# *In Vitro* Screening for Population Variability in Chemical Toxicity

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Immortalized human lymphoblastoid cell lines have been used to demonstrate that it is possible to use an *in vitro* model system to identify genetic factors that affect responses to xenobiotics. To extend the application of such studies to investigative toxicology by assessing interindividual and population-wide variability and heritability of chemical-induced toxicity phenotypes, we have used cell lines from the Centre d'Etude du Polymorphisme Humain (CEPH) trios assembled by the HapMap Consortium. Our goal is to aid in the development of predictive *in vitro* genetics-anchored models of chemical-induced toxicity. Cell lines from the CEPH trios were exposed to three concentrations of 14 environmental chemicals. We assessed ATP production and caspase-3/7 activity 24 h after treatment. Replicate analyses were used to evaluate experimental variability and classify responses. We show that variability of response across the cell lines exists for some, but not all, chemicals, with perfluorooctanoic acid (PFOA) and phenobarbital eliciting the greatest degree of interindividual variability. Although the data for the chemicals used here do not show evidence for broad-sense heritability of toxicity response phenotypes, substantial cell line variation was found, and candidate genetic factors contributing to the variability in response to PFOA were investigated using genome-wide association analysis. The approach of screening chemicals for toxicity in a genetically defined yet diverse *in vitro* human cell-based system is potentially useful for identification of chemicals that may pose a highest risk, the extent of within-species variability in the population, and genetic loci of interest that potentially contribute to chemical susceptibility.

*Key Words:* chemical toxicity; human; lymphoblasts; population variability.

Environmental and public health agencies are faced with the overwhelming task of assessing the human risk from tens of thousands of chemicals that are released in the environment each year (Judson *et al.*, 2009). Traditional approaches to toxicity characterization rely on extensive animal testing, cost millions of dollars, can take many years per chemical, and have significant uncertainty factors. Because of the time consuming and costly nature of *in vivo* testing, data currently exist for only a small fraction of the chemicals of potential concern (Judson *et al.*, 2009). The National Research Council report (NRC, 2007) argues for a transition from *in vivo* models to *in vitro/in silico* methods in order to capitalize on the advances in technology and computational biology to more efficiently and effectively predict human health risk and reduce the time and cost of testing. Governmental agencies in the United States have responded by initiating large-scale high-throughput screening programs, devising methods to coordinate data, and considering modified approaches to risk assessment (Collins *et al.*, 2008).

To address the pressures for more efficient risk assessment and prioritization strategies, there is a need for *in vitro* toxicity testing in population-based models (NRC, 2008). Although genetic information can play a key role in understanding and quantifying human susceptibility, an essential step in many of the risk assessments used to shape policy (Cullen *et al.*, 2008), traditional toxicity testing paradigms do not address this critical need. Toxicity data derived from studies on lower organisms (such as yeast), only a single cell line, or rodent strain cannot be used to understand how constitutional genetic variation between individuals in a human population may affect toxicity (Rusyn *et al.*, 2010). In the absence of an understanding of population genetic variation, default uncertainty factors, rather than scientific data, drive risk assessment decisions on most chemicals. Better population-based, genetically defined models are needed to fill these data gaps and to develop more meaningful uncertainty factors.

Recent studies using immortalized human lymphoblastoid cell lines (LCLs) from the International HapMap Consortium show promise in filling this gap with meaningful data. This *in vitro* population-based model has been used successfully to evaluate interindividual and gender-specific differences in responses to drugs (Dolan *et al.*, 2004; Watters *et al.*, 2004) and to identify genes in the transcriptional response to radiation, including genes involved in cell cycle control, DNA repair, and cell death (Jen and Cheung, 2003). HapMap cell lines of Northern and Western European ancestry (CEU) are arranged in family trios, have been densely genotyped

(Frazer *et al.*, 2007), and provide large, renewable sample sets for a wide range of studies (Meucci *et al.*, 2005). Although HapMap cell lines have important limitations (Choy *et al.*, 2008), we hypothesized that a population-based human *in vitro* model may be used for assessment of interindividual and population-wide variability in chemical-induced toxicity phenotypes and provide information for the biological interpretation of the variability.

The Centre d'Etude du Polymorphisme Humain (CEPH) panel of LCL trios was treated with 14 model environmental toxicants, and two widely used markers (Huang *et al.*, 2008; Xia *et al.*, 2008) of cell death/viability (ATP production and caspase-3/7 activity) were used to assess toxicity. We show that this *in vitro* genetics-anchored human model system can be used to demonstrate (1) the utility of a population-based approach to screening for chemical toxicity and (2) the potential to identify the genetic susceptibility factors that can be used as candidates in genotype-phenotype studies.

## MATERIALS AND METHODS

*Chemicals.* A set of 14 chemicals includes model chemicals representing a wide range of classes of toxicants. Chemicals were obtained from the U.S. Environmental Protection Agency National Center for Computational Toxicology and dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 20mM, aliquoted to a 96-well plate, and stored at −20°C. Chemical concentrations for *in vitro* experiments were chosen from the reports in the literature. Dilutions (100×) were prepared using dimethyl sulfoxide according to the necessary final concentration, and a 96-well working plate was kept at room temperature for no longer than 10 days. The 96-well treatment plates were prepared by aliquoting 1 µl each of 14 chemicals at three concentrations (with a replicate of each concentration) using Biomek 3000 (Beckman Coulter, Fullerton, CA) robot. Wells containing vehicle alone (1% final concentration), cells in vehicle, and a positive control (tetraoctyl ammonium bromide) were also included. Final chemical concentrations and plate design are shown in Supplementary figure 1.

