

A Quantitative Toxicogenomics Assay for High-throughput and Mechanistic Genotoxicity Assessment and Screening of Environmental Pollutants

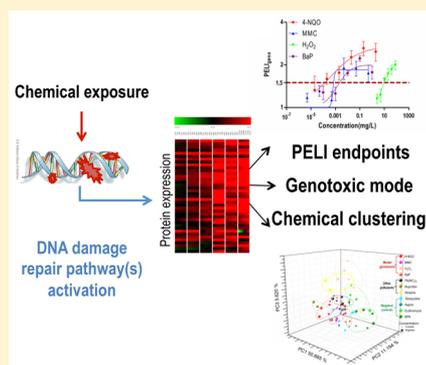
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S Supporting Information

ABSTRACT: The ecological and health concern of mutagenicity and carcinogenicity potentially associated with an overwhelmingly large and ever-increasing number of chemicals demands for cost-effective and feasible method for genotoxicity screening and risk assessment. This study proposed a genotoxicity assay using GFP-tagged yeast reporter strains, covering 38 selected protein biomarkers indicative of all the seven known DNA damage repair pathways. The assay was applied to assess four model genotoxic chemicals, eight environmental pollutants and four negative controls across six concentrations. Quantitative molecular genotoxicity end points were derived based on dose response modeling of a newly developed integrated molecular effect quantifier, Protein Effect Level Index (PELI). The molecular genotoxicity end points were consistent with multiple conventional *in vitro* genotoxicity assays, as well as with *in vivo* carcinogenicity assay results. Further more, the proposed genotoxicity end point PELI values quantitatively correlated with both comet assay in human cell and carcinogenicity potency assay in mice, providing promising evidence for linking the molecular disturbance measurements to adverse outcomes at a biological relevant level. In addition, the high-resolution DNA damaging repair pathway alternated protein expression profiles allowed for chemical clustering and classification. This toxicogenomics-based assay presents a promising alternative for fast, efficient and mechanistic genotoxicity screening and assessment of drugs, foods, and environmental contaminants.



INTRODUCTION

The ecological and health concern of an overwhelmingly large and ever-increasing number of chemicals (i.e., over 83 000 chemicals are in production by 2010) demands for toxicity screening and risk assessment of the potential toxicants.¹ It is recognized that, unless the approaches can be revised, the time and resources required to meet the demands of anticipated toxicity testing efforts will be measured in decades or beyond.² Genotoxicity is of particular importance because of its link to mutagenicity, carcinogenicity as well as cancer.^{3,4} Genotoxicity assays have been used to predict carcinogenic potential when carcinogenicity data are absent, or support carcinogenicity data in cancer risk assessment.^{4,8,9} Genotoxicity is caused by agents interacting with DNA and other cellular targets that control the integrity of the genetic materials, including induction of DNA adducts, strand breaks, point mutations, and structural and numerical chromosomal changes.^{5–7} Current genotoxicity assays, such as Ames test, comet test and micronucleus test (in *vitro* or in *vivo*), require relatively long testing time (up to days or weeks). And, depending on the detection target and mechanism of the genotoxicity assay, it identifies one or limited types of genetic material damage and cannot capture all DNA

damage effects, therefore may lead to inconsistency among test outcome and sometimes fail to capture potential genotoxicity. There is a pressing need for less costly and more rapid, yet informative and reliable genotoxicity screening and testing methods.

The Tox21 vision proposed by National Research Council (NRC) points out the promises of taking advantage of advances in genomics, computational toxicity, high throughput *in vitro* assay techniques to improve the ability to assess the impacts of chemically induced genetic damage in all its possible forms, to assess new and existing chemicals more efficiently, cost-effectively, and with less reliance on animal models.^{10,11} Batteries and/or tiered testing strategy that combine both *in vitro* and *in vivo* assays have been employed by U.S. EPA for evaluation of genotoxicity.¹² In recent years, high-throughput genotoxicity assessment based on single or a few biomarkers indicative of DNA damage recognition and repair have been

Received: October 19, 2015

Revised: January 21, 2016

Accepted: February 8, 2016

Published: February 8, 2016

Table 1. DNA Damage Type, Corresponding Repair Pathways and Biomarkers Selected for the Molecular Genotoxicity Assay Using GFP-Tagged Yeast Cells^{25,27,30}

DNA damage	repair pathway	proteins selected in the assay
general damage	DNA damage signaling (DDS)	CHK1, RAD9
DNA lesions	translesion synthesis (TLS)	RAD30
base alkylation	Direct reversal repair (DRR)	PHR1
base oxidation	base excision repair (BER)	OGG1
base alkylation and deamination		NTG1, NTG2, UNG1, MAG1, RAD27, APN1, APN2
single strand break		
cross-links	nucleotide excision repair (NER)	RAD1, RAD2, RAD4, RAD9, RAD14, RAD16, RAD23, RAD34
pyrimidine dimers		
bulky adduct		
mismatches	mismatch repair (MMR)	MSH1, MSH2, MSH3, MSH6, PMS1, MLH1, MLH2
double strand break (DSB)	DSB repair	XRS2, MRE11
	general response to DSB	
	homologous recombination (HR)	RFA1, RFA2, RFA3, RAD51, RAD52, RAD54, HTA1
	nonhomologous end joining (NHEJ)	LIF1, YKU70

demonstrated with RT-qPCR technique or in engineered cell reporter systems (e.g., recombinant bioluminescent bacteria or yeast).^{4,10,13–17} However, these assays are specific for certain targets and may not capture all types of genetic damage. For example, GreenScreen assay, which is listed in the Alternatives Assessment Program of ToxCast and uses yeast cells with GFP-infused single biomarker RAD54, can only detect genotoxins that lead to HR activation for DSB repair (implying lower assay sensitivity).^{15,16} Over the past decade, toxicogenomics, which examines the molecular-level activity of multiple biomarkers and pathways in response to environmental stressors, have shown promises to allow for rapid and sensitive evaluation of genotoxins, to more properly classify putative carcinogenicity and to reveal the potential mode of action (MOA).^{10,17–19} However, one of the major challenges is how to develop more quantitative molecular assay end points and link them to adverse effects,^{20,21} so-called phenotypic anchoring.^{22,23} Adverse outcome pathway (AOP) concept has been proposed to provide roadmap for establishing linkage between a molecular end point and an adverse outcome at a biological level of organization relevant to risk assessment.^{20,24}

