A New Approach Method (NAM) to Screen for the Impact of Endogenous Stress on Chemical Toxicity

Laura Word[1], Clinton Willis[1], Stephanie Padilla[1], Chelsea Weitekamp[1], Kelly Carstens[1], Logan Everett[1], Bridget Knapp[1,2], Katy Britton[1,3], Richard Judson[1], Joshua Harrill[1]

[1]US EPA/CCTE; [2]Oak Ridge Institute for Science and Education; [3]US EPA/ORAU

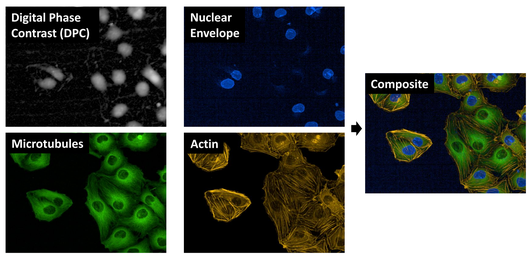
Background and Purpose

- Environmental justice affirms an individual's right to a healthy environment; however, people living in overburdened communities often simultaneously experience higher chemical exposures and higher levels of chronic stress, leading to poorer health[1]
- Thus, we are developing a new approach method (NAM) that can rapidly evaluate how increased cortisol levels from stress impacts chemical dose-response relationships
  - This will provide insight into cumulative impacts from chemical and non-chemical stressors and advance the understanding of chemical susceptibility in environmental justice communities

Methods Overview

- We are conducting high-throughput phenotypic profiling (HTPP) on human osteosarcoma cells that express fluorescent proteins for the nucleus and cytoskeleton (U-2 OS_{FP})
- **For the pilot experiment, U-2 OS_{FP} cells were co-exposed to cortisol and one of nine toxicants in concentration-response for rapid evaluation of phenotypic changes**
  - The cells were pre-treated with no, low (0.03 µM), or high (10 µM) cortisol (which was based on glucocorticoid receptor response to cortisol) for 24 hours prior to test chemical administration and imaging was captured after 24 hours of co-exposure to the test chemical and cortisol
  - 787 distinct phenotypic features were evaluated for the nine toxicants that were tested at 12 concentrations under conditions of low vs high cortisol

Image Acquisition for Phenotype Analysis



384-well plates, 20x water immersion objective, imaged 5 fields of view.

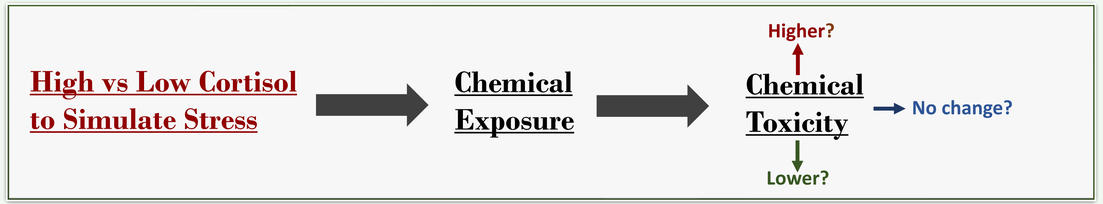
Note: The cells were transitioned to a serum-free media to allow for control over cortisol levels in the test system. During this process the cells struggled to proliferate, likely due to the lack of attachment factors in serum. When cells were cultured on collagen plates, their ability to proliferate was rescued.

Upcoming Work

- The upcoming **full screen will evaluate 147 chemicals**, including chemicals selected based on their relevance to environmental justice communities, their interaction with the glucocorticoid receptor pathway, and/or impact on known toxicity pathways. These chemicals cover a diverse array of mechanisms of action, which will provide additional insight into how cortisol-induced stress impacts chemical toxicity
- Future experimentation will include **zebrafish exposures**, which will provide further information on potential mixture effects of co-exposure to cortisol and chemicals in a developmental hazard model
- **High throughput transcriptomics** will be analyzed on the cell samples
- This multi-pronged approach will provide both *in vitro* and *in vivo* data for mechanistic insight into the quantitative impact of cortisol-induced stress on adverse outcome pathways (AOPs)

[1]Source: NEJAC 2004 report, available at www.epa.gov/environmentaljustice/ensuring-risk-reduction-communities-multiple-stressors-environmental-justice

We are developing a New Approach Method (NAM) using U-2 OS cells exposed to cortisol that is showing promising results for its ability to screen how chemical toxicity changes within stress-induced biological systems

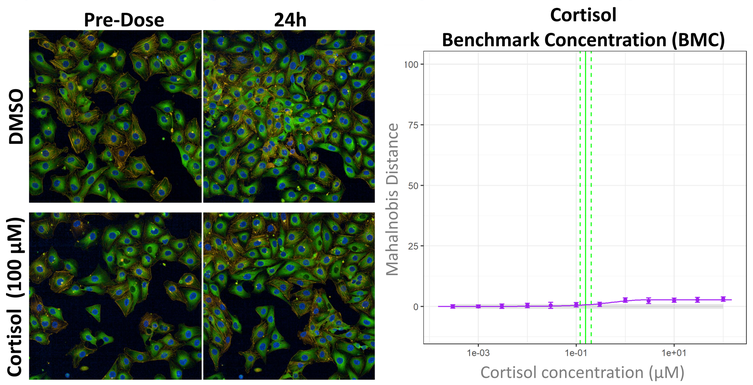


Conclusions:

