maximum acceptable concentrations of a fragrance material in different consumer products. This current NGRA is structured in three tiers and integrates NAM's and defined approaches (DA). **Methods:** The initial tier (Tier 0) involves a thorough review of the existing information including *in silico* predictions; existing data pertaining to skin sensitization hazard (historical or non-animal) and read across data. Based on the weight-of-evidence (WoE), a prediction is made whether a chemical or any of its metabolites are likely to be a sensitizer. If Tier 0 is insufficient for concluding that the chemical is a sensitizer or not, Tier 1 is initiated. In Tier 1, both the DPRA (OECD 442C) and h-CLAT (OECD 442E) *in vitro* tests are conducted and incorporated into the OECD 497 DA for hazard identification and GHS classification. If the chemical is found to be a sensitizer, Tier 2 is conducted where a GARDskin dose response assay is used for a NESIL prediction, which is then incorporated in QRA2. The systemic toxicity for fragrance materials was also addressed using NAMs. The acceptability of NAMs for addressing systemic toxicity may not lie in actually predicting the hazard but to provide appropriate risk management actions to protect human health. The European Partnership for Alternative Approaches (EPAA) recently started the NAM Designathon Challenge, which is a research exercise aiming to develop an appropriate risk management action based on the level of concern (high, medium and low) for systemic toxicity for chemicals based on NAMs. To address this, we first developed a hazard classification system based on the DNEL/ADI/RfD values of almost 150 industrial chemicals with varying degrees of toxicity (low, moderate and high). A low level-of-concern chemical is defined as one which can be used without any restriction (safety by design) and not needing any animal data for addressing systemic toxicity. Medium-risk chemicals would require a risk assesment for establishing safe use levels, while high-risk chemicals would be candidates for risk management. An IVIVE (In Vitro to In Vivo Extrapolation) workflow was then developed utilizing a publically available PBPK model (HTTK) and in vitro Points of Departure data to calculate the Equiavalent Administered Dose (EAD), which in conjunction with the classification system, is used to assign the "level of concern" to an unknown chemical. **Results:** Case studies were run to test our workflow. The data showed good reliability and accuracy (>80%) in predicting the "level of concern" for different industrial chemicals. The workflow was further validated for various fragrance materials and showed similar reliability and accuracy (>80%). The approach serves as a preliminary "screening-level" assessment and the workflow can be used as a starting point for working towards a NAM-based solution in a future for addressing systemic toxicity. **Conclusions:** In summary, this study represents a major step towards the establishment of a NGRA framework for conducting QRA2 evaluations for fragrance materials and addressing systemic toxicity.

## 3216 Microphysiological Systems as Predictive Tools for Kidney Nephrotoxicants: A Comparison Across Four Platforms

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**Background and Purpose:** The kidneys are crucial for eliminating drugs and chemicals from the body, with renal epithelial cells being particularly vulnerable to damage by xenobiotics and their metabolites. Complex *in vitro* models of the kidney, including Microphysiological Systems (MPS), can be used to faithfully predict clinical response to toxicants. However, their high cost and low throughput often limit their application in broad contexts of use, and constant technological development complicates the process of model selection. **Methods:** This study compared effects of 16 compounds in concentration response design using the TERT1-OAT1 renal proximal tubule epithelial cell (RPTEC) line cultured on four platforms: standard 96-well plates, static and fluidic Transwells (PhysioMimix® T12), and OrganoPlate® 3-lane 40. Cytotoxicity was monitored via LDH leakage activity and transepithelial electrical resistance (TEER, in Transwell-based models). Samples were collected after 2, 24, and 48 hours of exposure, and drug transport was measured by LC-MS/MS. Additionally, transcriptomic analysis was performed using the TempO-seq® S1500+ assay suite. **Results:** Cytotoxicity data showed that while RPTEC cultured in 96-well plates showed the lowest sensitivity, comparable effects were observed in the other platforms. When points of departure were derived comparing model predictions of human nephrotoxicity, we found that platforms incorporating fluid flow were more accurate, particularly in their sensitivity to detect "true positives". Drug transport analysis, conducted on the barrier models (static and fluidic Transwells, and OrganoPlate®) revealed similar transport behavior between the Transwell-based platforms. However, the OrganoPlate® exhibited significantly lower transport kinetics, compound movement being retarded by the presence of the gel channel. Finally, baseline gene expression analysis showed differences between cells cultured on Transwells and in 96-well plates or OrganoPlate®, however the presence of medium flow had minimal effects. **Conclusions:** This study demonstrates performance of different in vitro proximal tubule models with respect to their ability to address toxicity and pharmacokinetics. Based on gene expression data we found that culture platforms or presence of medium flow have relatively minor impacts on TERT-OAT1 RPTEC. Interestingly, the addition of fluid flow enhanced the sensitivity of nephrotoxicity detection but had little effect on drug transport. Our results provide clarity for optimizing platform selection for future studies of safety and pharmacokinetics and demonstrate the importance of carefully considering platform characteristics to balance physiological relevance, sensitivity, and throughput for specific contexts of use.