Based on the AOP concept and our current knowledge of DNA damage and repair pathways, we proposed and developed a new quantitative toxicogenomics assay, which detects and quantifies molecular level changes in proteins involved in known DNA damage repair pathways, for fast, sensitive and mechanistic genotoxicity evaluation of environmental pollutants. This genotoxicity assay employs a library of in frame GFP fusion proteins of *Saccharomyces cerevisiae* consisting of 38 reporter strains (key proteins) covering all the seven recognized DNA damage repair pathways, which measures in situ and real-time protein expression changes in exposure to any chemical, yielding chemical-specific temporal DNA damage repair response profiles (fingerprints) within 1–2 h.²⁵ By covering all the seven known DNA damage repair pathways and monitoring temporal protein expression levels, this approach aims to more comprehensively capture the impacts of chemically induced genetic damage in various forms, therefore improves the assay's sensitivity and reliability. Furthermore, in order to quantify the molecular-level effects, a Protein Effect Level Index (PELI) was proposed based on the concept from our previous work by quantifying the accumulative altered protein expression change over certain exposure period for a given protein, specific pathway, or selected multiple biomarkers

ensemble library (i.e., DNA damage and repair pathway biomarkers ensemble).^{25–27} The derived molecular end points based on PELI values quantitatively correlated (or phenotypically anchored) with conventional phenotypic genotoxicity end points, demonstrating that the proposed approach has potential to serve as an alternative high-throughput in vitro genotoxicity assay. The assay was tested against a number of model genotoxins as well as genotoxicity-negative control chemicals, in order to demonstrate its specificity and sensitivity. In addition, the information-rich and high-resolution data reveal potential DNA damaging mechanisms related to genotoxicity and were used for chemicals classification based on their distinct molecular responses in DNA damage and repair pathways.

MATERIALS AND METHODS

Chemicals. Twelve known genotoxic chemicals and four nongenotoxic negative controls (details in [Supporting Information \(SI\) Table S1](#)) were selected to evaluate the proposed genotoxicity assay. The selected model genotoxins include 4-nitroquinoline 1-oxide (4-NQO, a tumorigenic quinoline), mitomycin C (MMC, bioreactive alkylating agent), hydrogen peroxide (H₂O₂, oxidizing agent), and benzo[a]pyrene (BaP, enzymatically activated polycyclic aromatic hydrocarbon (PAH) genotoxicant); three environmental pollutants that were reported to exhibit genotoxicity: lead(II) nitrate (Pb(NO₃)₂), ibuprofen, and atrazine; five drinking water disinfection byproducts (DBPs) with genotoxicity reported: trichloroacetic acid (TCA), *N*-nitrosodimethylamine (NDMA), bromodichloromethane (BDCM), chlorodibromomethane (CDBM) and formaldehyde. Four negative controls are aspirin, tetracycline hydrochloride, erythromycin, and bisphenol A. They were selected as negative controls since they were reported negative in most genotoxicity assays and in vivo carcinogenicity assays (details in [Result and Discussion](#)).²⁸ All the chemicals were evaluated across approximately six-log concentration range (except H₂O₂) ([SI Table S1](#)), up to the pre-determined maximum noncytotoxic concentration (over 95% cell survival tested by growth inhibition in yeast for 24 h as shown in [SI Figure S1](#)).

Selection of Proteins as Biomarkers and Construction of Yeast Whole Cell Array for Genotoxicity Assessment. A library of 38 in frame GFP fusion proteins (selected proteins listed in [Table 1](#)) of *S. cerevisiae* (Invitrogen, no. 95702, ATCC

