Application of the TGx-28.65 Transcriptomic Biomarker to Classify Genotoxic and Non-Genotoxic Chemicals in Human TK6 Cells in the Presence of Rat Liver S9

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In vitro transcriptional signatures that predict toxicities can facilitate chemical screening. We previously developed a transcriptomic biomarker (known as TGx-28.65) for classifying agents as genotoxic [DNA damaging] and non-genotoxic in human lymphoblastoid TK6 cells. Because TK6 cells do not express cytochrome P450s, we confirmed accurate classification by the biomarker in cells co-exposed to 1% 5,6 benzoflavone/phenobarbital-induced rat liver S9 for metabolic activation. However, chemicals may require different types of S9 for activation. Here we investigated the response of TK6 cells to higher percentages of Aroclor-, benzoflavone/phenobarbital-, or ethanol-induced rat liver S9 to expand TGx-28.65 biomarker applicability. Transcriptional profiles were derived 3 to 4 hr following a 4 hr co-exposure of TK6 cells to test chemicals and S9. Preliminary studies established that 10% Aroclor- and 5% ethanol-induced S9 alone did not induce the TGx-28.65 biomarker genes. Seven genotoxic and two non-genotoxic chemicals (and concurrent solvent and positive controls) were then tested with one of the S9s (selected based on cell survival and micronucleus induction). Relative survival and micronucleus frequency was assessed by flow cytometry in cells 20 hr post-exposure. Genotoxic/non-genotoxic chemicals were accurately classified using the different S9s. One technical replicate of cells co-treated with dexamethasone and 10% Aroclor-induced S9 was falsely classified as genotoxic, suggesting caution in using high S9 concentrations. Even low concentrations of genotoxic chemicals (those not causing cytotoxicity) were correctly classified, demonstrating that TGx-28.65 is a sensitive biomarker of genotoxicity. A meta-analysis of datasets from 13 chemicals supports that different S9s can be used in TK6 cells, without impairing classification using the TGx-28.65 biomarker. 

Key words: genetic toxicology; biomarker; TGx-28.65 biomarker; toxicogenomics; micronucleus; furan; metabolic activation; S9
INTRODUCTION

There is great interest in the application of toxicogenomics in human cells for screening potential chemical toxicities. Higher throughput approaches for transcriptional profiling are being developed, and it is likely that in vitro global gene expression analysis will become a first screen to predict potential adverse health effects and modes of action for chemicals in the future [Lamb et al., 2006; Lamb, 2007; Patlewicz et al., 2013; Zhu et al., 2014]. A critical gap in the application of this approach is the availability of validated gene expression signatures that can be used to predict a chemical’s mode of action, or the probability that the chemical induces specific toxicities, that have been robustly tested across laboratories, cell culture models (including human models), gene expression platforms, and experimental designs. Although many studies have published transcriptional signatures to predict various toxicities [Uehara et al., 2011; Minowa et al., 2012; Cheng et al., 2013; Doktorova et al., 2013; Eichner et al., 2013; Thomas et al., 2013a; Yamada et al., 2013; Melis et al., 2014; Romer et al., 2014; Sahini et al., 2014; Wei et al., 2014; Oshida et al., 2015a,b; Schmeits et al., 2015; Shen et al., 2015], these have not been extensively validated or applied, and the majority of this work has been done on rodent cells or tissues. Therefore, accepted signatures capturing diverse toxicological targets and effects in human cells are needed for development of effective chemical screening approaches.