*Cell lines and culture conditions.* A panel of 87 immortalized human lymphoblast cell lines from the CEPH trios assembled by the HapMap Consortium was purchased from Coriell Cell Repository (Camden, NJ). Each of the CEU trios consists of a family of two parents and one child. Cell lines were cultured as a suspension in RPMI 1640 media (Gibco, Carlsbad, CA) supplemented with 15% Fetal Bovine Serum (HyClone, South Logan, UT) and 1% Pen-Strep (Gibco) as recommended by Coriell and maintained at 37°C and 5% $CO_2$. Cell density and viability were assessed prior to treatment using Cellometer Auto T4 Plus (Nexcelcom Bioscience, Lawrence, MA). Cells were grown to a concentration of $10^6$ cells per milliliter, volume of at least 12 ml, and viability of at least 85% before treatment. Cells (100 µl containing $10^4$ cells) were aliquoted to each well in a 96-well treatment plate (following addition of the chemicals, see above) and mixed using the Biomek 3000 robot. Plates were incubated for 24 h after treatment at 37°C and 0.5% $CO_2$.

*Cell viability and caspase-3/7 assays.* Following a 24-h incubation period, Cell-Titer-Glo Luminescent Cell Viability and Caspase-Glo 3/7 (Promega Corporation, Madison, WI) assays were used according to manufacturer's protocol. These assays assess ATP production, a marker of cell viability, and caspase-3/7 activity, a measure of apoptosis. Luminescence was measured and recorded using DTX880 (Beckman Coulter) plate reader. Replicate wells at each dose were averaged, and raw values were normalized relative to the baseline (cells in vehicle) and background (vehicle only) at each dose. These

normalized percentage of control values were used in all subsequent analyses. A subset of replicate cell viability (18 cell lines) and caspase-3/7 (31 cell lines) experiments were performed independently to evaluate experimental variability. Raw data are publicly available from PubChem (AID: 1976).

*Receiver operating characteristic curve analysis and response classification.* Values from the replicate experiments were used to determine experimental variability of chemical-elicited responses for each assay. Although the data are inherently quantitative, it is useful to develop thresholds for classification of individual cell lines as "responders" and "nonresponders." The interplate replicated values ("first run" and "second run") provide a useful comparison to establish thresholds to maximize reproducibility. Briefly, for each of the 14 chemicals, a threshold was applied to the quantitative response for the second run and used to provisionally classify individuals as responders/nonresponders. By comparing these provisional dichotomized values to the original quantitative response values for the first run, a receiver operating characteristic (ROC) curve analysis can be used as a measure of correspondence. Here the dichotomized response values serve the role of "true negative/positive" classification in the ROC calculation, although it should be recognized that our purpose is different than for typical ROC analysis. Each threshold resulted in a different ROC curve and an accompanying area underneath the curve. By examining various possible thresholds, we found the assay response value that gave the largest area underneath the ROC curve, and this value was used as the response/nonresponse threshold applied to the plate- and replicate-averaged response (see Supplementary table 1).

*Estimation and testing of heritability.* Heritability estimates for parent-child trios follow from a standard quantitative trait model: $y = \beta_0 + \beta_1 (a_m + a_p) + \varepsilon$ for normalized ATP or caspase-3/7 phenotypes $y$ as an average across plate and replicate measurements (see, e.g., similar expressions in Roy-Gagnon *et al.*, 2008), where $a_m$ and $a_p$ represent the separate maternal and paternal allelic contributions across all contributing loci, i.e., the polygenic effects. The normal error terms $\varepsilon$ were assumed to be independent across individuals. We did not include an extra family-specific environmental term, reasoning that the above model would potentially slightly overestimate heritability, and any apparently significant results would be subject to additional scrutiny and modeling. A straightforward variance component approach implemented in R (v. 2.10.1) fits each family separately as multivariate normal, with fixed effect mean $\beta_0$, overall variance $\sigma_g^2 + \sigma_\varepsilon^2$ (the genetic and error variances), covariances 0 between the unrelated parents, and covariances $\left(1/2\right)\sigma_g^2$ between parents and children. A likelihood ratio (LR) statistic with 1 degree of freedom was formed for the term $\sigma_g^2$, equivalent to testing $h^2 = \sigma_g^2/\left(\sigma_g^2 + \sigma_\varepsilon^2\right) > 0$. The positive constraint on the variance term implies that $2\log(\text{LR})$ is approximately distributed as a mixture of 0 and $\chi_1^2$, and this approximation was used to obtain $p$ values. The suitability of this approximation for a sample size of 30 trios was confirmed via additional simulations (data not shown).

*Modeling of cell line, plate, and replicate effects.* The availability of multiple plate measurements on a subset of individuals enabled dissection of the variance components because of each of the cell line, plate, and replicate effects for each chemical and each of the normalized ATP and caspase measurements. For the ATP assay, 16 cell lines and 34 plates were used for this modeling, with 2 plate replications for 14 cell lines and 3 plate replications for 2 cell lines. For the caspase-3/7 assay, 20 cell lines and 50 plates were used for this modeling, with 2 plate replications for 13 cell lines, 3 plate replications for 4 cell lines, and 4 plate replications for 3 cell lines. Using the terminology "north" and "south" to represent the upper and lower positions of replicates on the same plate (Supplementary fig. 1), we used the lme4 function in R v. 2.10.1 to fit the hierarchical model $y_{ijk} = \beta_0 + \beta_1 I\left(i = 2\right) + b_k^{(3)} + b_{jk}^{(2)} + \varepsilon_{ijk}$, where $\varepsilon_{ijk} \sim N\left(0, \sigma_1^2\right)$, $b_{jk}^{(2)} \sim N\left(0, \sigma_2^2\right)$, and $b_k^{(3)} \sim N\left(0, \sigma_3^2\right)$, for the $i$th replicate within plate ($i = 2$ corresponding to south), the $j$th plate, and the $k$th cell line. Here $\beta_0$ is a fixed intercept term, and $\beta_1$ is a fixed position effect, to account for possible systematic position bias. The terms $\sigma_1^2$, $\sigma_2^2$, $\sigma_3^2$ correspond to variance contributions from (replicate) error, plate, and cell line, respectively. Although

the $\beta_1$ term was significant for many chemicals, removal of the term did not appreciably affect the variance components (not shown). The models did not use family relationship information, as the heritability values were not significant, and the inclusion of the relationship information (which largely affects correlation structure in the error terms) would not appreciably change the estimates.