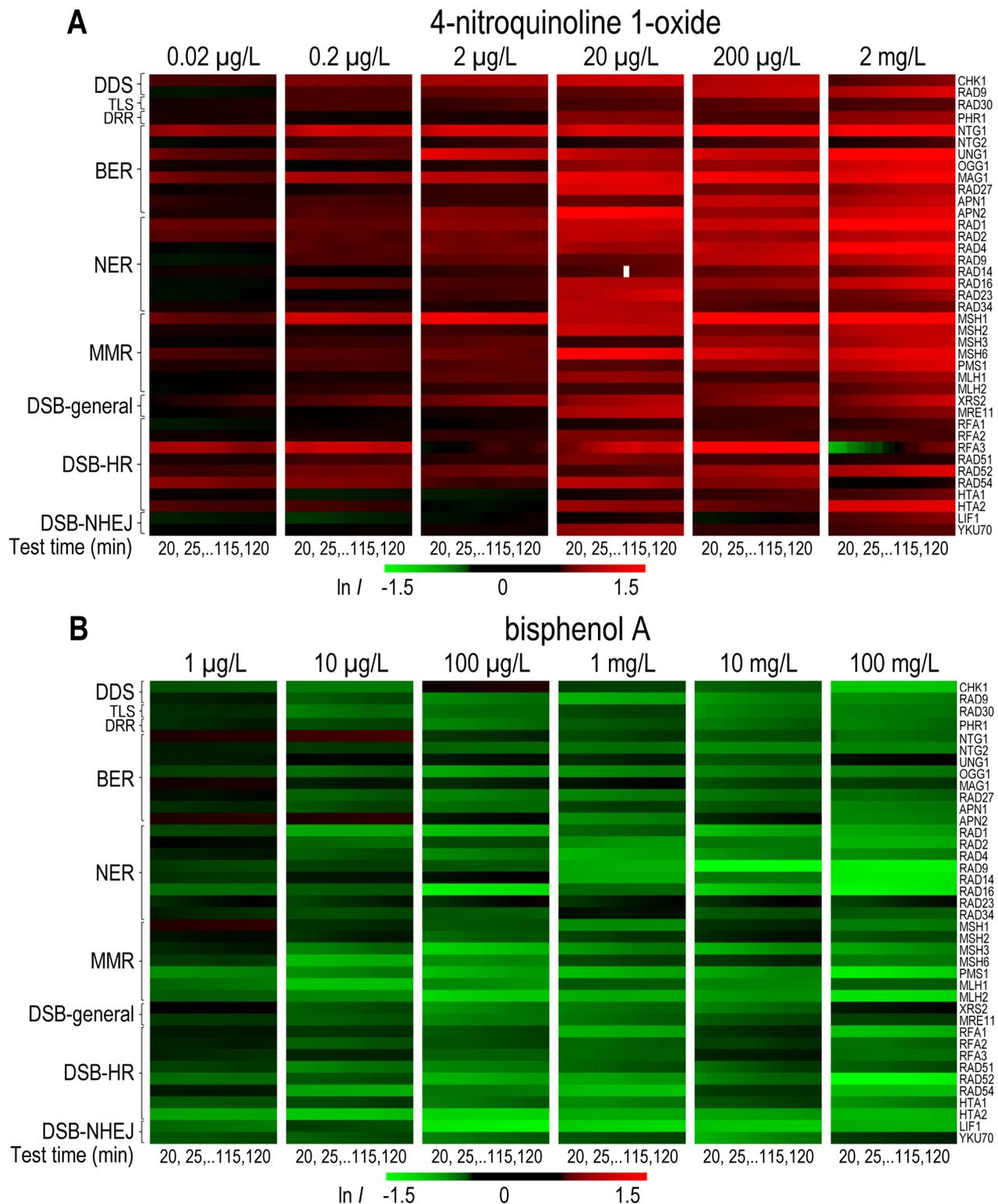


Figure 1. Temporal protein expression profiles of 38 biomarkers indicative of different DNA damage repair pathways upon exposure to A: 4-NQO (4-nitroquinoline 1-oxide, model genotoxicant), and B: BPA (bisphenol A, negative control),²⁵ across six concentrations. The mean natural log of induction factor ($\ln I$) indicates the magnitude of altered protein expression (represented by a green-black-red color scale at bottom. Red spectrum colors indicate up regulation, green spectrum colors indicate down regulation. Values beyond ± 1.5 are shown in the same color as ± 1.5). X-axis top: concentrations for each chemical, X-axis bottom: testing time in minutes, the first data point shown is at 20 min after exposure due to data smoothing with moving average of every five data points. Y-axis left: clusters of proteins by DNA damage repair pathways; Y-axis right: list of proteins (ORFs) tested, with details in Table 1. $n = 3$. Similar profiles for all other chemicals tested are shown in SI Figure S3.

201388), constructed by oligonucleotide-directed homologous recombination to tag each open reading frame (ORF) with

Aequorea victoria GFP (S65T) in its chromosomal location at the 3' end, was employed in this study.^{25,29,30} The selected

proteins were either specific for a certain type of DNA damage or play a pathway-specific role, therefore, changes in expression levels of these biomarkers would indicate the particular pathway responses associated with different DNA damages.^{31–41} A housekeeping gene PGK1 was selected as an internal control for plate normalization.⁴²

Real Time Protein Expression Analysis upon Chemical Exposures. Details of the proteomics assay for using GFP-tagged reporter yeast cells were described in our previous reports.^{25,27,30} Briefly, yeast strains selected for genotoxicity assessment (Table 1) were grown in clear bottom black 384-well plates (Costar) with SD medium for 4–6 h at 30 °C to reach early exponential growth (OD₆₀₀ about 0.2–0.4). 10 μL chemical (dissolved in PBS) or control (PBS only) was added to each well to reach the final concentrations (SI Table S1). For Benzo [a] pyrene (BaP), liver extract (S9 fractions, final concentration at 1.4%) (Sprague–Dawley Rat, Invitrogen, NY) was added for enzymatic bioactivation before exposure, with equal amount of S9 in PBS served as vehicle control.³⁰ The plates were then placed into a Micro plate Reader (Synergy H1 Multi-Mode, Biotec, Winooski, VT) for absorbance (OD₆₀₀ for cell growth) and GFP signal (filters with 485 nm excitation and 535 nm emission for protein expression) measurements every 5 min for 2 h after fast shake for 1 min. All tests were performed in dark in triplicate.

Protein Expression Profiling Data Processing and Quantitative Molecular End points Derivation. Protein expression profiling data of yeast library were processed as described previously.^{25,27,30} Temporal OD and GFP raw data were first corrected by background OD and GFP signal of medium control (with or without chemical). Protein expression P for each measurement was then normalized by cell number as $P = (\text{GFP}_{\text{corrected}}/\text{OD}_{\text{corrected}})$. The P values were also corrected with vehicle internal control (housekeeping gene PGK1) for plate normalization among replicates. The alteration in protein expression for a given protein at each time point due to chemical exposure, also referred as induction factor I , was represented by as $I = P_{\text{experiment}}/P_{\text{control}}$. Where, $P_{\text{experiment}} = (\text{GFP}_{\text{corrected}}/\text{OD}_{\text{corrected}})_{\text{experiment}}$ as the normalized gene expression GFP level in the experiments condition with chemical exposure, and $P_{\text{control}} = (\text{GFP}_{\text{corrected}}/\text{OD}_{\text{corrected}})_{\text{vehicle}}$ in the vehicle control condition without any chemical exposure.

To quantify chemical-induced protein expression level changes with consideration of exposure time, Protein Effect Level Index (PELI) was proposed and derived as a quantitative molecular end point.^{25,27,30} PELI can be derived to quantify the accumulative altered protein expression change averaged over the exposure period for a given protein (ORF_{*i*}) (PELI_{ORF_{*i*}}), a specific pathway (PELI_{pathway}) or for the overall DNA damage and repair pathway ensemble library (PELI_{geno}) as described in detail in the previous reports^{25,27,30} and in SI (Part 3). All tests were conducted in triplicates, and induction factor I , PELI_{ORF_{*i*}}, PELI_{pathway} and PELI_{geno} were evaluated by mean ± SD. For a given chemical, PELI_{geno} based dose–response pattern was modeled using Four Parameter Logistic (4PL) nonlinear regression model,^{43–45} which allowed the calculation of PELI_{max}. A chemical is considered genotoxicity positive if the PELI_{max} value derived from the PELI_{geno}-dose response curve is higher than 1.5, a predetermined threshold. The value of 1.5 was selected to reflect a statistically significant increase in protein expression levels compared to the untreated control, which is over $1 + 3 \times \text{SD}$ (SD refers to system standard deviation and was determined as 95% confidence interval for

the coefficient of variation (CV%) of PELI_{geno} values in this study, data not shown). The threshold of fold change in genes or proteins as 1.5 has been widely used and verified in the literature.^{46–51} Results from this study confirmed the appropriateness of the threshold value since all genotoxic negative chemicals exhibited PELI_{max} value less than 1.5, whereas all genotoxic positive chemicals yielded PELI_{max} values above 1.5.

DNA Damage Alkaline Comet Assay in Human A549 Cells for Phenotypic Confirmation. Alkaline comet assay in human A549 cells upon exposure to the 16 chemicals at selected concentrations (details in SI Table S1) or 1% FBS-F12 medium only (as untreated control) for 24 h was carried out according to the protocol of ITRC⁵³ using CometAssay 96 Kit of Trevigen Inc. (www.trevigen.com). All these procedures were performed in dark with triplicates. Twenty-five cells of each treatment were measured by software CASP randomly (University of Wroclaw, Institute of Theoretical Physics) and the damage was valued as % Tail DNA. Genotoxicity positive was defined as significant increase of tail DNA % compared to vehicle control with $p < 0.05$.

Clustering Analysis. Hierarchical clustering (HCL) was performed to cluster all the 16 chemicals across six concentrations (96 samples in total) based on protein expression levels of each chemical (ln I , average of triplicates) during 2 h exposure by software suite MeV (MultiExperiment Viewer) v4.8.⁵² The relationships were elucidated using the order of complete average linkage clustering based on euclidean distance.

Principal component analysis (PCA) was performed for all the chemicals too, to simplify the complex data sets of categories by analyzing the components with the greatest amount of variance based on their protein expression profiles. The analysis was conducted based on protein expression levels of each chemical (ln I , average of triplicates) during 2 h exposure by software suite MeV (MultiExperiment Viewer) v4.8,⁵² with centering mode as mean and number of neighbors for KNN imputation as 10.