Genotoxicity testing is a critical part of chemical risk assessment. The Health and Environmental Sciences Institute’s (HESI) Technical Committee for the Application of Genomics to Mechanism-Based Risk Assessment is currently undertaking a project to develop a transcriptomic biomarker that can be used to classify chemicals as either genotoxic (DNA damaging) or non-genotoxic [Goodsaid et al., 2010; Li et al., 2015] to address the needs described above. An initial study produced a reference database that consists of gene expression profiles from human TK6 cells exposed to 28 model compounds [Li et al., 2015], both genotoxic and non-genotoxic, representing various modes of action. Furthermore, a transcriptomic biomarker known as TGx-28.65 (this acronym represents toxicogenomics (TGx)), the 28 training agents used in biomarker development, and 65 genes in the signature), comprising genes from primarily DNA damage response pathways, was identified shown to differentiate genotoxic from non-genotoxic compounds [Li et al., 2015]. Within this study, the concentration of test chemicals used for monitoring the TGx-28.65 genes was determined by a dose setting experiment where gene expression changes of three prototypical stress response genes (ATF3, CDKN1A, and GADD45A) were measured across a wide concentration range of test chemicals by RT-qPCR [Li et al., 2015]. The selection of an appropriate concentration for microarray analysis by this means ensured that the experiments achieved maximal induction of transcriptional effects while reducing the complexity of the experimental approach to a single concentration of test chemical for the microarray analysis. Concentration selection is critical because some genotoxic chemicals disrupt transcriptional machinery resulting in attenuated transcription at high doses [Fornace et al., 1989; Li et al., 2007]. The ability of the TGx-28.65 biomarker to differentiate genotoxic and non-genotoxic compounds was then successfully demonstrated using three model agents. In these experiments, the genotoxicant isopropyl methanesulfonate was classified as genotoxic, and both the non-genotoxic compound 3-nitropropionic acid and the irrelevant positive caffeine (positive genotoxicity results in vitro and negative genotoxicity results in vivo) were classified as non-genotoxic [Li et al., 2015]. Human TK6 lymphoblastoid cells were selected for biomarker development because these cells are well characterized, are an accepted model for regulatory genetic toxicology testing, replicate rapidly as suspensions in culture, express wild type TP53, and demonstrate a robust response to cellular stresses [Amundson et al., 2005; Isleshi et al., 2005; Godderis et al., 2012; OECD 2014b; Li et al., 2015]. However, these cells lack cytochrome P450s and cannot metabolically activate chemicals. Given that many environmental chemicals require metabolic activation before they exert genotoxic effects, this limits application.

To broaden the utility of the biomarker, we previously investigated whether the TGx-28.65 transcriptomic biomarker could be used in the presence of a metabolic activation system [Buick et al., 2015]. Specifically, we explored the use of rat liver S9, which is the supernatant fraction derived following centrifugation of homogenized liver at 9000g for 20 minutes. S9 contains both cytosol and microsomes, the former containing enzymes involved in transferase activities (e.g., Phase II metabolism) and the latter containing cytochrome P450s [Greim and Snyder, 2008]. We found that a 4 hr exposure period in the presence of rat liver S9, replacement with fresh media, and sampling following a 3-4 hr recovery period resulted in the accurate classification of genotoxicity for benzo[a]pyrene and aflatoxin B1, and of non-genotoxicity for phenobarbital and dexamethasone, using the TGx-28.65 biomarker. The need for metabolic activation of these chemicals was confirmed through the demonstration that neither benzo[a]pyrene nor aflatoxin B1 activated the TGx-28.65 biomarker to indicate genotoxicity in the absence of S9. In addition, the TGx-28.65 biomarker accurately classified the genotoxicity of cisplatin, a chemical that does not need metabolic activation, in the presence and absence of S9.

S9s can be derived from animals that have been exposed to (mixtures of) particular chemicals that are
known to induce specific P450 families, or from uninduced animals. The biotransformation of xenobiotics by liver S9 is dictated by Michaelis-Menten enzyme kinetics and is driven by the $K_m$ and $V_{max}$ of the enzymes present in the homogenate toward their substrates. Our preliminary work was done using 1% 5,6 benzoflavone/phenobarbital-induced (BF/PB-induced) rat liver S9 [Buick et al., 2015], an S9 commonly used in regulatory testing. Our use of BF/PB-induced- over Aroclor 1254-induced S9 was done to address the disposal of animals contaminated with polychlorinated biphenyl mixtures since there are no qualitative differences between the two inducers with respect to performance in the genetic toxicology test battery [Callander et al., 1995]. Rat induced S9 mix contains a broad spectrum of CYP450s. Enzymatic activities of S9s are typically measured through analysis of alkoxresorufin O-dealkylation activities including EROD (CYPs 1A1 and 1A2), BROD (CYPs 2B1 and 3A), MROD (CYP1A2), and PROD (CYPs 2B1 and 3B2). These enzymatic activities are greatly upregulated in both Aroclor- and BF/PB-induced S9 (e.g., [Cox et al., 2015]). However, these S9s are not effective in activation of certain CYP2E1 substrates that are known carcinogens (e.g., nitrosamines). Some suggest that this is because of suppression of CYP2E1 by benzoflavone/phenobarbital or Aroclor 1254 in contrast to “super” induction of CYP450 1A1 by the same inducers [Burke et al., 1994]. In addition, there are known to be strong species differences in response to CYP2E1 substrates (e.g., butadiene, acrylamide, vinyl chloride, acrylonitrile, etc.). Thus, for agents that either have structural alerts or are known to be CYP2E1 substrates, scientific judgment should be exercised and an alternative S9 should be considered (e.g., non-induced S9 or ethanol-induced S9).