For the genome-wide scans, a single phenotype measurement was used by averaging across the plates and north/south replicates. The modeling approach described above can then be used to estimate the proportion of variability due to cell line for each assay and chemical. We have the new model for the $k$th cell line $y_k = \beta_0 + b_{0k} + \varepsilon_k$, where $y_k$ is the average assay value across north/south replicates and available plates and $b_{0k}$ is a random intercept with variance estimable from the previous variance component modeling. We have $\mathrm{var}(b_{0k}) = \sigma_3^2$, regardless of plate replication, and for cell lines with $r$ plate replicates ($r = 1, 2, 3,$ or $4$), we have $\mathrm{var}(y_k|r) = \sigma_3^2 + \sigma_2^2/r + \sigma_1^2/(2r)$. The proportion of variation due to cell line for the averaged phenotype is, finally, $\mathrm{var}(b_{0k})/\mathrm{var}(y_k)$, where the denominator is averaged over the number of replications.

***Genome-wide association study.*** Genotypes for the cell lines were obtained from phase II genotypes of the International HapMap Project (Frazer *et al.*, 2007). Quantitative transmission disequilibrium testing was applied to evaluate the association between genetic markers and chemical-elicited ATP production and caspase-3/7 activation response phenotypes using PLINK (Purcell *et al.*, 2007). For this analysis, quantile normalization was used on the assay response values to reduce the influence of extreme observations. The University of California-San Cruz Genome Browser (http://genome.ucsc.edu/index.html) (Kent *et al.*, 2002) NCBI Build 36.1 was utilized for examination of genomic loci of interest. To investigate networks related to genes of interest, we used Ingenuity Pathway Analysis Software (http://www.ingenuity.com; Ingenuity Systems, Mountain View, CA).

## RESULTS

### Interindividual Variability

We successfully screened 85 cell lines for cell viability and 83 cell lines for caspase-3/7 activity endpoints. These assays were selected based on their utility for *in vitro* screening of cytotoxicity in cell type- (Xia *et al.*, 2008) and individual-independent manner (Choy *et al.*, 2008). A combination of the two assays compensates for the limitations of the individual assays and therefore can provide sensitive and reliable estimation of cytotoxicity (Shi *et al.*, 2010).

Using "on the plate" duplicates and a subset of independent replicate experiments (containing duplicate wells for each chemical/concentration), we determined the extent of experimental variability. The thresholds established using this analysis (see Materials and Methods section and Supplementary table 1) were used to classify responder/nonresponder cell lines for each chemical and assay. Among the chemicals screened in this study, there was a range of interindividual variability in cytotoxicity across the population of cell lines. Even though three concentrations were tested for each chemical in all cell lines and assays, only the highest concentrations elicited robust responses (data not shown), and further analyses were performed on top concentrations. For cell viability assay, the intraplate correlations between responses to 14 chemicals ($r_{\mathrm{Pearson}} = 0.80 \pm 0.17$, $r_{\mathrm{Spearman}} =$

$0.68 \pm 0.22$) were generally higher than interplate correlations ($r_{\mathrm{Pearson}} = 0.65 \pm 0.28$, $r_{\mathrm{Spearman}} = 0.55 \pm 0.23$). For caspase-3/7 assay, the intraplate correlations ($r_{\mathrm{Pearson}} = 0.89 \pm 0.17$, $r_{\mathrm{Spearman}} = 0.64 \pm 0.14$) were as high as or even lower than those in interplate comparisons ($r_{\mathrm{Pearson}} = 0.88 \pm 0.13$, $r_{\mathrm{Spearman}} = 0.73 \pm 0.16$).

Interindividual (i.e., between cell lines) variability in responses was observed for some, but not all, chemicals in the panel of 14. For each chemical/assay, we used random-effect modeling to determine the portions of the variability due to cell line, plate replicates, and within-plate replicates (see Materials and Methods section and Supplementary tables 2A and 2B). The results indicate substantial portions of variability because of cell line for some chemicals, keeping in mind that the initial modeling reflected portions of variability for each measurement, not considering the impact of averaging across replicates. When the impact of replicate averaging is considered (Supplementary tables 2A and 2B), the proportion of variation because of cell line is even higher, ranging from 15 to 40% for several assay/chemical combinations. The averaged phenotype values are reflective of the individual "phenotype" value used for later association analysis, and such fractions of explained variability compare quite favorably to the cumulative effect size distributions for modern genome-wide association study data sets (Park *et al.*, 2010), although additional heritability investigation is warranted. Basal values for each of the assays tended to be correlated with the response values at the highest dose; thus, the analysis using normalized values was key to eliminate potentially spurious correlation because of the baseline differences between cell lines.

Representative examples of trends in responses that were seen across the population of cell lines are shown in Figure 1. The cell viability (ATP production) response (Fig. 1A) was most variable for perfluorooctanoic acid (PFOA) (100µM) and phenobarbital (3mM), and there is correlation between the two on the individual cell line level. At the same time, the loss of cell viability induced by rifampin (100µM) was very consistent across the panel. Induction of apoptosis (activation of caspase-3/7) phenotype (Fig. 1B) was most variable with phenobarbital; however, PFOA and propiconazole (100µM) did exhibit some but not as wide a range of responses.