## 3217 Comparative Analysis of Drug Permeability and Toxicity in Intestinal Segment-specific Membrane-Based Barrier Model Using Caco-2 and Human Jejunal and Duodenal Enteroid-Derived Cells

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**Background and Purpose:** Intestinal absorption is a critical factor in characterizing orally administered drugs. A well-established method for the assessment of intestinal pharmacokinetics involves the use of Caco-2 cells cultured on Transwell inserts. However, this model does not fully capture the roles of the different gut segments that play a role in differences of intestinal absorption of oral drugs. **Methods:** In this study, we used a panel of 14 reference compounds, using a concentration-response design, in three human segment-specific intestinal cell types: Caco-2 cells, and jejunal (J2) and duodenal (D109) enteroid-derived cells. The 14 tested compounds were selected for their known differences in absorption and toxicity within the gastrointestinal system (Benzophenone-4, Streptomycin, Buspirone, Nefazodone, Chlorpromazine, Gentamycin, Tenofovir Disoproxil Fumarate, Indomethacin, Ibuprofen, Sulforaphane, Perfluorooctane sulfonate, Staurosporine, Afatinib, and Idarubicin). Cells were seeded in 96-well Transwell plates to accommodate high-throughput testing of compounds, concentrations, and replicates. Cytotoxicity was assessed using lactate dehydrogenase (LDH) activity, and transepithelial electrical resistance (TEER) was measured to evaluate barrier integrity. Samples were collected at 2-, 24-, and 48-hours post-treatment, and drug transport was quantified using mass spectrometry. **Results:** TEER data revealed that Caco-2 cells were the most responsive to gut-specific toxic compounds (Staurosporine, Afatinib, and Idarubicin). These compounds disrupted TEER in both J2 and D109 cells after 48 hours of exposure at the highest concentrations. In contrast, LDH assays indicated that Caco-2 cells were less sensitive, while J2 and D109 cells exhibited cytotoxic responses to many of the tested compounds. Analysis of intestinal permeability data showed a broad range of absorption across the tested compounds, with segment-specific differences observed. For instance, Indomethacin was absorbed at the highest rate by J2 cells, aligning with known human data that show its primary absorption occurs in the small intestine rather than the colon. Similar results were observed for Ibuprofen, again aligning with known clinical data. Conversely, Benzophenone-4, a polar and highly water-soluble compound, showed very low absorption in all tested cell types. **Conclusions:** In summary, this study demonstrates the utility of intestinal segment-specific in vitro models for studying pharmacokinetics and toxicity. Our findings suggest that LDH is a sensitive marker of cytotoxicity in human intestinal enteroid-derived cells, while TEER is a more sensitive measure of toxicity for Caco-2 cells. Moreover, the observed differences in drug absorption among segment-specific cell types provide valuable data that can enhance the accuracy of in silico models of drug pharmacokinetics.