In view of the requirement for different S9s in chemical testing, in the present study we evaluated cells co-exposed to test chemicals and Aroclor 1254-induced or ethanol-induced S9, to confirm that other types of S9 are also appropriate for use with the TGx-28.65 biomarker. A brief review of the literature was first undertaken to identify test chemicals for this study, referring primarily to consolidated sources of information where available (e.g., IARC monographs and publicly available databases; Table I). Briefly, five of the test agents selected are mutagenic carcinogens in rodents, including: 2-aminoanthracene, cyclophosphamide, dibenzo[a,h]anthracene, dimethylnitrosamine, and 2-nitrofluorene. Following metabolic activation, the reactive metabolites of 2-aminoanthracene, dibenzo[a,h]anthracene, and 2-nitrofluorene form bulky DNA adducts. Cyclophosphamide is a nitrogen mustard alkylating agent, and dimethylnitrosamine is a mono-functional alkylating agent (causing primarily O-alkylation). Two test agents, sucrose and phenanthrene, do not induce mutagenicity in standard genotoxicity tests in vivo or in vitro. Phenanthrene was chosen because it is a non-genotoxic polycyclic aromatic hydrocarbon and serves as a point of comparison with the genotoxic polycyclic aromatic hydrocarbon dibenz[a,h]anthracene and benzo[a]pyrene. Acetaminophen was included because it is Ames negative and non-carcinogenic, but induces clastogenicity both in vitro and in vivo. Furan was selected as a test case because there is some debate as to its principle mode of action in cancer. Furan is Ames negative but induces clastogenicity at high doses, which is thought to occur indirectly because of increased oxidative stress following metabolism [Ding et al., 2012; McDaniel et al., 2012]. Benzo[a]pyrene, a polycyclic aromatic hydrocarbon that is genotoxic following metabolic activation, was used as a positive control. Finally, dexamethasone was used as a non-genotoxic agent in preliminary S9 experiments as it was also used in our previous work [Buick et al., 2015].

The chemicals selected require a diverse array of enzymes for their metabolic activation and detoxification. The majority of these chemicals are metabolically activated by enzymes found within standard Aroclor- or BF/PB-induced S9; exceptions to this include acetaminophen, dimethylnitrosamine, and furan, which are primarily metabolically activated by CYP2E1. In these cases, the use of an alternative S9 (e.g., uninduced rat liver S9 or ethanol-induced S9) that contains higher amounts of CYP2E1 (but much lower levels of the CYPs associated with EROD, PROD, BROD, and MROD activities) may provide an advantage. The genotoxic chemicals in this study are only genotoxic following metabolic activation; therefore, we only evaluated these agents in the presence of S9. Extensive previous work demonstrates that direct acting agents are readily identified in the absence of S9 [Li et al., 2015], and that genotoxic agents requiring metabolic activation do not induce the TGx-28.65 biomarker in the absence of S9 [Buick et al., 2015].

In this study, preliminary experiments were first conducted to explore the toxicity of various concentrations and types of S9, and their ability to increase micronucleus (MN) frequency in TK6 cells. The response of the TGx-28.65 biomarker in TK6 cells following exposure to dimethylnitrosamine, benzo[a]pyrene, and dexamethasone in the presence of 10% Aroclor-induced S9 was then assessed. An analysis of the effects of 5% ethanol-induced S9 on DMSO-treated cells was also undertaken. Following these preliminary studies, three concentrations were examined for each of the chemicals described in the paragraph above, in addition to concurrent solvent controls. Concentration responses were first done for all chemicals in the presence of 2% Aroclor-induced S9. Of the test agents that were expected to be genotoxic based on our literature review, only furan had no effects on relative survival and MN frequency using Aroclor-induced S9. TK6 cells were thus co-exposed to furan and 5% ethanol-induced S9 as it has a higher level of CYP2E1.