### Population-Wide Variability

Next, we assessed the ranges of variability in the population that were exhibited by the chemicals tested. Varying degrees of population-wide variability were exhibited in both phenotypes in response to the panel of 14 chemicals (Figs. 2A and 2B). Most of the chemicals tested showed rather narrow ranges of variability and little overall effect, except for PFOA and phenobarbital. The number of responder cell lines (see Materials and Methods section for a description of the classification) is shown in Figures 2C and 2D. Rifampin, myclobutanil (100µM), and propiconazole caused a robust decrease in ATP production (loss of cell viability) in half or
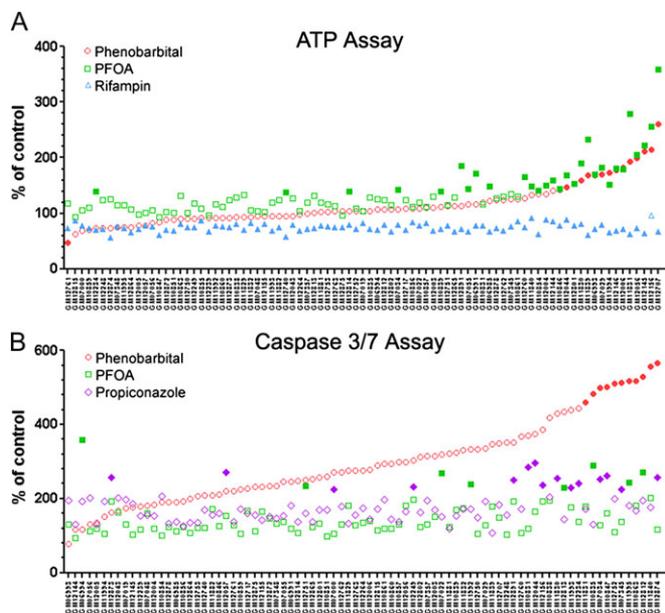
**FIG. 1.** Interindividual variability in response to toxicants in a panel of human lymphoblast cell lines. Production of ATP (A) and activation of caspase-3/7 (B) were assessed 24 h after treatment with phenobarbital (○, 3000μM), PFOA (□, 100mM), rifampin (▲, 100μM), or propiconazole (◇, 100μM). Percent change over the baseline (vehicle, 1% DMSO) in each cell line is shown. Cell lines are ordered (in each panel separately) according to the response to phenobarbital. Cell lines were considered responders (filled symbols) if percent change was exceeding the significance threshold (see Materials and Methods section ).

more of the cell lines even though there was little population-wide variability. Polychlorinated biphenyl-118, PFOA, and phenobarbital had an opposite effect on the ATP production phenotype. It has been reported previously that some cytotoxic treatments may lead to a transient increase in the intracellular ATP, an effect attributed to enhancement in energy production when cells are undergoing self-repair in response to toxic challenge shortly after exposure (Shi *et al.*, 2010). Propiconazole, and phenobarbital also caused the most increases in activation of caspase-3/7, even though the fraction of responder cell lines was much smaller, only 10–20%. Interestingly, whereas rifampin exhibited a uniform effect of loss of ATP production in this panel of cell lines and thus produced the highest number of responders, chemicals that showed great variability in responses (e.g., PFOA, phenobarbital, and propiconazole) also elicited a number of robust responses in both assays. It should be noted that cytotoxicity was observed in at least one individual in response to most of the chemicals (except for mono-ethyl-hexyl phthalate, PFOA, and perfluorooctanesulfonic acid for the ATP assay, and di-ethyl-hexyl phthalate and diethyl ether for the caspase-3/7 assay), further arguing for the utility of screening in a genetically diverse panel of cells whereby both the population-wide and the individual's responses can be evaluated.

## Comparison of Chemical-Elicited Responses

Similarities and/or differences in response to chemicals in two assays probing divergent modes of cell death in a population may provide additional clues for mode of action analysis. We compared compound effects both within and between assays by calculating correlations between compounds with respect to their population-wide effects. A heat map (Fig. 3) of Spearman correlation coefficients (see sidebar scale for reference) shows that strong concordance of responses across a population exists for some chemicals known to have similar modes of action (or a parent-metabolite pair) within each assay. However, some closely related chemicals, such as PFOA and perfluorooctanesulfonic acid, did not elicit highly similar responses within either assay. In addition (as is also shown on Fig. 1), responses to PFOA and phenobarbital were highly correlated in the ATP production assay, but not in the caspase-3/7 assay. Correlation of compound's activity between the two assays was also calculated; yet, no significant correlations between chemical-elicited responses in ATP production and caspase-3/7 activity were found (data not shown).

In addition to evaluating similarities in responses across chemicals, we assessed similarities among cell lines in the population. Correlations between responses to the 14 compounds in these cell lines were evaluated and unsupervised clustering performed (average linkage hierarchical clustering using the correlation metric) using the data from ATP production (Fig. 4A) and caspase-3/7 activation (Fig. 4B) assays. Similar to the observations in Figure 3, chemicals of the same class showed similar responses and clustered together. Across the individual cell lines, subclusters of response are apparent; however, no familial clustering of cell lines was evident for responses to this panel of chemicals.

As a more formal approach to assess the impact of familial relationships, we performed variance components heritability testing in the trios for each chemical/assay (see Materials and Methods section  and Supplementary table 3). None of these results was significant, an observation which is consistent with informal examination of mid-parent assay values compared with those of the offspring (data not shown).