- Cortisol co-exposure shifted the potency of some of the chemicals and had little or no effect in others
- The degree and direction of potency shifts were chemical specific and often grouped by mechanism
- When there was a shift, higher cortisol tended to increase the potency of the chemical (lower BMC)

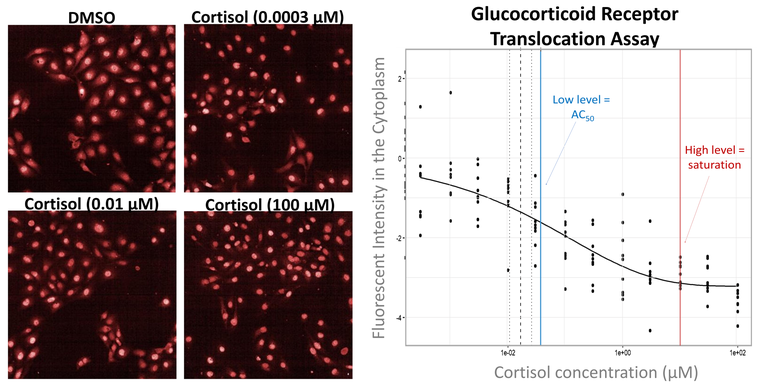
Pilot Study Results

A) Cortisol is Not Cytotoxic and the Impact on Morphology is Minor



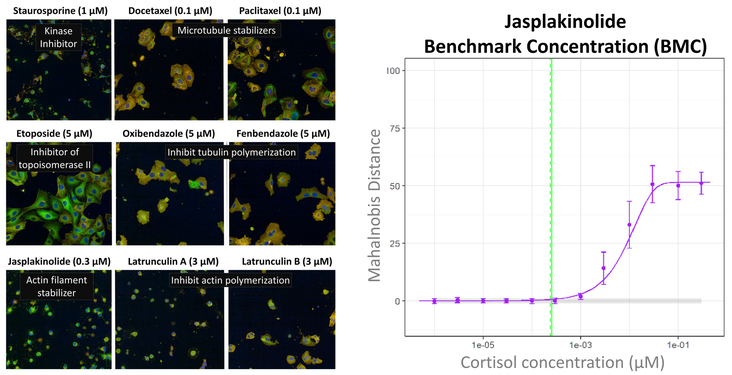
- Statistical analysis using Mahalanobis distance showed that cortisol had only a minor effect on cell morphology at 100 µM
- This was expected, since cortisol should not be overtly cytotoxic to cells

B) Glucocorticoid Receptor (GR) Responds to Cortisol in the Cell Media



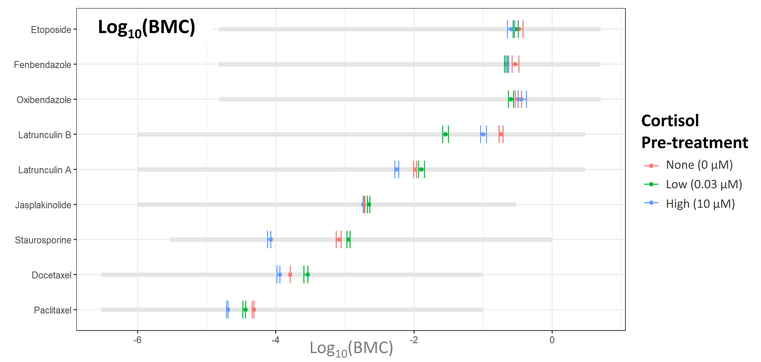
- We performed a glucocorticoid receptor (GR) translocation assay that showed a decrease in cytoplasm fluorescent intensity with increased cortisol, which confirmed that the cells respond to cortisol in the media
- This determined the cortisol dose levels for pilot co-exposures shown in Panel D: the low cortisol level (0.03 µM) was set to the active concentration 50% (AC_{50}) and the high cortisol level (10 µM) was set to the point at which assay saturation was reached, which indicates maximal glucocorticoid receptor response

C) Selected Cytoskeletal Toxicants Greatly Impact Cell Morphology



- We have conducted a pilot screen on nine toxicants that impact the cytoskeleton, the images above demonstrate the types of impact these chemicals have on cell morphology following 48-hours of chemical exposure at high doses (before cortisol co-exposure)
- Chemical mechanisms of action are overlaid on the cell images (images on the left)
- These chemical-only exposures were used to limit the chemical concentrations to non-cytotoxic doses
- An exemplar benchmark concentration curve is shown for Jasplakinolide (right)

D) Effects of 24 HR Cortisol Pre-Treatment on Benchmark Concentrations (BMCs) Varies by Reference Chemical, Tending Towards Higher Potency



- Features were reduced into a single latent variable (global Mahalanobis distance) for calculating benchmark concentrations (BMCs); larger distances indicate greater divergence in cellular phenotypes compared to control (see Nyffeler et al, 2021, PMID: 32862757)
- The thick gray lines are the range of concentrations tested for each chemical (12 conc, ½ log spacing)
- **When there was an effect from cortisol, high cortisol levels tended to cause chemical activity to occur at lower concentrations (a left-shift in the BMC), indicating higher chemical potency** compared to low or no cortisol conditions

The views are the authors' and do not necessarily represent the views of the U.S.EPA. The authors declare no conflicts of interest. Cell phenotyping images are by Clinton Willis.