| Chemical                  | Ames | In Vitro Chrom Abs (CA or MN) | In Vitro Mouse lymphoma assay or gene mutation | In Vivo Chrom Abs | In Vivo MN | In Vivo Other | IARC classification/ CARC overall | Web resource | Additional notes |
|---------------------------|------|-----------------------------|---------------------------------------------|------------------|-----------|--------------|----------------------------------|--------------|-----------------|
| Acetaminophen             | −    | −                           | +                                           | +                | +         | 3            | 1999 IARC Monograph 73            |              | Overall, acetaminophen was genotoxic in mammalian cells in vivo and in vitro. It was not mutagenic in any standard assay in bacteria (IARC 1999). |
| 2-Aminoanthracene         | +    | +                           | +                                           | +                | +         | +            | EURL ECVAM Mutait                  |              | Class: Genotoxic |
| Cyclophosphamide          | +    | +                           | +                                           | +                | +         | +            | EURL ECVAM Mutait                  |              | Class: Genotoxic |
| Dibenz[a]anthracene       | +    | +                           | +                                           | +                | +         | +            | EURL ECVAM Mutait                  |              | Class: Genotoxic |
| Dimethylnitrosamine       | +    | +                           | +                                           | Not done or equivocal | +         | 2A           | Mutait                            |              | Class: Genotoxic |
| Furan                     | −    | +                           | −                                           | +                | −         | +            | IARC 1995 (Monograph 63)          |              | Prediction: Genotoxic (?)|
| Phenanthrene              | −    | −                           | −                                           | +                | +         | 2B           | IARC 2013 (Monograph 105)         |              | Class: Genotoxic |

Sources:
1. http://monographs.iarc.fr/ENG/Monographs/PDFs/.
2. https://eurl-ecvam.jrc.ec.europa.eu/databases/genotoxicity-carcinogenicity-db.
3. http://mutait.swansea.ac.uk/transgenicDB/Custom/GenotoxcarcinogenCustomQuery.php.
4. http://www.epa.gov/iris/index.html.
5. http://toxnet.nlm.nih.gov/.

CARC: Cancer Assessment Review Committee
MATERIALS AND METHODS

All experiments were performed as described previously, with any modifications noted below [Buick et al., 2015]. Detailed descriptions of the chemicals used in developing the training set and the genes in the TGx-28.65 biomarker are found in the original publications [Buick et al., 2015; Li et al., 2015].

Chemicals

The sources and CAS numbers for the chemicals used in this experiment are shown in Table II. All chemicals were dissolved and diluted in dimethylsulfoxide (DMSO) except for sucrose, which was dissolved in water. Chemical exposures were run concurrently with matched vehicle controls. Published information on the genotoxicity of the test chemicals is shown in Table I.

Cell Culture

TK6 cells were obtained from American Type Culture Collection (ATCC© CRL-8015; ATCC, Manassas, VA). TK6 cell culture and maintenance is described elsewhere [Ellinger-Zieglerbauer et al., 2009], in addition to our previous publication [Buick et al., 2015]. Prior to exposure, cells were seeded at a density of 4 (± 0.5) × 10⁶ cells/ml in twelve-well plates with a final volume of 3 ml per well. Chemical exposures were conducted in the presence of either Aroclor 1254-induced, ethanol-induced, or 5,6 benzoflavone/phenobarbital-induced (BEPB-induced) male Sprague Dawley rat liver S9 (Moltox, Boone, NC, USA) with NADPH generating system cofactors for 4 hr. TK6 cell exposures and associated flow cytometry were performed by Integrated Laboratory Systems, Inc. (ILS; Research Triangle Park, NC) and are described below.

### Table II. Test Chemicals, Source, CAS Number, and the Type and Concentration of S9 Used in the Experiments

| Test Article          | Source                          | CAS No.                  | Type of S9 Used        |
|-----------------------|---------------------------------|--------------------------|------------------------|
| Acetaminophen         | Sigma-Aldrich (St. Louis, MO, USA) | 103-90-2                 | 2% Aroclor 1254-induced |
| 2-Aminoanthracene     | Sigma-Aldrich (St. Louis, MO, USA) | 613-13-8                 | 2% Aroclor 1254-induced |
| Cyclophosphamide      | Sigma-Aldrich (St. Louis, MO, USA) | 6055-19-2                | 2% Aroclor 1254-induced |
| Dibenzo[a,h]anthracene| Santa Cruz Biotechnology (Dallas, TX, USA) | 53-70-3                  | 2% Aroclor 1254-induced |
| Dimethylnitroamine    | Sigma-Aldrich (St. Louis, MO, USA) | 62-75-9                  | 2% Aroclor 1254-induced* |
| Furan                 | Sigma-Aldrich (St. Louis, MO, USA) | 110-00-9                 | 5% ethanol-induced     |
| 2-Nitrofluorene        | Sigma-Aldrich (St. Louis, MO, USA) | 607-57-8                 | 2% Aroclor 1254-induced |
| Phenanthrene          | Sigma-Aldrich (St. Louis, MO, USA) | 83-01-8                  | 2% Aroclor 1254-induced |
| Sucrose               | Sigma-Aldrich (St. Louis, MO, USA) | 57-50-1                  | 2% Aroclor 1254-induced |

*Dimethylnitrosamine was tested with 10% Aroclor 1254-induced S9 in the experiments exploring the effects of different S9s on % RS, % MN and classification by TGx-28.65, but was repeated for the final definitive study using 2% Aroclor 1254-induced S9.

activity, which is critical for furan’s metabolism. Gene expression profiles were measured 3–4 hr following a 4 hr exposure of cells to the chemicals and S9. The TGx-28.65 biomarker was used to classify treatments as genotoxic or non-genotoxic. The toxicogenomics data were considered alongside flow cytometry evaluation of relative survival, apoptosis/necrosis, and MN frequency from TK6 cells collected 20 hr after the 4 hr exposure, and in parallel with published genotoxicity information for these chemicals. The data were combined with the results of previous work to explore the relationships between the measured endpoints.

