with a constellation of biomarkers that comment on cytotoxicity (i.e., relative increased nuclei count, cleaved parp, and EMA-positive chromatin) and genotoxic mode of action (i.e., gamma-H2AX, phospho-histone H3, p53, and polyploidy). For these proof-of-principle experiments, TK6 cells were exposed to test chemicals in 96-well plates for 24 continuous hr. Eleven test chemicals were evaluated over a range of concentrations in the presence and absence of rat liver S9. The test chemicals were: hydroxyurea, ethyl methanesulfonate, cyclophosphamide, dibenzo[a,l]pyrene, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (clastogens); vinblastine, colchicine, and AMG900 (aneugens); and D-mannitol, cycloheximide, and brefeldin A (non-genotoxicants). MultiFlow assay data were acquired at 4 and 24 hr, and micronuclei were scored at 24 hr. With the exception of D-mannitol and cycloheximide, each of the chemicals induced what appeared to be micronuclei. Six of the micronucleus-inducing agents were observed to affect two or more DNA damage response biomarkers. Brefeldin A was the clear exception. While it strongly affected each of the cytotoxicity endpoints, none of the DNA damage response biomarkers were significantly elevated. PROAST Benchmark Dose software was used to calculate potency metrics for each endpoint, and we describe a novel approach that utilized ToxPi software to synthesize the resulting data into readily interpretable visuals. In summary, each of the genotoxicants was correctly identified as such, and their genotoxic mode of action was evident from accompanying biomarker signatures. Furthermore, the results with brefeldin A suggest the system is able to highlight false positive micronucleus test results.

2816 Assessment of Chemical-Induced Mutagenesis in Mice and Rats Using Error-Corrected Next Generation Sequencing

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Recent advances in Error Corrected-Next Generation Sequencing (EC-NGS) technology has reduced the sequencing error rate of standard Next Generation Sequencing (NGS) by 100,000-fold. The high error rate of standard NGS has prevented its use in Genetic Toxicology to assess mutagenicity of test articles *in vivo*, thus requiring use of transgenic rodent (TGR) mutation assays to assess *in vivo* mutagenesis. Big Blue® (BB) transgenic rodent (TGR) mutation assays, the gold-standard for measuring *in vivo* mutagenicity (OECD Test Guideline 488), use a recoverable lambda phage shuttle vector and a phenotypic plaque-based assay to measure mutant frequency (MF). By using double stranded barcode tags on DNA fragments and consensus calling of bases in both strands separately before forming a true duplex consensus, Duplex Sequencing (DS) Technology, an EC-NGS method, provides highly accurate assessments of background and induced MF and mutation spectrum. This approach permits measurement of MF and mutant spectra in any species, tissue, or DNA segment. The error rate of DS, below the spontaneous mutant frequency of mammalian genomic DNA, permits detection of both background and chemical-induced increases in MF. Here, we compared the MF and spectra for the traditional *cII* gene plaque assay to DS in BB mice exposed to vehicle (olive oil), N-ethyl-N-nitrosourea and benzo(a)pyrene. This bridging study showed plaque and EC-NGS methods yielded similar fold increases in MF and shifts in spectra demonstrating equivalence. We then evaluated four endogenous genes in BB mice covering 15 KB of genomic DNA. We then analyzed rats with similar exposures but used a 50 KB probe set covering 20 genomic sequences across chromosomes. ENU and BaP produced similarly large increases in MF over vehicle controls in both species. Unsupervised cluster analysis of simple mutant spectra and trinucleotide spectra showed that each treatment condition produced similar patterns in mice and rats that were unique for each treatment condition. The data show that EC-NGS is equivalent at detecting mutations in the *cII* gene. Advantages are that EC-NGS is robust in detecting changes in both MF and mutant spectra in endogenous DNA in two rodent species using both a gene-based probe set in mice and a representative genomic approach used in rats. In summary, our results show that an EC-NGS technology can measure *in vivo* mutagenesis associated with nonclinical biomarkers of human cancer risk.