RESULTS AND DISCUSSION

Concentration-Dependent, Chemical-Specific Temporal Differential Protein Expression Profiles. The temporal altered protein expression profiles (Figure 1 and SI Figure S3) indicative of DNA damage and repair pathway activities were distinctive for the tested chemicals, suggesting compound-specific cellular responses likely resulted from their different DNA-damaging molecular mechanisms. For genotoxicants that interact with DNA directly such as 4-NQO (Figure 1A) and MMC²⁵ (SI Figure S3A), proteins involved in DNA damage and repair pathways were significantly up-regulated immediately upon exposure and sustained during the 2 h exposure, suggesting strong DNA-damaging nature of the chemicals. In comparison, for chemicals that likely cause DNA damage indirectly such as H₂O₂ and BaP³⁰ (SI Figure S3B and C), more temporally dynamic protein expression change was observed with the maximum up regulations occurred with delay. The diffusion of H₂O₂ across membranes may be delayed since extracellular and membrane structure of yeast play important role in ROS defense,^{54–56} and BaP genotoxicity was reported in a time-dependent manner, which requires enzymatic bioactivation.⁵⁷ For all the four negative controls (Figure 1B and SI Figure S3, L to N), most proteins were not up-regulated or with averaged changes in activities near

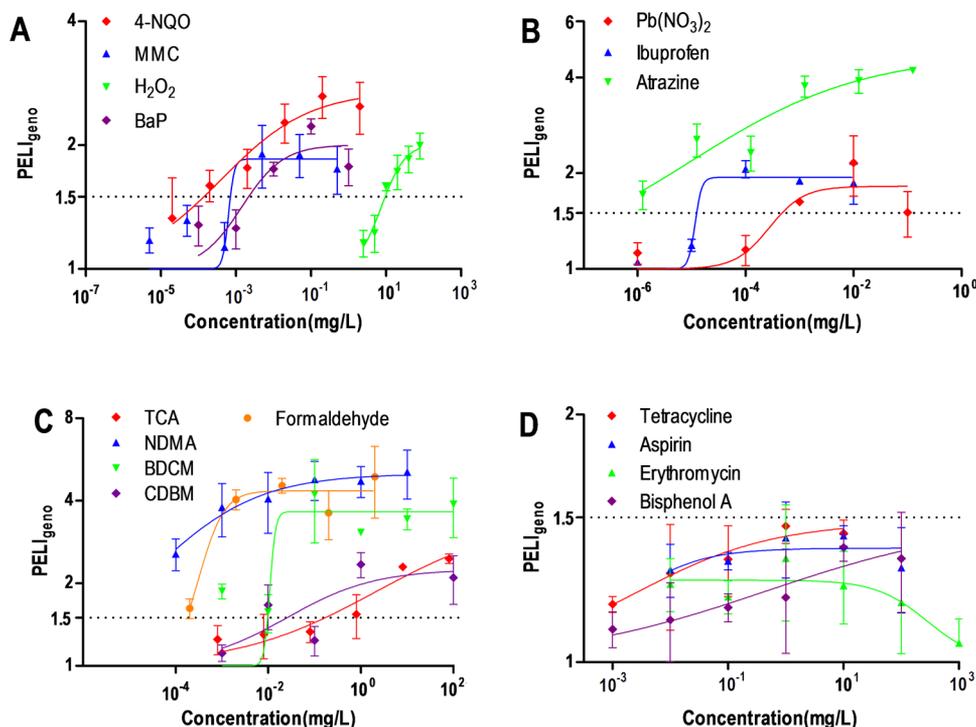


Figure 2. Dose–response curves of the 16 chemicals tested based on $PELI_{\text{geno}}$ values. A: model genotoxicants for positive control, B: environmental pollutants, C: DBPs; D: genotoxicity negative controls. Data points with an error bar represent the $PELI_{\text{geno}}$ value determined at each concentration. R^2 indicative of fitness are listed in Table 2. Genotoxicity positive is defined as having $PELI_{\text{max}}$ value (determined via model fitting dose response curves) greater than 1.5. X-axis: concentration for chemicals studied (mg/L). Y-axis: $PELI_{\text{geno}}$. Mean \pm SD, $n = 3$. For MMC and BPA, see ref 25.

threshold level, and also with many proteins down regulated, demonstrating that these genotoxicity-negative controls indeed have less effect on DNA repair pathways.

These chemical-specific response patterns were also concentration-dependent, which led to increase in magnitude of changes as concentration increases (e.g., 4-NQO and MMC), or up regulation of proteins that occur only at higher concentrations (e.g., H_2O_2 and BaP). For most chemicals tested, the highest exposure concentration led to decreases in the magnitude of up-regulation, or even shift from up-regulation to down-regulation in most of the tested proteins, which was likely caused by the transition from MOA-specific subcytotoxic effect to nonspecific cellular stress responses such as apoptosis, which led to overall cellular suppression effect.³⁰

Quantitative Molecular Genotoxicity End points Derivation. Using the newly proposed molecular effect quantifier PELI, we demonstrated that the molecular $PELI_{\text{geno}}$ values exhibited dose response with characteristic sigmoid-shaped patterns for all the 16 chemicals tested as shown in Figure 2. End point $PELI_{\text{max}}$ (the maximal PELI value determined based on the $PELI_{\text{geno}}$ -dose response curve using 4PL model fitting) can be derived (Table 2). $PELI_{\text{max}}$ quantifies the maximal protein expression effect for the overall DNA damage and repair pathway ensemble library that can be induced by a chemical in a 2 h exposure period, and indicates the limit of the dose–response relationship on the response axis for a certain chemical.^{37,38} The end point $PELI_{1.5}$ was derived based on the dose–response curve, which was defined as the corresponding concentration that causes the PELI value to reach 1.5, similar to the approach that has been applied for the umuC genotoxicity assay by Escher et al.⁵⁸ and our previous study.⁵⁹

Table 2. Summary of PELI Based Molecular End Points ($PELI_{\text{max}}$ and $PELI_{1.5}$, R^2 Indicates the Fitness of the Data to Four Parameter Logistic Models)^a

chemical	$PELI_{\text{max}}$	$PELI_{1.5}$ (mM)	R^2 (p value)
4-NQO	2.705	7.46×10^{-4}	0.9548 (0.0041)
mitomycin C	1.850	2.15×10^{-3}	0.7796 (0.0472)
H_2O_2	2.016	280.4	0.9791 (0.0013)
benzo [a] pyrene (with S9)	2.005	8.72×10^{-3}	0.7388 (0.1405)
lead(II) nitrate	1.818	1.43×10^{-3}	0.6708 (0.1810)
ibuprofen	1.941	6.00×10^{-5}	0.9699 (0.0152)
atrazine	4.720	9.80×10^{-7}	0.9082 (0.0121)
trichloroacetic acid (TCA)	3.479	1.06×10^{-3}	0.9313 (0.0078)
NDMA	5.021	1.97×10^{-9}	0.9631 (0.0030)
bromodichloromethane (BDCM)	3.653	6.00×10^{-5}	0.7356 (0.0631)
chlorodibromomethane (CDBM)	2.249	1.2×10^{-4}	0.6126 (0.2173)
formaldehyde	4.351	5.71×10^{-6}	0.8675 (0.0686)
tetracycline hydrochloride	1.481	NA	0.9439 (0.0285)
aspirin	1.376	NA	0.3258 (0.4292)
erythromycin	1.247	NA	0.7657 (0.0520)
bisphenol A	1.489	NA	0.8763 (0.0192)

^aNote: NA: not available. $PELI_{1.5}$ was not determined for negative control chemicals with $PELI_{\text{max}}$ less than 1.5.