### Pilot S9 Studies

Experiments were designed to explore the effects of different concentrations of three types of rat liver S9, alone or in combination, on TK6 cell survival and MN frequency both in the presence and absence of chemicals (all experiments were done in triplicate). In the first experiment, TK6 cells were exposed to increasing concentrations (1%–10%) of each of the three S9s individually (no test chemical exposure), to measure the effects of the S9 on % relative survival (RS) and MN frequency. Cells were exposed for 4 hr, and the media was then replaced for a 20 hr recovery period, prior to flow cytometry analysis (methods below). In the second experiment, cells were cultured in the presence of different mixtures of the S9s at different concentrations (concentrations and mixtures shown in Supporting Information Table I). In the third and fourth experiments, TK6 cells were exposed to increasing concentrations of dimethylnitrosamine and benzo[a]pyrene in the presence of different concentrations and mixtures of S9 (Supporting Information Table I) to determine effects on % RS and % MN, alongside concurrent DMSO controls. The experimental details for cell sampling and analysis of % RS and % MN are found below.

A final experiment was conducted in which TK6 cells were exposed to increasing concentrations of dimethylnitrosamine, 10 µg/ml benzo[a]pyrene, or 1 mM dexamethasone, in the presence of 10% Aroclor 1254-induced rat liver S9 for 4 hr concurrent with DMSO controls. The media was replaced and cells were allowed to recover for 4 hr (for RNA sampling and analysis using the TGx-28.65 biomarker; methods below) or 20 hr (for analysis of % RS and % MN).

### Range Finder Studies

Test chemicals were first run in range finder studies for concentration selection (Table II for the type of S9 used for each chemical). Cells were exposed to up to eight concentrations of the chemical agent (single replicates) or solvent for 4 hr, followed by a 20 hr recovery. TK6 cells exposed to 10 µg/ml benzo[a]pyrene served as the positive control. All exposures were done in the presence of either 2% Aroclor 1254-induced rat liver S9 with NADPH generating system cofactors. For known genotoxic agents, if there was no response in terms of declines in relative survival or increases in MN following treatment in the presence of Aroclor-induced S9, other S9s were considered. In this experiment, only furan did not show the expected response to chemical exposure. It is established that furan is metabolized by CYP2E1 and thus subsequent experiments used 5% ethanol-induced S9 for furan range finders. Following the 4 hr exposure, cells were collected by centrifugation and rinsed with phosphate buffered saline (PBS) prior to the replacement of fresh media. Cells were then incubated for an additional 20 hr, and harvested for cytotoxicity and MN analysis.

Test chemicals were measured endpoints.

The data were combined with the results of previous work to explore the relationships between the measured endpoints.

The sources and CAS numbers for the chemicals used in this experiment are shown in Table II. All chemicals were dissolved and diluted in dimethylsulfoxide (DMSO) except for sucrose, which was dissolved in water. Chemical exposures were run concurrently with matched vehicle controls. Published information on the genotoxicity of the test chemicals is shown in Table I.
Cytotoxicity, Apoptosis/Necrosis, and MN Frequency

The In Vitro MicroFlow™ kit (Liton Laboratories, Rochester, NY) was used for flow cytometry assessment of TK6 cells. This assay can be used to simultaneously determine the % RS, percentage of apoptotic/necrotic cells, and MN frequency. Reagent preparation, cell harvest, sequential staining, and flow cytometric analysis were performed according to the In Vitro MicroFlow™ instructional manual. RS was determined by the addition of 6 µm latex microspheres (as an internal standard) to the initial cell preparation, which were counted in the cells sampled 24 hr later by flow cytometry on a Becton-Dickinson FACSCalibur™ 2 laser 4-color instrument (Becton Dickinson, San Jose, CA). RS was calculated using the intact viable nuclei to bead ratios in treated versus control cells. Apoptotic/necrotic cells were identified by ethidium monoazide (Nucleic Acid Dye A contained in the kit) staining, which crosses the outer membrane of necrotic and apoptotic cells. MN were scored in treated and control cells by analyzing 20,000 (± 2,000) cells from each sample using the double staining procedure described in the In Vitro MicroFlow™ kit instruction manual [Avlasevich et al., 2006].