2817 Modification of DNA Damage and Repair Pathways Reveals Detailed Mechanistic Information on the Genotoxicity of Clastogens

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The miniaturized and automated *in vitro* assay platform - MultiFlow - has demonstrated its utility for deriving genotoxic mode of action information. Recent adaptations of this approach yield additional levels of insight into aneugenic and clastogenic mechanisms. Here we report on the latest advancement that combines established biomarkers of genotoxicity with agents known to alter specific DNA damage and repair pathways. TK6 cells were preincubated with the following agents to modify their respective targets - olaparib a PARP inhibitor that affects base excision repair (BER), NU7441 a DNA-PK inhibitor that affects non-homologous end joining (NHEJ), MK8776 a CHK1 inhibitor, and finally a cocktail of reactive oxygen species (ROS) scavengers for investigating oxidative stress-mediated damage. Cell aliquots from each of these conditions were then exposed to genotoxic agents with varied clastogenic activities, i.e. the ROS generators hydrogen peroxide (H2O2) and menadione (Mna), a DNA synthesis inhibitor hydroxyurea (HU), camptothecin (Camp) and etoposide (Eto) as topoisomerase I and II inhibitors respectively and methyl methanesulfonate (MMS) as an alkylator. Cells were collected at 4 and 24 hrs of exposure and examined for γH2AX and p53 responses. Dose-response data were converted to area under the curve (AUC) values. AUC values for Mna and H2O2 were dramatically reduced in the presence of ROS scavengers, while none of the other test articles showed such attenuation. Eto responses were potentiated by NU7441 consistent with the role of NHEJ in response to topo II poisons. HU effects were potentiated by MK8776 an agent known to magnify DNA synthesis inhibition. Camp- and MMS-induced responses were potentiated with olaparib, highlighting the role of BER in response to these agents. Overall, these varied profiles of response potentiation and attenuation provided a means to differentiate between the multiple classes of clastogens. In addition to the AUC approach, we are investigating other data analysis approaches and assessing their performance. The use of specific modifiers of DNA repair pathways and ROS-mediated damage to the existing MultiFlow assay provides signatures of test article activity that can be used to investigate mechanisms and molecular targets. This information will be of great utility for risk assessment tools such as adverse outcome pathways and *in vitro* to *in vivo* extrapolation.

2818 High-Throughput Screening for Resistance to Colon Cancer-Associated Carcinogen in Budding Yeast Identifies Both DNA Repair and Ribosomal Protein Genes

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Risk factors for colon cancer include both diet and genetics; charred meat contains heterocyclic aromatic amines, such as 2-amino-3-methylimidazo [4,5-f]quinoline (IQ). However, few IQ resistance genes have been identified. We used yeast as a model organism to determine genetic susceptibility to IQ by introducing expression vectors that contain both human CYP1A2 and N-acetyl transferase (NAT2) genes into the yeast deletion library. To identify resistance genes, we used a high throughput approach for screening the "humanized" and the original yeast deletion library. Pooled cells from both libraries were exposed to 400 μM and 800 μM IQ, the DNA barcodes were sequenced using the Illumina platform, and statistical significance was determined for exactly matched barcodes. We identified 117 IQ resistance genes from the yeast library expressing human genes. Using EIXIR STRING database, we identified a highly interacting set of ribosomal protein genes, including *RPL26A, RPL26B, RPL4A, RPL23, RPL33, RPL4A, RPS16B,* and *RPS6B*. Upregulation of these genes are correlated with multidrug resistance while downregulation confers sensitivity to several cross-linking DNA damaging agents, including oxaliplatin. In a separate screen, DNA repair genes, *RAD18* and *NTG1*, were identified. Interestingly, polymorphic alleles of *RAD18* and *NTG1* have been documented to be risk factors for colon cancer and *ntg1* knock out mice develop colon cancer. These studies thus suggest that activated IQ targets housekeeping genes involved in genome stability as well as protein synthesis. Future experiments are planned to knock-down human orthologues of the yeast genes and conduct similar screens in human cell lines.