## 3218 Effect of Peracetic Acid Vapor on THP-1 Macrophages in an Air-Liquid-Interface Culture

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**Background and Purpose:** Peracetic acid (PAA), a highly reactive, low-molecular-weight organic oxidant, is widely used as a sanitizing agent in the healthcare, food, and wastewater recycling industries. Occupational exposure to PAA can result in irritation of the eyes and respiratory tract. In addition, acute or chronic exposure to PAA can lead to incidental diagnoses of work-related asthma (WRA). However, the mechanistic basis underlying PAA-induced WRA is unknown. Macrophages play a crucial role in lung homeostasis, immune activation, and the pathophysiology of asthma, an obstructive lung disease. In this study, we determined the effect of vapor containing PAA on the viability and function of macrophages in an air-liquid-interface (ALI) culture. **Methods:** THP-1 cells, a human monocytic cell line, differentiate into cells exhibiting an alveolar macrophage-like phenotype following exposure to phorbol 12-myristate 13-acetate (PMA). In this study, THP-1 monocytes were cultured in RPMI-1640 medium, and following treatment with PMA for forty-eight hours, they differentiated into macrophages. Two days later, the macrophages were plated on transwell inserts and subsequently air-lifted. Twenty-four hours following air-lift, THP-1 cells exhibited phenotypic features of macrophages, which was confirmed using fluorescent imaging of biomarkers CD11b and CD14 and were acutely exposed for one hour to either filtered room air (air) or vapor containing PAA (1.5, 3, 6, or 12 ppm). Immediately or twenty-four hours following cessation of exposure, cellular apoptosis and necrosis, culture supernatant lactate dehydrogenase (LDH), and phagocytosis of zymosan particles were quantified using high content imaging and traditional biochemical assays. **Results:** Fluorescent imaging of biomarkers CD11b

and CD14 confirmed THP-1 monocyte differentiation to macrophages, which was maintained under ALI conditions. Regardless of the time following cessation of exposure, air- and PAA-exposed THP-1 macrophages stained positive for apoptosis while only PAA exposure caused necrosis. No differences in culture supernatant LDH existed between air- and PAA-exposed macrophages. Exposure to PAA inhibited phagocytotic activity immediately and twenty-four hours following cessation of exposure. **Conclusions:** Air- and PAA-exposed THP-1 macrophages were successfully imaged for biomarkers of apoptosis and necrosis and phagocytotic activity using high content imaging on an ALI platform. Both air- and PAA-exposed macrophages exhibited features of apoptosis; however, only PAA-exposed cells exhibited features of necrosis and diminished phagocytotic activity. THP-1 macrophages face unique challenges when placed in an ALI mono-culture, and to prevent air-exposed cells from exhibiting features of apoptosis, we need to optimize culture conditions to improve the viability of these cells.

### 3219 hiPSC-derived GlutaNeurons and Astrocytes Enhances Seizure Liability Assessment and Detection with MEA Technology