Definitive Studies

Based on range finder studies, three concentrations per chemical (low, medium, and high) were selected for definitive studies: (1) a low concentration that caused little or moderate cytotoxicity and did not cause an increase in MN frequency; (2) a medium concentration that was between the low and the high concentrations; and (3) a high concentration that approached 55% cytotoxicity (i.e., 40%–50% RS), as recommended in OECD test guideline 487 [OECD 2010]. In the absence of cytotoxicity or genotoxicity, the top concentration selected was 10 mM or 5 mg/ml, whichever was lower in both cases, when solubility of cytotoxicity or genotoxicity, the top concentration selected was the top concentration that increases the expression of at least one of these genes (gene symbol). Because of revised annotation on the Agilent database under accession number GSE74216. Statistical and bioinformatics analyses are described in detail in our previous papers [Buick et al., 2015; Li et al., 2015]. Briefly, error-weighted averages for each TGx28.65 gene were generated by merging Agilent probe ID signal intensities for the Genbank accession number associated with the classifier genes (gene symbol). RT-qPCR methods were described in detail previously [Buick et al., 2015]. Briefly, RT-qPCR was performed using TaqMan® Gene Expression Assays (TaqMan® MGB probes, FAM™ dye-labeled), as per the manufacturer’s instructions, on a Bio-Rad CFX96™ Real-Time PCR Detection system (n = 3 replicates per condition). TaqMan® Gene Expression Assay IDs (Life Technologies Inc., Burlington, ON, Canada) were: Hs00231069_m1 (ATF3); Hs00355782_m1 (CDKN1A); Hs00169255_m1 (GADD45A). GAPDH was used as the reference gene, and was not affected by treatment. RT-qPCR analysis was performed on TK6 cells exposed to the chemicals for 4 hr and sampled after the 3-4 hr recovery period.

The relative expression (RE) ratio was calculated as follows:

\[ \text{RE} = \frac{(E_{\text{target}}^{\Delta\Delta C_{\text{target}}/\text{control} - \text{sample}})}{(E_{\text{ref}}^{\Delta\Delta C_{\text{ref}}/\text{control} - \text{sample}})} \]

Where \( E \) represents the PCR efficiency, target is the gene of interest, ref is the reference gene, and \( C(i) \) is the crossing threshold [Fafit et al., 2002]. A one-way ANOVA was applied to identify significant changes relative to vehicle (<59) controls. Results were back-transformed and the fold changes linearized. Standard errors for the back transformed results were obtained using the delta method.

Microarray Analysis

Transcriptional profiles were derived on Agilent DNA microarrays using a two-color dye swap design [Kerr and Churchill 2001]. Microarray analysis was performed on pooled technical replicates from samples collected 3–4 hr post-exposure and on concurrent pooled vehicle controls. Cyanine-3 and cyanine-5 labeled cRNA was created from 100 ng of total RNA, using Agilent Low-Input Quick Amp Labelling kits according to the manufacturer’s instructions (Agilent Technologies, Mississauga, ON, Canada). Labeled cRNA samples (325 ng of cyanine-3 labeled cRNA and 325 ng of cyanine-5 labeled cRNA) were hybridized to Agilent SurePrint G3 Human GE 8x60K oligonucleotide microarrays at 65°C for 17 hr. Slides were washed according to the manufacturer’s instructions and scanned on an Agilent DNA microarray scanner at 3 µm resolution. Agilent Feature Extraction (version 11.0.1.1) was used for data extraction and quality control analysis.

Statistical and Bioinformatic Analyses

Generalized estimating equations (GEEs) [Liang and Zeger 1986; Prentice and Zhao 1991] were applied for the analysis of RS and MN. A normal distribution for the RS data and a binomial distribution for MN data were assumed for the error terms. This analysis was conducted using the gee pack library ([Yan, 2002; Yan and Fine 2004; Højsgaard et al., 2005]) in R. GEEs are semi-parametric and provide an alternative to generalized linear models. GEEs only require specification of the first two moments, the mean and the variance. In this analysis, a log link function was used for the MN analysis. Here, the results were back transformed to the original scale using the delta method. Statistically significant increases in MN frequency were considered to be a positive result if the changes were at least twofold above matched solvent vehicle controls + 59.