## Phenotype-Genotype Association Mapping

The cell-based model system used in this study is not only genetically diverse but is also genetically defined, with dense genotyping information (several million single nucleotide polymorphisms [SNPs]) publicly available (Frazer *et al.*, 2007). Thus, it is possible to examine associations between the chemical-induced phenotypes (ATP production and caspase-3/7 activity) and the genetic markers, investigating potential genetic contributions to the observed variability in responses. Although phenotype-genotype relationships were assessed using genome-wide association scans (see Materials and Methods section ) on all chemical/assay combinations, we report here a detailed analysis of the effect of PFOA
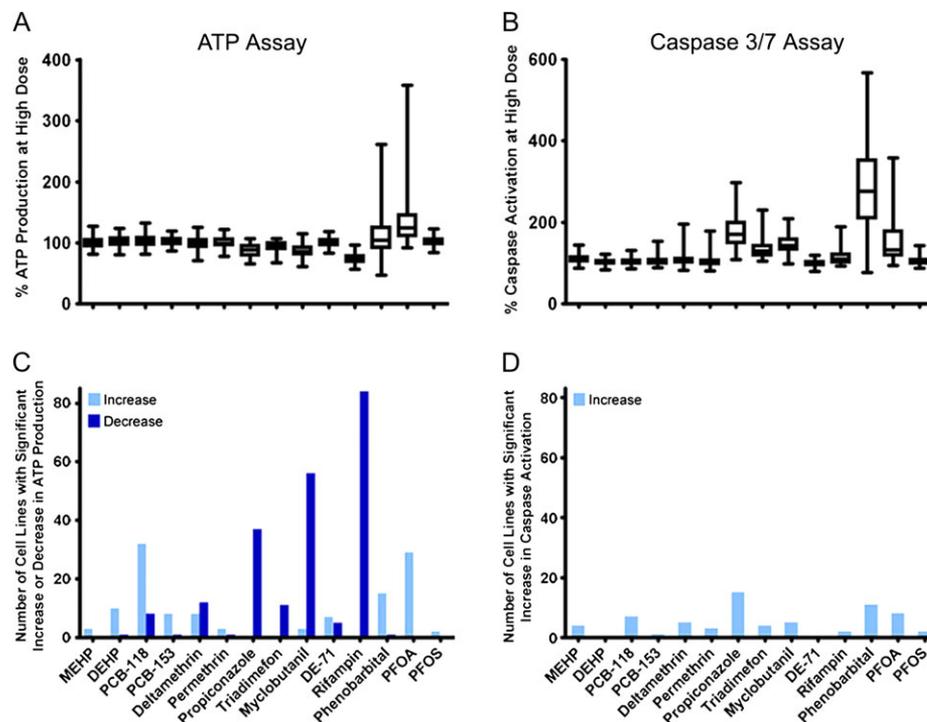
**FIG. 2.** Population-wide variability in response to 14 model toxicants. Production of ATP (A and C) and activation of caspase-3/7 (B and D) in a panel of human lymphoblast cells were assessed 24 h after treatment with the highest dose (see Supplementary fig. 1) of each compound. Box and whiskers plots (A and B) were used to exhibit the variability in responses across the population. Bar graphs (C and D) demonstrate the number of cell lines with a significant response above (light bars) or below (dark bars) the vehicle control.

in ATP production (cell viability) assay. PFOA elicited the widest range of interindividual variability in this phenotype, with close to 35% of cell lines classified as responders, across the population of cells. Both pharmacokinetics and toxicity of PFOA are known to be highly variable in many species, as evidenced, e.g., by studies with controlled exposures in nonhuman primates (Butenhoff *et al.*, 2002). In addition, PFOA was estimated to exhibit approximately 20% variation due to cell line for the averaged response (Supplementary table 2), although as described earlier there was no significant evidence of heritability. In such circumstances, the power to detect significant associations is likely to be limited, but a genome scan for these samples can nonetheless be performed as the genotypes of these cell lines are publicly available, potentially identifying candidate regions for follow-up analyses.

The genome-wide plot in Figure 5A shows ~2.2 million SNPs and their strengths of association with PFOA-elicited ATP production phenotype. Using a genome-wide threshold of $p < 10^{-6}$ as suggestive evidence identifies loci on chromosomes 4 (Fig. 5B) and 14 (Fig. 5C). Within the 500-kb loci flanking SNPs with highest association, there are several potential candidate genes for susceptibility to PFOA. On chromosome 4, FAT tumor suppressor homolog 1 (*FAT1*) is a human gene whose homolog in the rat has been shown to be responsive to PFOA treatment (in the liver) (Guruge *et al.*,

2006). On chromosome 14, three genes were located in the candidate quantitative trait locus (QTL): solute carrier family 24 (sodium/potassium/calcium exchanger), member 4 (*SLC24A4*); cleavage and polyadenylation specific factor 2 (*CPSF2*); and Ras and Rab interactor 3 (*RIN3*). *SLC24A4* is a sodium ion carrier, and numerous genes from the solute ion carrier family have been identified as responsive to PFOA treatment in rat liver, rat kidney, and mouse liver (Guruge *et al.*, 2006; Kudo *et al.*, 2002; Rosen *et al.*, 2007). Although *CPSF2* and *RIN3* have not been shown in previous studies to be responsive to treatment with PFOA, they are tightly linked through a gene network to genes that have been observed as responsive to PFOA treatment in other species. Networks for *CPSF2* (Fig. 5D) and *RIN3* (Fig. 5E) show the interactions with immunoglobulin heavy constant mu and RAB5A/B, member of RAS oncogene family (*RAB5A* and *RAB5B*), respectively, which are responsive to PFOA in rat and chicken liver (Guruge *et al.*, 2006; Yeung *et al.*, 2007).

## DISCUSSION

Although the utility and relevance of the cell-based models with regard to species, metabolism capacity, concentration selection, and other important factors are still under intense debate (Coecke *et al.*, 2006; Hartung and Daston, 2009),
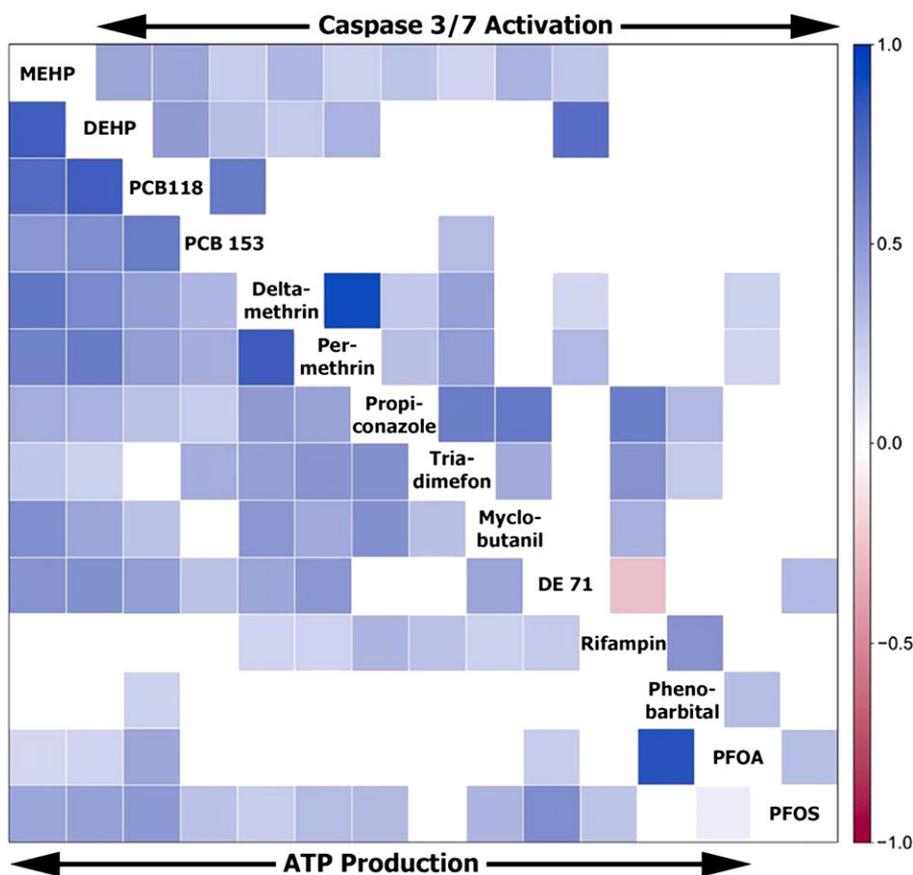
**FIG. 3.** Within-assay correlation map of compound activity across the population. Correlations between compounds with respect to their population-wide effects in the ATP production (bottom left) or caspase-3/7 activation (upper right) assays are shown as a heat map using Spearman correlation coefficient values (see sidebar scale for reference). Only DE 71 and Rifampin (Caspase 3/7 activation) showed negative correlation.