2819 Evaluation of Telomere Length and Markers of Neurodegeneration after Welding Fume Exposure

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Inhalation of welding fume (WF) can result in the deposition of toxic metals, such as manganese (Mn), in the brain and may cause neurological changes in exposed workers. Alterations in telomere length are indicative of cellular aging and, possibly, neurodegeneration. Here, we investigated the effect of WF inhalation on telomere length and markers of neurodegeneration in rat brain. Male Fischer-344 (F-344) rats were exposed by inhalation to stainless steel WF (20 mg/m3 x 3 hr/d x 4 d/wk x 5 wk) or filtered air (control). Telomere length, DNA-methylation, gene expression of Trf1, Trf2, ATM, and APP, pro-

tein expression of p-Tau, α-synuclein, and presenilin 1 and 2 were assessed in brain tissue at 12 wk after WF exposure ended. Results suggest that WF inhalation increased telomere length without affecting telomerase in whole brain. Moreover, we observed that components of the shelterin complex, Trf1 and Trf2, play an important role in telomere end protection, and their regulation may be responsible for the increase in telomere length. In addition, expression of different neurodegeneration markers, such as p-Tau, presenilin 1-2 and α-synuclein proteins, were increased in brain tissue from the WF-exposed rats as compared to control. These findings suggest a possible correlation between epigenetic modifications, telomere length alteration, and neurodegeneration because of the presence of factors in serum after WF exposure that may cause extra-pulmonary effects as well as the translocation of potentially neurotoxic metals associated with WF to the central nervous system (CNS). Further studies are needed to investigate the brain region specificity and temporal response of these effects.

PS 2820 Duplex Sequencing Provides a Sensitive Method for Early Detection of Mutations Induced by Chemical Exposure *In Vitro*: A Concentration Response and Time Course Experiment in Human TK6 Cells Exposed to N-ethyl-N-nitrosourea

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Duplex Sequencing (DuplexSeq), a form of error-corrected DNA sequencing, has an ultra-low error rate, allowing for detection of rare mutations within individual samples. This technology has wide-ranging applicability in diverse genomics fields, from paleo-genomics to forensics, and is especially useful in clinical applications such as early detection of diseases, e.g., cancer, and monitoring response to treatment over time. In a collaborative effort among the National Toxicology Program, TwinStrand Biosciences, and Integrated Laboratory Systems, the application of DuplexSeq to mutagenicity testing was evaluated *in vitro*. Human TK6 lymphoblastoid cells (TK6 cells) were exposed to five concentrations (25 - 200 micromolar) of N-ethyl-N-nitrosourea (ENU) for 24, 48, 72, or 96 hours. Cell samples collected at each time point were evaluated for mutation frequency (MF) and mutational spectra using the Duplex Sequencing Human Mutagenesis Panel developed at TwinStrand Biosciences. Genotoxicity, measured by micronucleus frequency, was also evaluated in the same ENU-exposed TK6 cells to provide confirmation of ENU-induced genetic alterations. The performance of DuplexSeq was excellent in the TK6 cell samples, with a yield of over 1 billion duplex bases in 98% of the samples and high concordance between duplicates. As expected, MF increased with increasing concentrations of ENU and frequencies were higher at the later time points in samples exposed to the 3 highest ENU concentrations. An increase in MF relative to vehicle control was observed even at the lowest concentration of ENU. The mutational spectrum did not vary with ENU concentration or exposure duration. Interestingly, there was a non-canonical increase in C to T mutations across all the samples; however, increased C to T substitution following exposure to ENU is consistent with previous reports in both TK6 cells and in tissues collected at early timepoints (less than 96-hours) in ENU-exposed rats. These results indicate considerable potential for applying DuplexSeq to *in vitro* genetic toxicology testing.

PS 2821 Scoring of the *In Vitro* Micronucleus Assay Using Imaging Flow Cytometry and Deep Learning