Gene expression data were deposited in the National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database under accession number GSE74216. Statistical and bioinformatics analyses are described in detail in our previous papers [Buick et al., 2015; Li et al., 2015]. Briefly, error-weighted averages for each TGx28.65 gene were generated by merging Agilent probe ID signal intensities for the Genbank accession number associated with the classifier genes (gene symbol). Because of revised annotation on the Agilent array, the biomarker contained 64 genes in the present study. The hclust function in R (www.r-project.org) was used for hierarchical clustering; agglomerative clustering was based on complete linkage with \( 1 - p^2 \) as the dissimilarity measure. Statistical significance was determined by the permutation test implemented in the *Hmisc* package in R.
the dissimilarity metric, where \( p^\circ \) is the error-weighted Pearson’s correlation coefficient for two treatments or two genes. The Nearest Shrunken Centroids (NSC) method in the pamr function of R (www.bioconductor.org) was used to classify the gene expression profiles of the test agents based on similarity to the reference chemical profiles in the database [Li et al., 2015] using statistical and bioinformatics tools [Tibshirani et al., 2002]. More specifically, the standardized centroid (SC) was computed for each class of the training set. The SC was then shrunken in the direction of the overall centroid to create the NSC. Experimental samples were classified through comparison of their gene expression profile to the class of NSCs. Samples were assigned to a class (genotoxic or non-genotoxic) that was closest to it in squared distance when probability of class membership was greater than 0.90.

Heatmaps, hierarchical clustering, and principal component analyses (PCA) were conducted to aid in the interpretation of the results. Hierarchical cluster analysis was conducted using Euclidean distances with average linkage. These analyses were conducted using the hclust [Becker et al., 1988] function in the R software. The PCAs were performed using the prcomp function [Venables and Ripley, 2002] in R. Only the training set data [Li et al., 2015] were used to estimate the principle components. The PCA loadings obtained from this analysis were applied to the new data. A scatterplot with the training data and new data was generated to visualize the results.

RESULTS

S9 Analysis

Three different types of rat liver S9 were assessed for their effects on cytotoxicity and MN frequency (Supporting Information Table I A,B). All of the S9s, alone or in a mixture, caused a decrease in RS in TK6 cells, but had no effect on % MN up to the 10% S9 applied. Ethanol-induced S9 had the greatest effect on RS, causing a decline to 82% RS at 1%, and 64% RS at 10% S9 concentrations. However, all of the S9s, alone or in combination, were generally similar in terms of their impacts on cytotoxicity and MN in untreated cells, causing approximately 20%–35% declines in RS for concentrations spanning 2%–10% S9.

TK6 cells were then exposed to increasing concentrations of either dimethylnitrosamine or benzo[a]pyrene to measure the efficacy of the various S9s in metabolically activating these chemicals (Supporting Information Table I C,D). This analysis considered 10% Aroclor- and 10% BF/PB-induced rat liver S9, and 5% ethanol-induced S9 (reduced to 5% because of the greater cytotoxic effects of this S9). We also considered mixtures of the Aroclor- and BF/PB-induced S9s with the ethanol-induced S9. The analysis revealed that the largest increase in % MN following chemical exposure (i.e., the most effective metabolic activation) was observed for 10% Aroclor-induced for both benzo[a]pyrene and dimethylnitrosamine, but most S9 preparations were effective to a certain degree in activating these chemicals, with the exception that ethanol-induced S9 did not activate benzo[a]pyrene. There was an inverse relationship between % MN and % RS, with 10% Aroclor-induced S9 also causing the greatest decline in RS.

A final study on S9 was done to explore the use of the TGx-28.65 biomarker in combination with a high % S9. For this analysis, we exposed cells to 10% S9 in combination with increasing concentrations of dimethylnitrosamine (1, 5, and 10 mM), 10 \( \mu \)g/ml benzo[a]pyrene, or 1 mM dexamethasone (Supporting Information Table IIE). Significant increases in % MN and declines in RS were found for all concentrations of dimethylnitrosamine and for benzo[a]pyrene relative to DMSO controls. No effect on MN was observed for dexamethasone relative to controls, but a slight increase in RS was observed. NSC application of the TGx-28.65 biomarker correctly classified all concentrations, and each replicate (n = 3 per condition), of dimethylnitrosamine and benzo[a]pyrene as genotoxic (Fig. 1a).