high-throughput *in vitro* screening methods are destined to become the major means for addressing numerous gaps in chemical toxicity data (Andersen and Krewski, 2009). One important dimension that is yet to be comprehensively considered in chemical testing is the role of the genetic variability that plays a major role in differential susceptibility to chemicals. Indeed, an *in vitro* model system that is genetically defined, representative of a diverse population, and amenable to high-throughput screening could significantly aid in chemical prioritization, identification of susceptibility factors, population-wide uncertainty analysis, and predictive toxicity testing.

The human population-based *in vitro* resource provided by the HapMap Consortium allows generation of phenotype data in a cell-based model system, which contains genetic information representative of a diverse human population (Meucci *et al.*, 2005). The toxicity data obtained in this model system can potentially be combined with high-density genotype information to enable the discovery of genetic causes of susceptibility and variability in response (Watters *et al.*, 2004). Combining genotype and phenotype information for both molecular profiles and complex traits is a promising strategy for understanding which genes, pathways, and

biological processes are also under the influence of a given QTL (Harrill and Rusyn, 2008). Additional advantages of using HapMap LCLs are ease of experimental manipulation and ability to control the experimental environment, allowing the genetic contributions toward a specific chemical-induced phenotype to be tested (Huang *et al.*, 2007).

Some (Choy *et al.*, 2008) have argued that nongenetic factors for the LCL cell lines could offer alternative explanations for cell line variability, hampering efforts to elucidate genetic variation. Although we have taken steps to minimize alternate sources of variation, the question will remain open until clear genetic susceptibility variants have been identified. The establishment of apparent cell line variation indicates the need for caution in interpreting a potential population-wide risk for a chemical, both because of the potential for underlying genetic susceptibility as well as the possibility that genomic assays performed by other researchers on much more limited sample sets have also been subject to such variation. Nonetheless, we point out clear differences in the scientific goals of toxicogenetic testing approaches compared with that of other genetic subdisciplines (e.g., pharmacogenomics). In toxicology, merely establishing
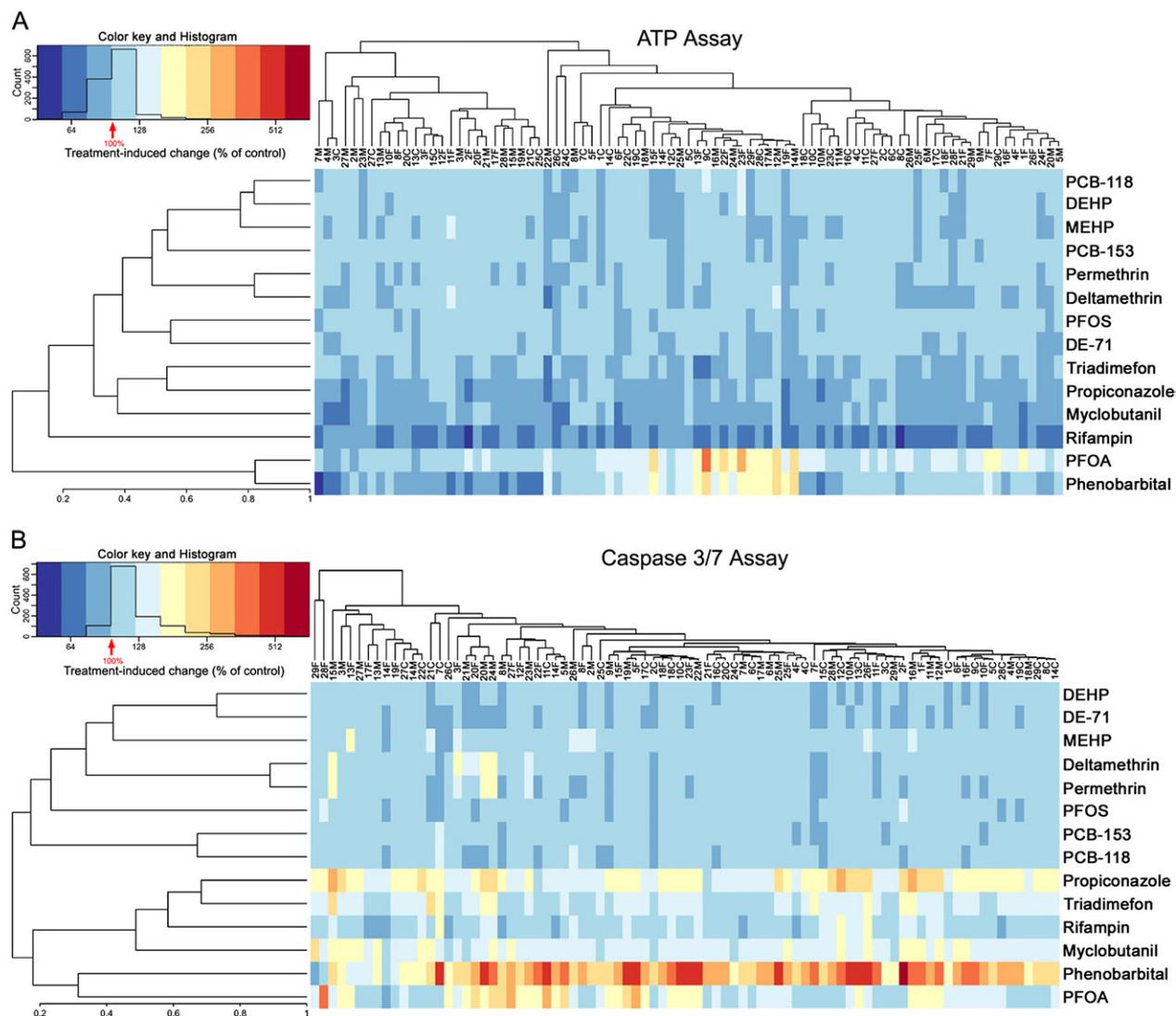
**FIG. 4.** Correlation of responses between chemicals and individuals. Heat maps were generated by clustering (average linkage on the correlation metric) the data on percent ATP production and percent caspase activation at highest dose across all chemicals and individuals. The heat maps are ordered both vertically and horizontally with the most correlated participants and chemicals being displayed near to one another. Data were mean centered and transformed as indicated in the corresponding histograms.