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The *in vitro* micronucleus (MN) assay is well-established for evaluating genotoxicity and cytotoxicity and is often scored by manual microscopy which is laborious and subject to low throughput and scorer variability. Automated scoring methods such as slide-scanning microscopy and flow cytometry have been developed but have drawbacks. Microscopy requires high quality slides to be created and flow cytometry lacks the ability to visually confirm the legitimacy of MN. The use of imaging flow cytometry (IFC) to perform the assay possesses the potential to overcome these limitations by combining the speed and statistical robustness of conventional flow cytometry with high resolution imagery capabilities of microscopy. The use of IFC to perform the MN assay is demonstrated using several cell lines and a number of common genotoxicants. Cells are imaged in suspension, eliminating the requirement to create microscope slides, permitting imagery of thousands of key events to be captured in just a few seconds. Traditionally, the MN assay performed by IFC has relied on feature-based image analysis to identify key events and quantify MN. In this study, we developed a classification model using convolutional neural networks (CNNs) to score the MN assay. Image classification by CNNs presents several advantages in comparison to traditional feature-based scoring methods, including elimination of complex image analysis strategies as well as translatability across multiple cell lines and test compounds. Here we show that a single CNN-based classification model is able to score both the cytokinesis blocked and unblocked versions of the MN assay using TK6 lymphoblastoid cells and three widely used test chemicals: Mitomycin C, Etoposide and Mannitol. All samples were also scored by manual microscopy and feature-based analysis software, permitting direct comparison of all scoring methods. We demonstrate that CNN-based scoring is able to correctly identify statistically significant increases in MN frequency, outperforms feature-based methods and compares well to manual slide microscopy. The use of IFC and deep learning presents a further step towards a fully automated scoring solution for the MN assay. This combined approach introduces a number of novel elements and potential improvements towards overcoming many challenges inherent in conventional techniques.

PS 2822 Next Generation Genotoxicity Assessment in Human Hepatocyte Models: CometChip and Micronucleus Assay in Metabolically Competent HepaRG Cells

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We are developing medium throughput genotoxicity assays using human-relevant metabolically competent hepatocytes to reduce, replace and refine the use of animals in the practice of genetic toxicology. We are combining HepaRG™ cells with CometChip® technology, a single cell array platform developed at MIT, and flow cytometry-based micronucleus (MN) assay to develop a New Alternative Methodology (NAM) aimed at reducing reliance on the *in vivo* comet and micronucleus assay. CometChip® facilitates rapid processing of 96 samples with unbiased-automated image-based scoring of the comet assay that can replace 30 yr old slide-by-slide one cell at a time scoring. Each imaged well of the 96 well plates have ≥ 200 scorable comets with the entire plate scored in less than 45 minutes compared to days needed to score 96 samples using traditional comet assay scoring. The *in vitro* and *in vivo* MN assay are part of the ICH S2R1 genetic toxicology test battery and we have adapted the flow cytometry-based micronucleus (MN) assay for use in HepaRG™ cells. To qualify this approach as a NAM we have: developed an initial basic protocol using a 3-day repeat exposure regimen, established qRT-PCR assays for functional assessment of HepaRG™ metabolic competency, conducted "power" studies to determine optimal number of comets scored per each sample, trained external collaborators at ILS, completed testing of an initial "training set" of negative and positive control test articles for use in the qualifying the HepaRG™CometChip® assay, and integrated HepaRG™CometChip® assay with MN assay to follow up *in vitro* MN positive responses. Multiple endpoint genotoxicity assessments in human hepatocyte models can serve as alternatives to animals for equivalent or better human-relevant safety and risk assessments than relying solely on rodent models. *This work is funded by NIEHS SBIR 4R44ES024698-02.*

PS 2823 Impact of DNA Polymerase ζ in the Mutation Signature of Methylating Agents

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Impact of DNA Polymerase ζ in the mutation signature of methylating agentsDNA Polymerase ζ (Pol ζ) is a low fidelity B-family polymerase involved in DNA damage tolerance by mediating replication after DNA adducts are formed from exposure to genotoxic chemicals. While it can be kinetically inefficient at inserting a base opposite DNA adducts, it is proficient in extending from base modifications or mismatches, thus it's expression and function can influence the likelihood that mutations arise following chemical modification of DNA. This model for Pol ζ function, however is primarily based on bulky or oxidation-induced DNA adducts, while little is known concerning it's role in replicating highly mutagentic alkyl DNA adducts such as O^6-methylguanine (O^6-meG), nor how the genomic location of modifications influences resulting mutation profiles. O^6-meG arises from exposure to methylating agents such as methyl nitrosamines and gives rise to patterns of G>A transitions

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The Toxicologist

Supplement to *Toxicological Sciences*

Toxicological Sciences

ISSN 1096-6080
Volume 180, Issue S1
March 2021

The Official Journal of the Society of Toxicology

OXFORD UNIVERSITY PRESS

SOT | Society of Toxicology

www.academic.oup.com/toxsci

Publication Date:
March 12, 2020