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**Background and Purpose:** Drug-induced seizures are severe adverse reactions that can occur during drug development. Current methods for assessing seizure liability rely on behavioral evaluations of animals exposed to drugs. Although animal models with electroencephalogram (EEG) telemetry measurements can provide high sensitivity and predictivity, these in vivo methods are low-throughput and can yield controversial outcomes due to species differences, and do not meet the mandate of reducing animal use in pre-clinical studies.To address these limitations, we propose the use of human-induced pluripotent stem cell (hiPSC)-derived cells to assess the risk of drugs on human toxicity. Co-culturing hiPSC-neurons and hiPSC-astrocytes can mimic seizure-like hyperactivity in neural systems, providing an ideal solution for detecting the seizurogenic potential of drug compounds. **Methods:** In our study, we cultured hiPSC-neurons and astrocytes (iCell® glutaneurons and iCell® astrocytes; both from FUJIFILM Cellular Dynamics, Inc.) and recorded their extracellular field potential using a multi-electrode array (MEA) system (Maestro Pro, Axion Biosystems, Inc.). This system captures synchronized neural activity as network bursts and detects seizure-like hyperactivity by observing an increase in network burst frequency following drug exposure. **Results:** Notably, we consistently observed at least a 30% increase in network burst frequency with GABA-A receptor antagonists (picrotoxin, gabazine, and bicuculline) across several manufacturing batches of cells. Additionally, we confirmed that the system can detect various pharmacological effects on synchronized neural activity using 12 compounds with known pharmacological effects. The results obtained were comparable to previously published findings. We also analyzed the differences in the concentration range for inducing seizure-like increases in network burst frequencies among different in vitro systems, considering species differences and analytical methods for acquiring neural activities, which warrant further discussion. **Conclusions:** In conclusion, we have established a robust in vitro assay using a co-culture of hiPSC-glutaneurons and astrocytes with an MEA system. This assay accurately detects known pharmacological effects of drug compounds that induce seizures. By reducing the risk of encountering seizurogenic issues at a later stage, this assay contributes to the more successful development of drugs.

### 3220 Retinoic Acid Pathway Activation Using a CRISPRa-Mediated Approach in U-2 OS Cells

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**Background and Purpose:** The US EPA is heavily invested in developing higher-throughput, lower-cost New Approach Methodologies (NAMs) to identify potential hazards of thousands of environmental chemicals with unknown impacts to human health. Genetic editing techniques can mediate specific changes in gene pathway targets of toxicological interest. These can be used to generate molecular *in vitro* transcriptomic or phenotypic profiles, aiding NAMs data interpretation for mechanism and potential hazards. In this study, we targeted specific activation of nuclear receptors of the retinoic acid (RA) pathway, a critical regulator of cellular development, differentiation, and proliferation, using a CRISPRa-dCas9 strategy in human osteosarcoma cells (U-2 OS). **Goal:** Refine transcriptomic and phenotypic profiles for RA pathway activation. **Methods:** In order to activate the retinoic acid pathway by purely genetic manipulation (Figure 1), we transfected U-2 OS cells stably expressing the CRISPRa-dCas9-VPR system with CRISPR RNAs (crRNAs) individually targeting retinoid receptors RARA, RARB, RXRA, and RXRB or targeting the RARA/RXRA heterodimer. Addition of RARA ligand, *all-trans* retinoic acid (ATRA) was used to further augment pathway activation. Based on prior results, we used *RARB* expression as an indicator of pathway activation. Real-time quantitative PCR (qPCR) measured the expression of different RA pathway genes and organelle morphological changes were measured by Cell Painting. Due to differences in target gene expression and downstream gene expression, a time course study was done to determine the maximum point of expression. See more details on digital copy of poster. **Results:** CRISPRa-dCas9-VPR increased expression of targeted RA pathway receptors (*RARA, RARB, RXRA, RXRB*) compared to non-targeting control crRNAs ($p<0.01$, two-way Student's t-test), but did not increase RA pathway activation as indicated by increased expression of the RA pathway-sensitive gene, *RARB* (with the exception when *RARB* was specifically targeted by crRNA). Exposure to the RA receptor ligand, ATRA, dose-dependently increased *RARB* expression. The combination of CRISPRa of RARA receptor and 10 nM ATRA exposure augmented *RARB* expression over ATRA exposure alone (~3.2-fold, $p<0.005$). CRISPRa of the RARA/RXRA heterodimer (1:1) resulted in suppressed RA pathway activity, with or without ATRA exposure. Cell painting measures indicated previously described mitochondrial compaction due to ATRA exposure, however added CRISPRa of *RARA* did not further influence this phenotype. The time course study revealed that gene expression changes are highest at the 18-hour time point, setting a new window for experimental exposures. **Conclusions:** Based on this study, genetic editing techniques can mediate specific gene pathway targets of toxicological interest, and aid in NAMs data interpretation, but not necessarily increase total pathway activation. More work is needed to optimize the timing of the CRISPRa, and to confirm that target protein is also increasing due to our CRISPRa. This further work will elucidate feedback mechanisms within the pathway and further refine this as a tool for screening chemicals of toxicological interest.