ranges of variability is important, both because such ranges may reflect true underlying genetic variability but also because they may reflect other sources of variability, the extent of which may have gone unrecognized.

The freshly isolated lymphocytes (generally described as peripheral blood mononucleated cells) are widely used as a surrogate tissue for toxicity studies and as a source of DNA for genotyping. Numerous reports in the literature have examined the metabolic capacity of the total lymphocyte preparations, as well as fractions of T and B lymphocytes (Sempoux *et al.*, 1999; Siest *et al.*, 2008; Vanden Heuvel *et al.*, 1993), and their capacity for induction of the metabolism genes by xenobiotics (Krovat *et al.*, 2000). Whereas lymphocytes do not have the metabolic capacity of the liver, or even that of freshly isolated hepatocytes, they do express a number of

nuclear receptors, as well as most genes of the phase I and II metabolism, and transporters (Siest *et al.*, 2008). Thus, even though the metabolism-dependent toxicity may not be detected in cultured lymphocytes and lymphoblasts, this model system is comparable to many other transformed cells; yet, it offers a critical advantage of the defined genetic variability.

This study shows that data produced by testing a set of chemicals in a genetically defined yet diverse human population-based *in vitro* system can be useful in many ways. The examination of interindividual variability shows that for some chemicals, such as phenobarbital and PFOA, there is an appreciable interindividual variability in response across the population, whereas for others, such as rifampin, toxicity response is uniform across the population. This suggests that there is utility of using this population-based *in vitro* system for
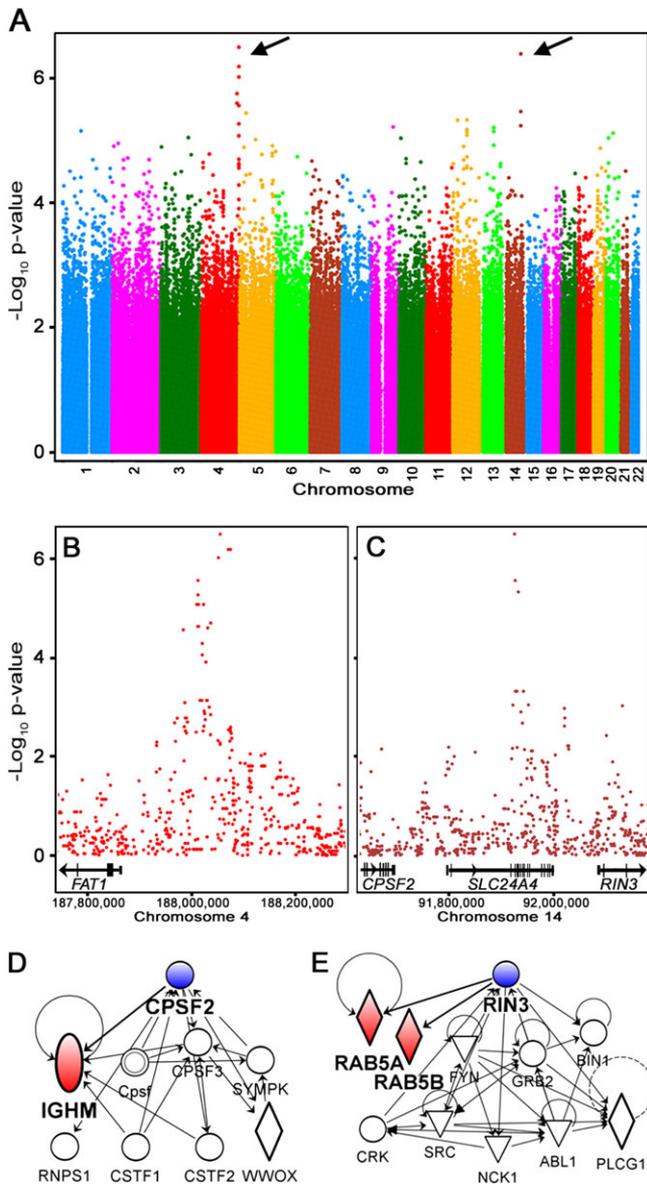
**FIG. 5.** Genome-wide association scan of ATP production response to PFOA. (A) Genome-wide plot of SNPs and their degree of association with PFOA-elicited ATP production phenotype. Arrows indicate the loci with highest association. (B and C) Loci (500 kb) on chromosomes 4 and 14 flanking SNPs that are highly associated ($p < 0.000001$) with PFOA-elicited phenotype. Genes located in these loci are shown as well as gene networks for *CPSF2* (D) and *RIN3* (E). Shaded symbols (IGHM, RAB5A and RAB5B) indicate genes that have been identified as responsive to PFOA treatment in rat and chicken liver (Guruge *et al.*, 2006; Yeung *et al.*, 2007).

both prioritization of chemicals for more in-depth screening (for those exhibiting great variability across the population) and understanding the potential genetic causes of individual susceptibility. In addition, these data represent a measure of the degree of variability in toxicity-relevant phenotypes, information which may be useful in defining the extent of a population-wide uncertainty for risk assessment.

