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In vitro screening for population variability in toxicity of pesticide-containing mixtures

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ABSTRACT

Population-based human in vitro models offer exceptional opportunities for evaluating the potential hazard and mode of action of chemicals, as well as variability in responses to toxic insults among individuals. This study was designed to test the hypothesis that comparative population genomics with efficient in vitro experimental design can be used for evaluation of the potential for hazard, mode of action, and the extent of population variability in responses to chemical mixtures. We selected 146 lymphoblast cell lines from 4 ancestrally and geographically diverse human populations based on the availability of genome sequence and basal RNA-seq data. Cells were exposed to two pesticide mixtures – an environmental surface water sample comprised primarily of organochlorine pesticides and a laboratory-prepared mixture of 36 currently used pesticides – in concentration response and evaluated for cytotoxicity. On average, the two mixtures exhibited a similar range of in vitro cytotoxicity and showed considerable inter-individual variability across screened cell lines. However, when in vitro-to-in vivo extrapolation (IVIVE) coupled with reverse dosimetry was employed to convert the in vitro cytotoxic concentrations to oral equivalent doses and compared to the upper bound of predicted human exposure, we found that a nominally more cytotoxic chlorinated pesticide mixture is expected to have greater margin of safety (more than 5 orders of magnitude) as compared to the current use pesticide mixture (less than 2 orders of magnitude) due primarily to differences in exposure predictions. Multivariate genome-wide association mapping revealed an association between the toxicity of current use pesticide mixture and a polymorphism in rs1947825 in *C17orf54*. We conclude that a combination of in vitro human population-based cytotoxicity screening followed by dosimetric adjustment and comparative population genomics analyses enables quantitative evaluation of human health hazard from complex environmental mixtures. Additionally, such an approach yields testable hypotheses regarding potential toxicity mechanisms.

1. Introduction

Pesticides are chemicals that are used to kill, repel, or control certain forms of plant or animal life that are considered to be pests (Krieger, 2010). Adverse health effects of pesticides can range from mild skin and mucous membrane irritation to more severe outcomes such as neurotoxicity and cancer (Bassil et al., 2007; Rother, 2014; Sanborn et al., 2007). Moreover, potential for adverse effects following exposure may be higher among vulnerable individuals, life stages or sub-populations (Jurewicz and Hanke, 2008; Perry et al., 2014). There are several challenges in the evaluation of the human health hazard of pesticides. First, pesticides have variable modes of action (MOA) dependent on use and activity, and are meant to be harmful and toxic to pests, but not humans. Second, because they are widely used in agricultural and household settings, people are frequently exposed to pesticide residues. Third, humans are typically exposed to mixtures of pesticides, creating challenges in hazard evaluation (Feron et al., 1998; Manikkam et al., 2012).

While safety testing of the individual pesticides is conducted according to established regulatory guidelines (Babut et al., 2013), evaluation of the toxicity of mixtures is less structured (U.S. EPA, 2002). The cumulative risk assessment is conducted for mixtures of chemicals with common mechanisms of toxicity, even though data are usually available only for individual chemicals. Indeed, current toxicity testing paradigms have been questioned for their failure to consider commonly occurring co-exposures and the magnitude of human population variability in response to chemicals (National Research Council, 2009).

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Whole animal testing is difficult to employ for evaluating the hazards of chemical mixtures. In contrast, in vitro testing allows greater flexibility, as chemicals can be grouped according to their effects on key biologic pathways or tested over a broad range of concentrations to capture varied exposure scenarios in a rapid and inexpensive manner (Andersen and Krewski, 2009). The resulting data could enable an informed and focused approach to the problem of assessing hazard in risk-relevant manner in human populations that are exposed to mixtures. Furthermore, with an experimental in vitro design that represents a human population, we are able to explore not only the hazard, but also its intrinsic variability across different concentration ranges (Lock et al., 2012; O'Shea et al., 2011). Such information would be valuable to inform regulatory decisions that could more fully protect public health and sensitive subpopulations (Abdo et al., 2015).

In this study, we addressed the hypothesis that comparative population genomics with efficient in vitro experimental design can be used for evaluation of the potential for hazard, mode of action, and the extent of population variability in responses to chemical mixtures. Specifically, we aimed to address two important issues, mixtures toxicology and exploration of genetically-based inter-individual variation using in vitro cytotoxicity study design. While the emphasis was on genetic variability, the use of the two mixtures as testing agents allowed for a greater exploration of the genetic space because, for example, polymorphisms in different pathways may be the determinants of the variability for different chemicals. The overall study design is depicted in Supplemental Fig. 1. We screened 146 lymphoblast cell lines (LCLs) from four ancestrally and geographically diverse populations with publicly available genotypes and sequencing data from the 1000 Genomes Project (1000 Genomes Project Consortium, 2010). Cells were exposed to two pesticide mixtures (an environmental sample, comprised primarily of a mixture of organochlorines extracted from a passive surface water sampling device, and a mixture of 36 currently used pesticides) at 8 concentrations. Cytotoxic response was assessed using an effective concentration threshold of 10% (EC_{10}), designed to be relevant to the dose–response evaluation commonly used in quantitative risk assessment practice and to meaningfully capture ranges of variation in response across individuals. Genome-wide association mapping was performed to evaluate the genetic determinants of susceptibility. Furthermore, in vitro-to-in-vivo extrapolation with reverse dosimetry was utilized to translate the in vitro concentrations to oral equivalents, which were then compared to predicted human cumulative exposures.