### 3221 Transcriptomic profiles to support read-across for dinitrobenzene and dinitrotoluene analogs

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**Background and Purpose:** Read-across relies on the assumption that two chemicals with similar chemical structure will have similar biological targets and activity. Of course, there are examples in which chemicals with small structural differences have different toxicity. Transcriptomics provides a comprehensive way of evaluating biological activity. In this study we test the ability of transcriptomics to distinguish between similar chemicals with different toxicity and determined the extent to which it can group chemicals. **Methods:** We performed two studies. In the first we compared the effects on gene expression of dinitrobenzene (DNB) isomers: 1,3-DNB, a testicular toxicant that causes Sertoli cell death and 1,2-DNB, which does not. We compared these in the following cell types: MCF7, A549, HepG2 and iPS-derived cardiomyocytes. Gene expression was evaluated using the TempoSeq platform and responses compared using connectivity mapping. Chemicals were evaluated at three concentrations in triplicate, with cells exposed for 6 hours in a 96-well plate format. Because HepG2 cells were the most responsive in the first study, in a second experiment we used HepaRG cells, an iPS-derived hepatocyte line, to investigate gene expression after exposure to 1,2-, 1,3-, 1,4-DNB, 2,3-, 2,4-, 2,5-, 2,6-, 3,5-dinitrotoluene (DNT), 3-nitroacetanilide or 3-nitroaniline. The DNT isomers were included as analogs to the DNB isomers and the other two compounds as putative 1,3-DNB metabolites. **Results:** HepG2 cells were the most responsive to the DNB isomers in the first study. These cells retain some xenobiotic metabolism capability, suggesting that metabolism to an active form is important in the toxicity. The responses of 1,2- and 1,3-DNB were markedly different, with 1,3-DNB connecting to HIF activators and HSP inhibitors. Similar results were obtained with DNB isomers in the second study with HepaRG cells: 1,2- and 1,4-DNB produced equivalent patterns of gene expression which were very different from 1,3-DNB. Chemicals that are suitable analogs for each other affected gene expression similarly: 1,2 and 1-4-DNB and 2,3-DNT had similar patterns of gene expression, as did 1,3-DNB and 2,4-, 2,5-, 2,6-, and 3,5-DNT. 3-Nitroacetanilide and 3-nitroaniline, presumptive metabolites of 1,3-DNB, did not connect strongly to 1,3-DNB in this experiment suggesting they may not be responsible for the toxicity of 1,3-DNB. 3-Nitroaniline caused few changes in gene expression. **Conclusions:** These results indicate that transcriptomics in the appropriate cell type can distinguish between closely related chemicals that have different toxicity profiles and can be used to group chemicals by biological activity. Interestingly, that cell type need not be the same cell type that characterizes the in vivo toxicity.

### 3222 Using Cell Painting to Predict Ames Outcomes Through Machine Learning

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**Background and Purpose:** Cell Painting is a high-content imaging assay that enables comprehensive profiling of cellular phenotypes. This technique involves staining treated cells with a combination of fluorescent dyes that stain specific cellular components, including the nucleus, cytoskeleton, and cytoplasmic organelles. From the imaging data, approximately twelve hundred phenotypic features can be extracted per cell, via an image processing tool, Harmony®, resulting in a significant data yield that is suitable for machine learning purposes. The Ames assay is a regulatory requirement for agrichemical registration to assess for mutagenicity, and it was hypothesized that the outcomes of this assay would be suitable for machine learning predictions using the phenotypic features extracted from the Cell Painting image

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