Identifying chemicals with the widest variability in response in a genetically diverse population signifies the need to further examine the mechanisms behind interindividual responses. First, the comparisons of the adverse effects for a panel of chemicals and across a population can generate hypotheses concerning modes of action that may explain the similarities and/or differences within the population and between chemicals. For example, responses to PFOA and phenobarbital were highly correlated in the ATP production assay, but not in the caspase-3/7 assay, indicating similarities in their modes of action with regard to the effect on general cell metabolism and cell proliferation, but not on the activation of apoptosis. Second, the lack of significant correlations between chemical-elicited responses in ATP production and caspase-3/7 activity may indicate that none of the chemicals that we tested reduces cell viability through the apoptotic pathway at the concentrations used. Third, comparing responses to chemicals across the population is another important assessment that provides information on population-wide effects. Lack of familial clustering may indicate that ATP production and caspase-3/7 activation responses to chemicals in this panel of cells are not heritable, but because of the complexity of the traits that we observed and the small number of chemicals, concentrations, and time points tested, further studies may be needed.

In addition, this study identified several potential candidate susceptibility genes for PFOA-induced adverse effects. The genes in the susceptibility loci can be linked either directly or indirectly to the known effects of PFOA in other tissues and species (Guruge *et al.*, 2006; Kudo *et al.*, 2002; Rosen *et al.*, 2007). Existence of previous reports from the *in vivo* model systems provides biological plausibility to the discovered associations and serves as an illustrative example supporting the potential of using high-throughput phenotype and genotype data from an *in vitro* human population-based resource to discover genetic loci that may be relevant to observable trends in phenotypes across the population. In addition, the candidate regions can be further elucidated using existing expression QTL (eQTL) data under the hypothesis that regional SNP variants may act on susceptibility via expression-based regulation. Whereas a search of the eQTL browser (eqtl. uchicago.edu) found no cis-eQTL associations in the *FAT1* region with the requisite evidence for display (*cis*-eQTL evidence with $p < 10^{-3}$), two SNPs (rs12586368 and rs7159431) in intron 1 of *SLC24A4* were reported as having evidence of cis-eQTL action on transcript *CCDC88* and one SNP (rs17783660) in the exon 2 of *RIN3* was reported as having evidence of *cis*-eQTL action on *BTBD7*. All the SNPs and transcripts are in the same 11q32 region based on the RNA-seq data (Montgomery *et al.*, 2010). Although the tissue sources for examination of eQTL evidence are currently limited, larger and richer eQTL data sets will soon be available, with organ-specific evidence of genetic regulation of transcription. Further studies are needed to firmly address the potential biological role of these genes in PFOA-induced adverse effects

and to test these and other candidates in human or animal studies to verify their mechanistic relevance to interindividual susceptibility.

It is important to consider that *in vitro* systems have many limitations. Culture conditions of many cell-based *in vitro* systems do not mimic physiological conditions, are not homeostatic, and are often oxygen deprived. Cell densities are often lower than tissue densities, impairing normal intracellular signaling, and multiple cell types usually are not represented, limiting cell-cell interactions. Many *in vitro* cell-based systems are of cancer origin, which is problematic because it is known that cancerous cells have thousands of mutations and even chromosomal aberrations which can affect phenotypes (Hartung and Daston, 2009), and these limitations make prediction of human toxicity based on *in vitro* toxicity results very challenging. The HapMap cell lines used in this study are not devoid of many of these limitations. In addition, the CEPH panel consists of cell lines derived from a human population that have been immortalized via Epstein-Barr virus transformation to induce long-term growth (Tosato and Cohen, 2007). A concern was expressed that Epstein-Barr virus copy number as well as cellular growth rate and ATP levels play a confounding role in studies using these cell lines to detect genetic contributions to phenotypic traits (Choy *et al.*, 2008).

Although the HapMap cell lines have important limitations, we were able use these cell lines to generate data that can be used for hazard identification in a population context. Our study was limited in size and throughput but serves as an important proof of concept that may be scaled to larger samples. In addition, high-throughput studies should be done to explore a wider range of chemicals and time points to fully explore the ability of this system to provide a measure of population-based dose-response for risk assessment purposes. Nevertheless, our work does demonstrate that a genetically defined, diverse *in vitro* model system can be used to generate data that are useful for exploring population-wide interindividual variability in response, chemical mode of action characteristics, and genetic contributions to observed phenotypes.

## CONCLUSIONS

This study aimed to investigate the utility of using a genetically defined yet diverse human *in vitro* model system to screen for adverse effects of environmental chemicals. A population of cell lines was exposed to a panel of model toxicants, and cell viability and caspase activation were assessed. The interpretation of the data from *in vitro* screening in the population-based cell culture models, rather than collections of unrelated cell lines from multiple species and tissues, is affording several advantages. First, our results show that some, but not all, chemicals elicit interindividual variation

in response to treatment. Chemicals that vary in their effects across the population may need to be prioritized for further testing using additional *in vitro* or *in vivo* approaches. Second, this screening method provides a population-wide measure of uncertainty, information which may be crucial for the future risk assessment approaches that will rely heavily on *in vitro* data (Andersen and Krewski, 2009). Third, such data may be used to explore potential differences/similarities in modes of action between chemicals on the population-wide level. Lastly, by combining the toxicity data and publicly available genetic information, it is possible to probe the contribution of genetics to adverse phenotypes and select the candidate genes and regulatory networks for further studies to verify their mechanistic relevance with regard to differences in susceptibility to chemical treatment.

## SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci .oxfordjournals.org/.

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