Both genotoxic positive and negative model chemicals were selected in this study to demonstrate the validity of our proposed molecular genotoxicity assay. All the chemicals that are known to be genotoxic showed $PELI_{\text{max}}$ value higher than 1.5, ranging from 1.850 to 2.705. The four negative controls had $PELI_{\text{max}}$ value below 1.5 (ranging from 1.247 to 1.489). The results demonstrated that our quantitative toxicogenomics assay indeed captured the genotoxicity potential of the

chemicals tested and the genotoxicity threshold we chose is appropriate. Of course, further testing with a larger and wider range of chemicals is warranted.

NDMA is a pro-mutagen and requires S9 metabolic activation to become genotoxic. However, strong genotoxicity was observed for NDMA without S9 added in this assay, with $PEL_{\text{max}} = 5.021$, which may be related to the cytochrome of yeast. Several cytochrome monooxygenase enzymes in yeast (including *S. cerevisiae* of this study) can perform Phase I metabolism on some compounds in a manner analogous to mammalian microsomes, but less efficiently.^{60–62}

Correlation of Molecular End Points with Conventional Genotoxicity End Points-Phenotypic Anchoring. One key challenge for wide application of a protein expression based-assay is to establish the relationship between protein expression-based end points to conventional phenotypic end points at relevant biological level and then further incorporate these in vitro assays into toxicity and risk assessment framework.²³ We evaluated the correlation of quantitative genotoxicity molecular end points derived from the yeast assay with the results from a conventionally accepted genotoxicity assay, comet test in human A549 cells. In addition, we also examined the ability of our assay for predicting in vivo carcinogenicity in mice. Figure 3 shows the correlation of

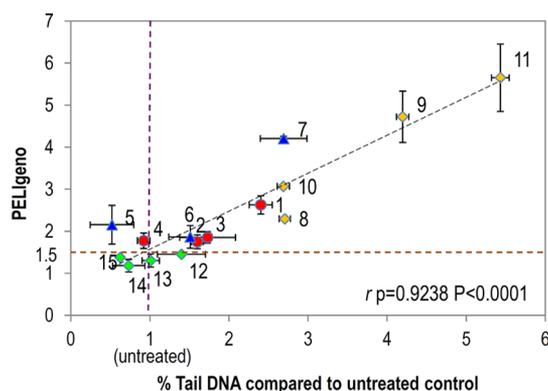


Figure 3. Correlation of molecular end point PEL_{geno} with phenotypic end point of DNA damage induced in alkaline comet assay for selected concentrations shown in SI Table S1. The brown dotted line (horizontal) indicates cutoff line of PEL_{geno} (1.5). The purple dashed line (vertical) indicates %Tail DNA of untreated control (ratio = 1). Chemicals: model genotoxicants, 1:4-NQO, 2: MMC, 3: H_2O_2 , 4: BaP; environmental pollutants, 5: $Pb(NO_3)_2$, 6: ibuprofen, 7: atrazine; DBPs, 8: TCA, 9: NDMA, 10: BDCM; 11: formaldehyde; negative controls, 12: aspirin, 13: tetracycline hydrochloride, 14: erythromycin and 15: bisphenol A. X-axis: 24-h DNA damage measured by % Tail DNA compared to vehicle control in human A549 cells (Details in SI Figure S4); Y-axis: PEL_{geno} . r_p indicates Pearson correlation coefficient of PEL_{geno} to DNA damage comet assay phenotypic end points (% Tail DNA). Mean \pm SD, $n = 3$.

PEL_{geno} values with the % Tail DNA valued from the comet assay for 15 chemicals at their selected concentrations (see details of comet assay in SI Figure S4). A statistically strong correlation ($r_p = 0.9238$, $P < 0.0001$) was observed between molecular genotoxicity end point (PEL_{geno}) and the phenotypic DNA damage end point (% Tail DNA), suggesting that the quantitative molecular disturbance quantifier based on altered protein expression of key proteins involved in various known DNA damage and repair pathways is able to successfully capture the DNA damage potential, and therefore possibly

quantitatively predict phenotypical outcome in terms of DNA damage. Importantly, a statistically strong correlation between molecular end point $PEL_{1.5}$ and in vivo carcinogenic end point TD50 (mice) was observed (Figure 4), demonstrating

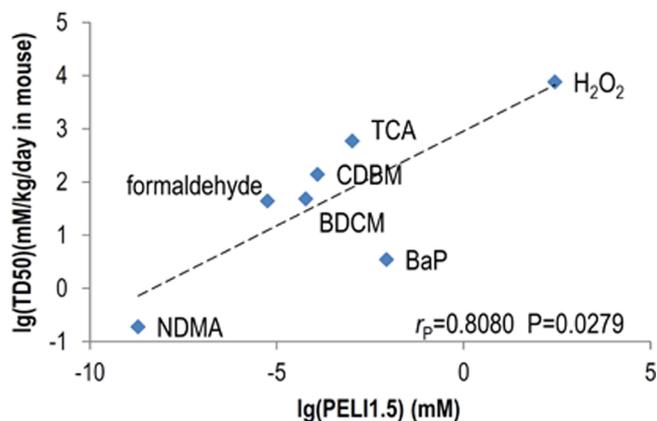


Figure 4. Correlation of molecular end point— $PEL_{1.5}$ with phenotypic end point—carcinogenic potency from 2-year carcinogenesis test in mice (SI Table S1), X-axis: $PEL_{1.5}$ determined via model fitting dose response curves ($\lg(PEL_{1.5})$, mM); Y-axis: carcinogenic potency in mice ($\lg(TD50)$, mM/kg/day). r_p indicates Pearson correlation coefficient of $PEL_{1.5}$ to carcinogenic potency. $n = 7$.

the consistency between our genotoxicity molecular assay end points and in vivo carcinogenic potency, suggesting the potential ability of our assay for carcinogenicity prediction. Current in vivo rodent carcinogenicity bioassay is resource-intensive and time-consuming, which makes it infeasible for evaluating a large number of chemicals.^{1,63}

To further evaluate the sensitivity and specificity of the proposed molecular genotoxicity assay, we qualitatively (positive or negative) compared our genotoxicity end point with more conventional in vitro genotoxicity results from literature for the chemicals studied (Table 3). Since genotoxicity potentially leads to carcinogenicity,^{3,4} we also included in vivo carcinogenicity according to the assessment approach reported in literature. Sensitivity is estimated as positive correctly classified over the total positives, specificity is defined as negatives correctly classified over the total negatives and accuracy is determined as the sum of both positive and negatives correctly classified over the total sum of positives and negatives.⁶⁴ Comparing to other accepted in vitro genotoxicity assays, our assay showed higher consistency to predict in vivo carcinogenicity with high sensitivity (100%) and accuracy (93.75%) (Table 4). The assay results also exhibited good specificity (80%) for the 16 chemicals tested.

