoints of interest include: changes in epithelial barrier function, cytotoxicity, and enrichment of gene ontologies for pathways associated with allergic airway inflammation. In AE-OCM + IL-13, we found significant reductions in epithelial barrier function, as measured by transepithelial electrical resistance (TEER; ohm×cm2), after AgNP treatment (25 and 50 µg/mL) at 5×4 and 24 hours compared to untreated control at day in vitro 25. We also found significant increases in cytotoxicity, as measured by LDH Release (%), after AgNP treatment (12.5, 25, and 50 µg/mL) at 5×4 and 24 hours compared to untreated control. We found few significant differences in cytotoxicity across strains, with A/J showing increased susceptibility compared to C57BL/6J. Compared to preliminary data for AE-OCM, AE-OCM + IL-13 shows reduced epithelial barrier function at baseline, and increased cytotoxicity after AgNP treatment, suggesting that differentiation state may mediate AgNP-induced toxicity.

Attendee Presenter

In Silico Modeling of Layered Systems: Case Studies with Skin and Gut

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In vitro models often provide valuable approximations to *in vivo* responses that are less invasive, faster, and lower cost. We take modeling one step further by developing virtual *in silico* 3D models that capture mechanisms of action at cell-resolution and experimental scale. Simulations of these models using high-performance computer systems approximate *in vitro* responses to interventions at even higher speed and lower cost than *in vitro* models. We aim to develop a reusable infrastructure for layered biological systems; e.g., those that include epithelial tissues. We aim to demonstrate applicability to product efficacy and safety testing by recapitulating skin and gut behaviors at the tissue scale of an *in vitro* test. Our models are agent-based and implemented using Biocellion (biocellion.com), a software platform that facilitates integration of biological, chemical and mechanical submodels into one high-fidelity whole-system model. E.g., our skin model integrates pre-existing models for cell proliferation/differentiation; transepidermal water loss; skin penetration; cell migration and mechanical force interaction models. Skin behaviors can be tested under varying environmental conditions and stimuli, such as application of toxic chemicals that inhibit cell proliferation. Though only recently developed, our models are able already to recapitulate many aspects of tissue growth, homeostasis and response to some interventions. Using Biocellion, they can be incrementally extended and improved to become increasingly predictive under an ever broadening spectrum of interventions.

Attendee Presenter

Multicellular Human Bronchial Models Exposed to Diesel Exhaust Particles Induce Inflammation, Oxidative Stress and Macrophage Polarization

Jie Ji[1], Swapna Upadhyay[1], Xiaomiao Xiong[1], Maria Malmlöf[2], Per Gerde[2], Lena Palmberg[1]
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Introduction
Human airway epithelium, forms the first line of defense in lung, and is a primary target of diesel exhaust particles (DEPs). Macrophages, with polarization as main feature, functioned as phagocytic cells. We have reported that both bronchial epithelial cells and macrophages are equipped with Toll-like receptors (TLRs). In this study, we exposed a multicellular model consisting of primary bronchial epithelial cells (PBEC) and macrophages (MQ; THP-1 derived macrophage) cultured at air-liquid interface (ALI) with DEPs. We aim to identify the effects of DEPs in mono- or multicellular airway models.
Method
PBEC cultured at ALI were co-cultured without and with MQ (PBEC-ALI and PBEC-ALI/MQ, respectively) and exposed to 63.5µg/ cm2 DEPs aerosols via XposeALI®. Control exposures to clean air were performed. After exposure, the models were incubated for 24h. The secretion of CXCL8 and IL-6 in basal medium were detected by ELISA. The mRNA expression CXCL8, IL6, TNFα, NFKB, HO1, GPx, TLR2/4, IL10, IL4, IL13, MRC1, MRC2 and RETNLA were analyzed by qRT-PCR. The surface expression of TLR2/4 were detected by FACS. Cell viability and apoptosis rate were analyzed with LDH-assay and FACS.
Result
In PBEC-ALI, the secretion of CXCL8, mRNA expression of inflammatory markers (CXCL8, TNFα) and oxidative stress markers (NFKB, HO1, GPx) were significantly induced by DEPs exposure. However, after DEPs exposure, mRNA expression of these markers (CXCL8, IL6, NFKB, HO1) were reduced in PBEC-ALI/MQ compared to PBEC-ALI. After sham exposure, the surface expression of TLR2 and TLR4 on PBEC in PBEC-ALI/MQ was significantly attenuated compared to in PBEC-ALI. After DEPs exposure, surface expression of TLR2 was increased while TLR4 was decreased on PBEC in both PBEC-ALI and PBEC-ALI/MQ. DEPs exposure resulted in similar expression pattern of TLR2 and TLR4 on MQ in PBEC-ALI/MQ. In PBEC-ALI/MQ, DEPs exposure increased M2 macrophage markers (IL10, IL4, IL13, MRC1, MRC2) transcription.
Conclusion
DEPs induced an inflammatory and oxidative stress response in PBEC-ALI models which was attenuated in the presentence of MQ. In both PBEC and MQ, DEPs exposure increased TLR2 but decrease TLR4 surface expressions. The combination of DEPs exposure and co-culture with PBEC drove the polarization of MQ to M2 phenotype.