2. Materials and methods

2.1. Experimental design

2.1.1. Cell lines

A set of 146 immortalized LCLs was acquired from Coriell Cell Repositories (Camden, NJ). The 146 cell lines represent 4 ancestrally and geographically diverse populations (Table 1): Utah residents with Northern & European ancestry (CEU); Tuscan in Italy (TSI); Yoruban in Ibadan, Nigeria (YRI); and British from England & Scotland (GBR). Cell lines were chosen based on the availability of dense genotyping information (1000 Genomes Project Consortium et al., 2012). Screening was conducted in two batches, and cell lines were randomly divided into batches without regard to family structure, but with equal representation of population and gender. Cells were cultured in RPMI 1640 media (Gibco, Carlsbad, CA) supplemented with 15% fetal bovine serum (HyClone, South Logan, UT) and 1% penicillin-streptomycin (Gibco) and cultured at 37 °C with 5% CO_2. Media was changed every 3 days. Cell count and viability were assessed once a day for five days for all cell lines using Cellometer Auto T4 Plus (Nexcelom Bioscience, Lawrence, MA). Cells were grown to a concentration of up to 10^6 cells/ml, volume of at least 100 ml, and viability of >85% before exposures. After centrifugation, the cells were re-suspended in fresh media. Cells (100 μl containing 10^4 cells) were aliquoted to each well in a 96-well treatment plate (following the addition of the chemicals) and mixed using the Biomek 3000 robot. Plates were incubated for 24 h after treatment at 37 °C and 0.5% CO_2.To increase the robustness of the data and to evaluate reproducibility, each cell line was seeded in at least two plates so that each compound would be screened in each cell line on 2 or more plates.

Table 1
Human populations from which lymphoblast cell lines were selected for this study.

Population	# of cell lines screened	% of total	N males	N females
CEU: Utah residents with Northern & Western European ancestry	47	32.2	24	23
YRI: Yoruban in Ibadan, Nigeria	40	27.4	19	21
TSI: Tuscan in Italy	32	21.9	16	16
GBR: British from England & Scotland	27	18.5	14	13
Total	146	100	73	73

2.1.2. Chemical mixtures

Cells were exposed to two environmental chemical mixtures. First mixture, referred to as "chlorinated pesticide mixture" throughout the manuscript, is an environmental sample obtained from a universal passive sampling device deployed for 30 days in surface water next to a chlorinated pesticide storage facility. In this extract, 10 pesticides were present in detectable quantities in the post-collection laboratory analysis (see Table 2 for a complete list of pesticide chemicals identified by mass spectrometry). The second mixture, referred to as "current use pesticide mixture", was a laboratory-generated mixture of 36 currently used pesticides with relative concentrations selected to mimic fractional composition of the pesticide exposures in Eastern North Carolina (Table 3). Stock solutions of each mixture were further diluted with dimethyl sulfoxide (DMSO) 8-fold in ½-log step-wise manner. Final cumulative concentrations ranged from 0.032 to 370.4 μM for the current use pesticide mixture and from 0.022 to 65.7 μM for the chlorinated pesticide mixture in 0.5% (vol/vol) DMSO. The mixtures were aliquoted to 96-well plate format using Biomek 3000 robot (Beckman Coulter, Inc., Brea CA). The negative control was DMSO at 0.5%; the positive control was tetra-octyl ammonium bromide at 46 μM.

2.1.3. Cytotoxicity profiling

The CellTiter-Glo Luminescent Cell Viability (Promega, Madison, WI) assay was used to assess intracellular ATP concentration, a marker for cytotoxicity, 40 h post treatment. Time points were selected based on previous experiments at the National Institutes of Health Chemical Genomics Center (Xia et al., 2008). A ViewLux plate reader (PerkinElmer, Shelton, CT) was used to detect luminescent intensity.

2.2. Data processing

2.2.1. Cytotoxicity EC_{10} estimation and outlier detection

Cytotoxicity data were normalized relative to positive/negative controls as described elsewhere (Abdo et al., 2015). We derived an effective concentration 10th percentile (EC_{10}) to provide a single cytotoxicity dose summary per chemical and cell line. The derivation of EC_{10} was based on the logit model:

$$\log\left(\frac{\eta-\theta_{\min}}{\theta_{\max}-\theta_{\min}}\right)=\beta_0+\beta_1 d,$$

with $y=\eta+\varepsilon, \varepsilon \sim N(0,\sigma^2)$, where y is the observed normalized signal representing proportion of surviving cells (which we term the "cytotoxicity value"), d is the log(concentration) for each chemical, and θ_{max} is the limiting mean cytotoxicity value for the zero concentration. θ_{min} was set to zero, to avoid difficulties in estimating the minimum cytotoxicity value for chemicals with low cytotoxicity. An exception was made for chemicals in which the cytotoxicity value at the highest concentration was higher than 0.4, as a very few number of plates/chemicals did