Note that comparisons among different in vitro assays are not necessarily appropriate as each conventional assay only detects a specific type of DNA damage. For example, Ames test targets on frame shift or point mutations,⁶⁵ comet assay detects strand breaks⁶⁶ and micronucleus assay detects chromosome damages.⁶⁷ The discrepancy between in vitro genotoxicity assays and in vivo rodent carcinogenic potency may come from the inherent limitations in in vitro assay: pharmacokinetics and metabolic aspects of chemicals cannot be reflected in in vitro assays. For example, in vivo metabolism may alter genotoxicity compared to in vitro tests because of metabolism of the compound (e.g., the negative response of ibuprofen in

Table 3. Comparison of End Points (Genotoxicity Positive or Negative) From Our Proposed Quantitative Toxicogenomics Based Genotoxicity Assay with Conventional Genotoxicity and in Vivo Carcinogenesis Assays Across Different Species

chemical	yeast assay in this study ^a	genotoxicity assay			in vivo carcinogenesis assay ^{21,55}
		bacteria	mammalian cells		
		Ames test ^{68–74}	comet test ^{57,74–81}	micronucleus test ^{2,57,74,80,82–90}	
4-NQO	+	+	+	+	+
mitomycin C	+	+	+	+	+
H ₂ O ₂	+	+	+	+	+
benzo [a] pyrene (with S9)	+	+	+	+	+
lead(II) nitrate	+	+	+	+	+
ibuprofen	+	–	+	+	–
atrazine	+	–	+	+	+
trichloroacetic acid	+	–	+	–	+
N-nitrosodimethylamine (NDMA)	+	+	+	+	+
bromodichloromethane	+	+	–	–	+
chlorodibromomethane	+	+	–	–	+
formaldehyde	+	+	+	+	+
tetracycline hydrochloride	–	–	+	–	–
aspirin	–	–	+	–	–
erythromycin	–	–	+	+	–
bisphenol A	–	–	+	–	–

^aGenotoxicity positive (+) in our assay is defined as PELI_{max} value greater than 1.5, based on the comparison of the PELI_{max} value derived from the PELI-dose response curve with both positive and negative genotoxic chemicals, the signal-to-noise ratio for similar systems according to literature, as well as the standard deviation range of our testing systems (see details in [Materials and Methods](#) section).

Table 4. Comparison of Our Quantitative Toxicogenomics Based Genotoxicity Assay with Other in Vitro Conventional Assays for Predicting in Vivo Carcinogenicity^a

	sensitivity	specificity	accuracy
yeast assay in this study	11/11 (100%)	4/5 (80%)	15/16 (93.75%)
Ames test	9/11 (81.82%)	5/5 (100%)	13/16(81.25%)
comet test	9/11 (81.82%)	0/5 (0%)	9/16 (56.25%)
micronucleus test	8/11 (72.73%)	3/5 (60%)	11/16 (68.75%)

^aNote: Sensitivity, specificity and accuracy of the assays were determined by comparing the results against in vivo 2-year rodent carcinogenic potency, according to the approach described in previous literature as shown in [Table 3](#).⁶⁴

carcinogenesis may be due to its metabolism). S9 fraction has been used to simulate possible metabolic activation, to partially overcome the limitation of in vitro cell-based assays. Furthermore, nongenotoxic carcinogens such as endocrine-modifiers cannot be detected by the genotoxicity assays such as those in vitro assays or our assay. At last, the variations in the genotoxicity results associated with varying testing conditions studies should also be taken into consideration when comparing independent studies. Nevertheless, the comparison and correlation of PELI based molecular end points with conventional in vitro genotoxicity end points and in vivo carcinogenic end point provided preliminary validation of the proposed toxicogenomics based genotoxicity assay approach, and its potential for carcinogenicity prediction. Of course, the assay needs to be further tested and refined with more cumulative genotoxicity data for more and variety of chemicals.

Great challenges remain for understanding the principles and developing approaches to convert toxicogenomics information into quantitative toxicity end points at biological and regulatory relevant levels. On a fundamental level, our results demonstrated two important points related to the challenges in molecular toxicology regarding molecular end point quantifi-

cation and validation. First, molecular changes (in this study, protein expression changes of DNA repair pathways) at pathway level with multiple biomarkers can be potentially quantified and they exhibit dose–response manner similar to phenotypic observations. Second, it is possible to phenotypically anchor the molecular end points to biological DNA damage end points, therefore possibly predict the genotoxicity, across different species and even from cellular to organism level. This provides promising information and evidence for the concept of adverse outcome pathways, to link the molecular initiating events to adverse outcomes at a biological relevant level.

Chemical-Specific Protein Expression Profiling and Pathway Activation Reveal Distinct Genotoxicity Mechanisms among Chemicals. The real-time protein expression profiling of key proteins indicative of various DNA damage and repair pathways provide insights into the detailed DNA damage mechanisms induced by the tested chemicals. [Table 5](#) summarized the various DNA damage repair pathway activation (quantified by PELI_{pathway} values) and suggested potential DNA damage mechanisms revealed by this study, as well as known genotoxicity mechanisms for the chemicals examined.