Attendee Presenter

Not All Particulate Matter Is Equal: Examining Toxicity by Source

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Exposure to particulate matter (PM) causes cardiopulmonary morbidity and mortality. PM exposures are generally classified by particle size because smaller particles can penetrate deeper into the lung, causing greater toxicity. But in addition to varying in size, PM from different sources also varies in composition. In order to screen for differential toxicity of PM from various sources, we conducted air-liquid interface exposures of primary bronchial epithelial cells from a panel of healthy human donors to identical particle masses of six different pollutants: diesel exhaust particles from two different sources (A-DEP and C-DEP), wood smoke particles (WSP), coarse (PM10), fine (PM2.5), and ultrafine (PM0.1) concentrated ambient particulates (CAPS) collected in Chapel Hill, North Carolina. We compared the relative ability of these materials to induce the expression of oxidative stress and antioxidant defense genes and assessed the inter-individual variability in exposure outcomes. Exposure to A-DEP and C-DEP caused changes in the expression of different genes, with more genes upregulated following exposure to A-DEP. This indicates that the engine from which diesel exhaust particles (DEP) are generated and how they are processed affects their toxicity. Second, the A-DEP and WSP caused strong upregulation in the same set of genes across all donors: NQO1, HMOX1, TXNRD1, GCLC, GCLM, and SRXN1. This suggests that similar mechanisms drive the toxicity of these two types of PM. Finally, coarse, fine, and ultrafine CAPS elicited different responses, with smaller CAPS causing more drastic changes in gene expression. Since the exposure setup used for these experiments does not model lung deposition, this is further evidence that differential toxicity between these particles is attributable not only to their size but also to their composition. Additionally, if a donor was less responsive to one exposure, then they tended to be less responsive to the others. This indicates that each donor may have an overall response phenotype that influences their sensitivity to an array of air pollutants. Overall, these data highlight how the degree of PM toxicity is influenced both by PM source and by inter-individual variability. Exploring these differences in PM exposure outcomes will improve our understanding of how these toxicants cause adverse health effects and help identify and protect susceptible populations. This abstract does not reflect US EPA policy.

Attendee Presenter

Optimization of Normal Human Bronchial Epithelial (NHBE) 3D Cell Cultures for *In Vitro* Lung Model Studies

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Several factors including cigarette smoke, pollutants, genetic mutations, and infection affect the function of the respiratory epithelium including mucociliary apparatus. Robust *in vitro* lung models and assays are required for risk assessment to reliably and consistently measure key events leading to respiratory diseases. A challenge with primary human epithelial cell-based models is limited characterization and biological variability among different isolates. To understand how the culture conditions affect the epithelial phenotype, we evaluated several passages of NHBE cells from three donors (one from Lonza® and two from in-house), cultured in an air-liquid interface (ALI) and assessed the bronchial epithelial phenotype, culture characteristics, cell integrity and differentiation. NHBE cells obtained from Lonza® were grown in three different expansion media in the presence and absence of Rho kinase inhibitor or 3T3 Swiss albino feeder cells. Cells then formed differentiated epithelial layers in ALI. Optimally differentiated cultures were obtained with cells seeded at $1.5x10^5$ cells/cm^2 in PneumaCult™ Ex-PLUS (expansion medium)/PneumaCult ALI (differentiation medium) (StemCell™ Technologies). Cells from all three donors were successfully cultured up to seven passages and differentiated epithelial layers could be grown from passages 1-6. However cells from passages 1-4 optimally differentiated into a ciliated pseudostratified columnar epithelium with goblet cells as evidenced by trans-epithelial electrical resistance (TEER) (>330 Ohms.cm^2), presence of columnar ciliated cells , and cystic fibrosis transmembrane conductance regulator-mediated short-circuit currents (>3μA/cm^2). Interestingly, amiloride-sensitive epithelial sodium channel short-circuit currents significantly declined at passage 2, but remained stable thereafter. A significant decline was observed in TEER in some donors, as well as ciliary beat frequency (CBF) after passage 4. No change in airway surface liquid (ASL) meniscus length was observed up to passage 6. These results demonstrate, for the first time, how epithelial cell phenotypic features are altered when cultured in different media, under different conditions and between cell passages, and highlight the criticality of evaluating phenotypic endpoints when developing *in vitro* lung models.

Attendee Presenter

Pre-Validation of an Acute Inhalation Toxicity Assay Using the EpiAirway *In Vitro* Human Airway Model

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Knowledge of acute inhalation toxicity potential is important for establishing safe use of chemicals and consumer products. Inhalation toxicity testing and classification procedures currently accepted within worldwide government regulatory systems require the use of tests based on lethal effects in animals. The goal of the current work was to develop and pre-validate a non-animal (*in vitro*) method for determining acute inhalation toxicity using the EpiAirway™ *in vitro* human airway model as a potential alternative for currently accepted animal tests. The *in vitro* test method exposes EpiAirway tissues to test chemicals for 3 hrs, followed by measurement of tissue viability as the test endpoint. Fifty-nine chemicals covering a broad range of toxicity classes, chemical structures and physical properties were evaluated. The *in vitro* toxicity data was utilized to establish a prediction model to classify the chemicals into regulatory categories: highly toxic/corrosive, moderately toxic and mild/non-toxic. The data demonstrate that the EpiAirway acute inhalation toxicity test is highly sensitive for identifying dangerous toxic chemicals and/or respiratory tract corrosives. Corrosivity, electrophilic and oxidative reactivity appear to be the predominate mechanisms of toxicity for the highly toxic chemicals. These results indicate that the EpiAirway test is a promising alternative to the currently accepted animal test for acute inhalation toxicity. Further validation of the EpiAirway prediction model with additional chemicals is warranted.

Attendee Presenter

The Role of Oxidative Stress in Dry Eye Disease: Investigations Using an *In Vitro* Organotypic Human Corneal Tissue Model (EpiCorneal)

Patrick J Hayden[1], Thoa Truong[1], Miriam Kinuthia[1], Allison Lapointe[1], Mitchell klausner[1], Yulia Kaluzhny[1]
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Oxidative damage plays an important role in many ocular diseases, including dry eye disease (DED). This study evaluated the utility of an *in vitro* reconstructed 3D tissue model to study oxidative stress (OS) and DED. OS was generated by UV or desiccating stress conditions (DSC) to stimulate changes relevant to DED. Reactive oxygen species (ROS), lipid oxidation, cytokine release, barrier function, tissue viability, histology, and gene expression were evaluated. UV and DSC caused increased ROS, oxidation of lipids, release of IL8 and upregulation of proinflammatory genes. Application of topical lubricants improved tissue morphology, barrier function, and normalized IL8 release. The effect of UV irradiation and DSC on expression of 84 genes related to OS were evaluated. 6 genes were >2-fold upregulated in UV-treated cultures, including genes involved in ROS metabolism, peroxidases, and serine peptidase inhibitor SPINK1. 15 genes were >2-fold upregulated in DED tissues, including antioxidants and PTGS2 (COX-2) peroxidases, SPINK1, OS responsive gene HMOX1, and other genes involved in superoxide metabolism - ALOX12 and NOS2. In both UV-irradiated and DED tissues OS pathway signature genes, SPINK1 and HMOX1 were upregulated. The EpiCorneal tissue model structurally and functionally reproduces OS and DED markers. Gene expression changes in OS-exposed tissues closely parallel *in vivo* changes associated with inflammatory response. This model is anticipated to be a useful tool to study molecular mechanisms of ocular surface damage, DED, and to evaluate new corneal drug formulations.

Attendee Presenter

Use of *In Vitro* Human Intestinal Microtissues as Relevant Models for Drug Permeation and Metabolism in the Gut

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The goal of this study was to validate the relevance of an *in vitro* organotypic human small intestinal (SMI) microtissue model (EpiIntestinal) for predicting intestinal drug safety, permeability and metabolism. Permeability coefficients of 16 drugs for which *in vivo* data are available were evaluated. Toxicity was assessed using transepithelial electrical resistance, MTT viability, and Lucifer Yellow leakage assays. Bioavailability or efflux transport of drugs was analyzed using LC-MS/MS. The metabolic activity of the SMI microtissue model was also assessed and metabolites were analyzed using LC/MS or using isozyme-specific fluorogenic CYP450 substrates. The SMI microtissue model categorized the test drugs as high permeability and low permeability with a sensitivity of 100%, specificity of 89%, and accuracy of 94% compared to historical human absorption data. Efflux transporter Inhibitors increased bioavailability and decreased efflux ratio. Efflux ratios for substrates talinolol, digoxin and loperamide were reduced by 45%, 40%, and 60%, respectively, in the presence of the P-gp inhibitor verapamil. The efflux ratio of nitrofurantoin (BCRP substrate) was reduced by 63% in the presence of the BCRP inhibitor, novobiocin. Midazolam (CPY3A substrate) was metabolized (6.5% conversion) by the SMI microtissue model. Furthermore, Metabolism by CYP2C9, CYP2C19, CYP3A4, and CYP3A5 was observed using isozyme-specific fluorogenic CYP450 substrates. In conclusion, the SMI microtissue model appear to be promising new tool for evaluation of drug safety, permeability, and metabolism.

Attendee Presenter