For the four negative controls, DNA damage was below or near detection threshold level, suggesting little DNA-damaging related genotoxicity for these chemicals as expected.^{73,77,84,101} DNA damage mechanisms of all the known genotoxic chemicals in this study were generally consistent with their reported mechanisms. For example, model genotoxicant 4-NQO induced base-pair substitutions, bulky adducts, base damage, and mismatches¹⁰³ indicated by activation of nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR). MMC induced similar DNA base damage and mismatches, cross-links and bulky adducts as well as severe double strand breaks (DSB), which is consistent with its reported genotoxic mechanism.⁹¹ H₂O₂ induced severe damage of DSBs, by its strong oxidative damage as a strong

Table 5. Summary of DNA Damage Repair Pathway-Activation^a and Potential Genotoxicity Mechanisms Revealed

Chemical	Concentration	DNA repair pathway							Damage suggested by our assay	Reported genotoxic mechanisms
		DSS	TLS	DRR	BER	NER	MMR	DSB		
4-NQO	0.02 µg/L 2 mg/L								Strong base damage, bulky damage, and mismatches; DSBs	A pre-carcinogen activated by cellular machinery, covalently bind to DNA, specific for base-pair substitutions ⁸⁰
MMC	0.005 µg/L 500 µg/L								Base damage; bulky damage, mismatches and DSBs	Bio-reactive alkylating agent, produces base alkylation, crosslink, and double strand break ⁹¹
H ₂ O ₂	2.5 µg/L 80 mg/L								Base damage (oxidative damage) and DSBs	Inducing oxidation related DNA damage including strand breaks ^{92, 93}
BaP	0.1 µg/L 10 mg/L								Base damage (oxidative damage); bulky damage, mismatches and DSBs	Enzymatically activated PAH inducing DNA adducts and oxidative DNA damage ⁹⁴
Pb(NO ₃) ₂	0.001 µg/L 100 µg/L								Base damage and mismatches	May affect the fidelity of DNA synthesis in vitro; covalently interact with tertiary phosphate ions in nucleic acids ⁸⁵
ibuprofen	0.001 µg/L 100 µg/L								Base damage, mismatches, DSBs; weak bulky damage	Mechanism not clear ⁹⁵
atrazine	0.00125 µg/L 125 µg/L								Strong base damage and DSBs, bulky damage, mismatches	Mechanism not clear, might be related to oxidative stress ⁹⁶
trichloroacetic acid	0.8 µg/L 80 mg/L								Strong base damage, mismatches and DSBs	oxidative damage related DNA damage ⁹⁷
NDMA	0.1 µg/L 10 mg/L								Strong base damage and DSBs, bulky damage, mismatches	Act as alkylating agents after metabolism ⁹⁸
BDCM	10 µg/L 1 g/L								Strong base damage and DSBs, bulky damage, mismatches	Mechanism not clear
CDBM	10 µg/L 1 g/L								Base damages, mismatches and DSBs	Mechanism not clear
formaldehyde	0.2 µg/L 20 mg/L								Strong base damage and DSBs, bulky damage, mismatches	Form DNA-protein crosslinks ⁹⁹
tetracycline	1 µg/L 100 mg/L								Weak base damage	Negative in Ames, chromosome aberrations and carcinogenicity tests, positive in some in vitro tests. Mechanism not clear. ^{28, 100, 101}
aspirin	1 g/L								BDT (below detection threshold)	No reported genotoxicity ¹⁰²
erythromycin	10 µg/L 1 g/L								BDT	Negative in Ames and carcinogenicity tests. Potential genotoxicity probably related to oxidative stress. ⁸⁸
BPA	10 µg/L 1 g/L								BDT	No reported genotoxicity ⁵⁷

^aDNA damage and repair pathway activation indicated by mean natural log value of $PEL_{pathway}$ of triplicate tests. Red spectrum colors indicate activation, with black–red color scale from 0 to 1.5 (values greater than 1.5 shown as same color as 1.5). X-axis top: seven known pathways of DNA damage repair (see Table 1 for details). Y-axis left: chemical and six concentrations from lowest to highest (top to bottom). Aberrations for DNA repair pathways: DDS: DNA damage signaling; TLS: translesion synthesis; DRR: direct reversal repair; BER: base excision repair; NER: nucleotide excision repair; MMR: mismatch repair; DSB: double strand break. For MMC and BPA, see reference.²⁵

model oxidant. Genotoxicant BaP induced DNA adducts, oxidative damage and apurinic sites indicated by the activation of NER,⁸⁶ BER,⁸⁷ MMR,¹⁰⁴ and direct reversal repair (DRR). The consistency of DNA damage repair pathway activation with reported genotoxic mechanisms of both known genotoxicants and negative controls demonstrated the validity of our proposed assay. Biomarkers of DSB (for example, RAD51 and RAD54) were also up-regulated by BaP exposure at high concentration (>100 µg/L), although DSB has not been reported for BaP. Previous study has reported that DNA strand breaks may be introduced directly by genotoxic compounds, through the induction of apoptosis or necrosis, secondarily through the interaction with oxygen radicals or other reactive intermediates, or as a consequence of excision repair enzymes.¹⁰⁵ The DSB led by BaP may be indirect consequences of other cellular toxic effects or DNA damages.

The information obtained from our assay also provides insights into potential genotoxic mechanisms of chemicals that have not been well studied. The genotoxicity of Pb(NO₃)₂ is still not clear with conflicting reports, which may be related to oxidative stress, DNA repair inhibition and interaction with related proteins.^{85,106} Pb(NO₃)₂ exposure moderately activated BER and MMR in this assay, suggesting that it may cause DNA base damage to produce mismatches. Ibuprofen seemed to affect various DNA repair pathways, likely as a result of its direct membrane activity or oxidative stress.^{107,108} Atrazine induced wide activation across all the DNA damage repair pathways similar to H₂O₂, suggesting the damage may be contributed by ROS production as reported previously.⁹⁶ TCA,

NDMA, and formaldehyde also induced wide activation of DNA repair pathways, suggesting strong DNA damage related to oxidative damage, alkylation or cross-link respectively as reported.^{97–99} According to the strong DNA repair pathway activation, BDCM and CDBM may also induce oxidative damage, base damage and double strand DNA break. Since there has been no report for their genotoxic mode, the results of our study could provide more information to better understand their genotoxic mechanisms. Although more investigation is needed to confirm these hypotheses, the results can provide mechanistic insight for further exploration.

Chemical Clustering Based on Their DNA Damage Repair Pathway Protein Expression Profiles. The chemical-specific and concentration-dependent real time protein expression profiles may serve as fingerprints to allow chemicals clustering and classification based on their distinct biological responses and underlying DNA damaging mechanisms in a dose-sensitive manner, as shown in Figure 5. In general, the same chemical at varying concentrations clustered mostly together (i.e., bisphenol A, H₂O₂ and CDBM) as a result of the conserved DNA-damaging nature of each individual chemical. Although, some chemicals seemed to show more dose-sensitive separation (i.e., BaP, MMC) as results of changing DNA-damaging effects at varying concentrations. The chemicals that shared more similar DNA damaging nature had closer distance such as MMC, Pb(NO₃)₂, 4-NQO and BaP. The negative control chemicals with weak DNA damaging effects clustered together. A principal component analysis (PCA) based on the temporal differential

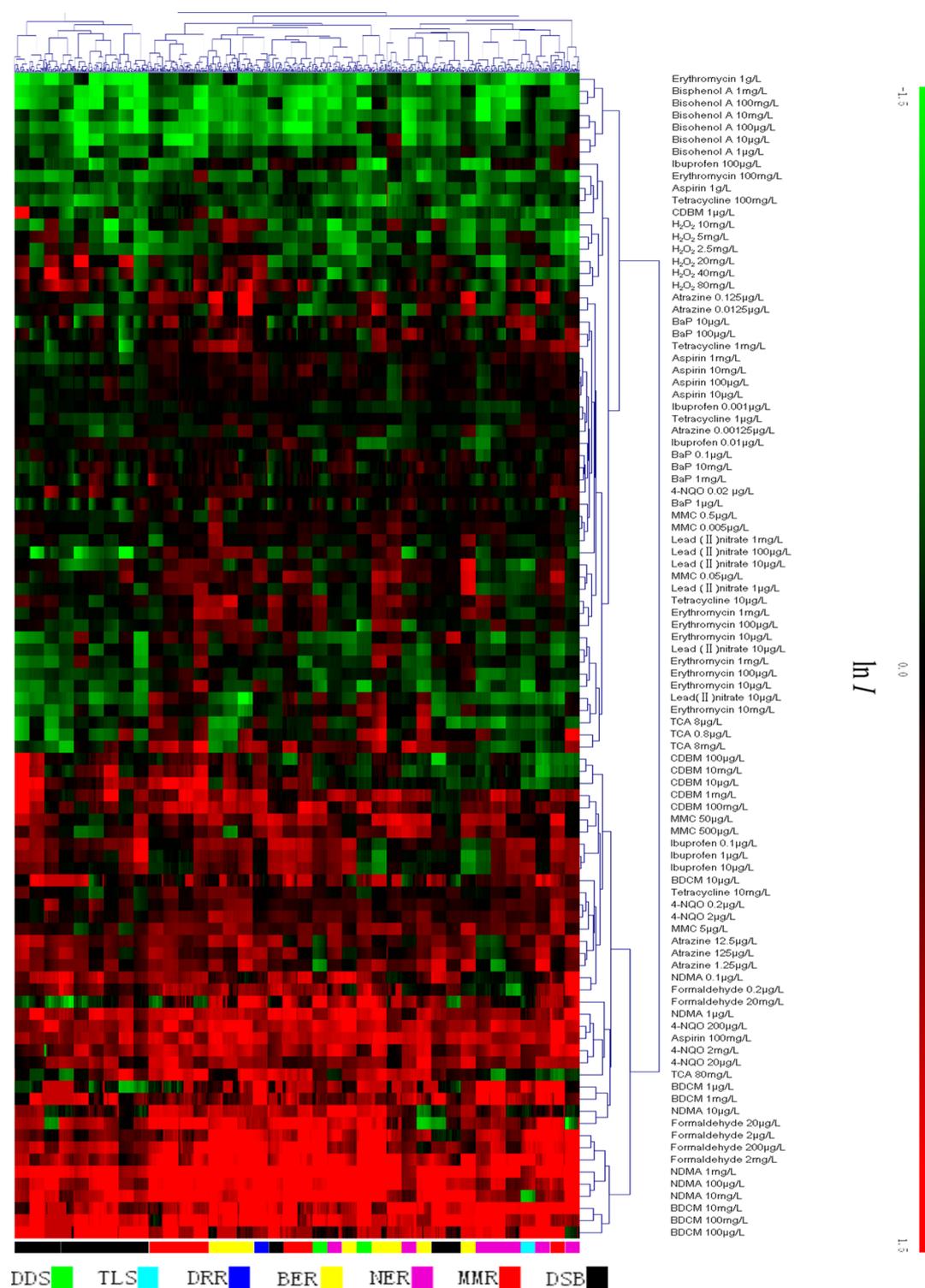


Figure 5. Hierarchical Cluster (HCL) analysis diagram based on $\ln I$ values of the 16 chemicals across six concentrations in this study (Euclidean distance, complete linkage clustering). The magnitude of altered protein expression ($\ln I$) is represented by a green-black–red color spectrum. Red spectrum colors indicate up regulation, green spectrum colors indicate down regulation. Values beyond ± 1.5 are shown in the same color as ± 1.5 . X-axis top: cluster roots of protein biomarkers used in this study; X-axis bottom: DNA damage and repair pathways with color codes; Y-axis right: cluster roots and list of chemicals tested. Aberrations for DNA repair pathways: DDS: DNA damage signaling; TLS: translesion synthesis; DRR: direct reversal repair; BER: base excision repair; NER: nucleotide excision repair; MMR: mismatch repair; DSB: double strand break.

protein expression profiles (SI Figure S5) also suggested the profiles had high resolution to distinguish various chemicals and toxic mechanisms. The clustering analysis suggested the high-resolution altered protein expression profiles were sensitive enough to differentiate chemicals based on their

genotoxic mechanisms, therefore potentially allows for genotoxicity-based chemical classification and identification.

This study demonstrated a sensitive, rapid, mechanistic and quantitative toxicogenomics-based approach for genotoxicity assessment based on DNA damage and repair pathway

ensemble activities using GFP-tagged yeast library. This assay can potentially be applied as an alternative method or as part of the tiered testing system for genotoxicity screening and evaluation for large number of chemicals and environmental pollutants. Compared to current available in vitro genotoxicity assays that take several days (e.g., over 2 days for Ames test and comet assay, 2–3 days for in vivo micronucleus test), our high throughput assay is faster (2 h), more cost-effective (yeast can be easily cultured with minimal cost) and yet yields more information regarding the underlying mechanisms. Compared to existing biomarkers-based molecular genotoxicity assays that employ single or a limited number of biomarkers, the proposed assay covers most of known biomarkers involved in DNA damage repair pathways, and therefore provides improved performance with at least equivalent or higher sensitivity, specificity and accuracy for genotoxicity assessment, and possible in vivo carcinogenicity prediction. In addition, current PCR-based assays measure transcriptional activities, whereas our assay uses in frame GFP fusion that detects protein expression directly, therefore more accurately reflecting cellular responses beyond genetic activation. Most importantly, the proposed new quantitative molecular genotoxicity end points were shown to correlate well with the phenotypic genotoxicity end points, suggesting possible phenotypically anchoring to higher organism, therefore making the assay applicable in health and environmental risk assessment. At last, the pathway ensemble-based assay approach can be potentially extended to other types of toxicity detection, such as oxidative, membrane, and protein damage.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05097.

Details for chemicals and their concentration range in the study; 24-h cytotoxicity of chemicals in yeast cells for concentration range selection; data processing procedure for real-time protein expression profiles of yeast cell array; real-time protein expression profiles of all the other 14 chemicals tested in this study; results of comet assay in human A549 cells; principal component analysis (PCA) result of the 16 chemicals (PDF).

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was supported by National Science Foundation (NSF) (EEC-0926284, CAREER CBET-0953633, CBET-1437257), PROTECT (NIEHS P42ES017198) and CRECE (NIH P50ES026049). We are grateful to Professor Penny Beuning at Chemistry and Chemical Biology in Northeastern University for her advice and critique of the manuscript.

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