Analysis by PCA and hierarchical clustering placed dimethylnitrosamine and benzo[a]pyrene closest to genotoxic agents (Supporting Information Figs. 1a and 1b), except for the lowest concentration of dimethylnitrosamine. In contrast, two out of three replicates of dexamethasone were correctly classified as non-genotoxic, with one misclassification. Analysis by PCA and hierarchical clustering grouped dexamethasone with non-genotoxic agents, with one replicate on the borderline in the PCA (Supporting Information Fig. 1c). Finally, an analysis of TK6 cells treated with DMSO in the presence of S9 (either 10% Aroclor-induced or 5% ethanol-induced) versus TK6 cells treated with DMSO without S9 was conducted (Fig. 1b and Supporting Information Fig. 1d). This analysis did not result in a classification of genotoxicity using the TGx-28.65 biomarker for either of the S9s.

Toxicity Assessment of Chemicals in TK6 Cells

Range finder experiments (data not shown) were conducted to identify a low, medium, and high concentration for each test chemical for definitive studies (the study used for gene expression analysis), with the aim to have the top concentration achieve (55 ± 5%) cytotoxicity. With the exception of furan, all experiments were done using 2% Aroclor-induced S9. Because exposure of TK6 cells to the chemical furan did not cause a decline in RS as expected with 2% Aroclor-induced S9, we repeated range finders for furan using 5% ethanol-induced S9 (CYP2E1 is more active in ethanol-induced S9). The 5% ethanol-induced S9 had the expected effect on RS with concomitant increases in MN in the exposed TK6 cells (data not shown). Thus, 5% ethanol-induced S9 was used in subsequent definitive studies of furan (described below). The % S9 used in the definitive experiments is shown in Table II.

The % RS, % apoptotic cells, and % MN for the final definitive studies for each chemical in TK6 cells is shown in Figure 2. RS declined at the highest concentration to approximately 50% for every chemical except sucrose, with concomitant increases in apoptosis/necrosis. We considered MN frequency to be biologically relevant if
Fig. 1. (a) Heat maps of the TGx-28.65 gene signature following exposure to test chemicals in the presence of 10% Aroclor 1254-induced S9 (right hand side). The heat map on the left hand side shows the 28 reference compounds used to generate the TGx-28.65 biomarker [Li et al., 2015]. The labels on the right hand-side show gene symbols for the classifier genes in TGx-28.65. Upregulated genes are shown in red and down-regulated genes are shown in green. Genes that were similar to solvent controls are in black. The results of classification using the TGx-28.65 biomarker are shown in red (genotoxic) and blue (non-genotoxic) bars above each heat map. Genotoxicity based on the literature review is shown as 'Expectation based on literature' (red = genotoxic; blue = non-genotoxic) above the heat maps. Note: BaP, benzo[a]pyrene; DMN, dimethylnitrosamine (three concentrations: 1, 5, and 10 mM); DEX, dexamethasone. (b) Heatmap of the TGx-28.65 biomarker genes in TK6 cells treated with DMSO in the presence of 10% Aroclor-induced S9 or 5% ethanol-induced S9 versus non-S9 exposed DMSO-treated TK6 cells.
A P < 0.05 and the fold change relative to vehicle control (+S9) was greater than 2-fold. The largest increases in % MN were observed for the high concentrations of acetaminophen (14-fold increase above vehicle + S9 control) and cyclophosphamide (8-fold increase above control). Dibenz[a,h]anthracene, dimethylnitrosamine, furan, and 2-nitrofluorene all had statistically significant % MN increases of over twofold relative to controls for at least one concentration. Although RS for TK6 cells exposed to 2-aminoanthracene declined to 47%, there was only a 1.8-fold increase in MN above controls. There was no significant increase in % MN for phenanthrene or sucrose.

Fig. 2. Relative survival (% RS – ○ – left axis), apoptosis (% apoptotic – ▲ – left axis), and MN frequency (% micronuclei – ■ – right axis) in human TK6 cells (n = 3) following exposure to test chemicals. Standard error bars are shown for % MN and % apoptotic cells (if error bars are not visible, then the range was too narrow to be visible in the plot). Cells were exposed for 4 hr in the presence of S9, and sampled after a 20 hr recovery period. * denotes P < 0.05 compared with the vehicle control. Panels (a)–(i) are the different chemicals, listed at the bottom of each figure. Please note the different scales on the % micronuclei axes for acetaminophen and cyclophosphamide, which showed the largest responses.
The highest concentration of phenanthrene caused a decline in RS to ~40%. Exposure to 10 µg/ml benzo[a]pyrene (positive control) consistently caused declines in RS to ~30%–40%, and minimum increases in MN of ~threefold above controls (data not shown).

**Gene Expression Analysis and Genotoxicity Classification Using TGx-28.65**

RT-qPCR analysis (Supporting Information Fig. 2) showed that all of the test chemicals except for dimethylnitrosamine, phenanthrene, and sucrose induced the minimum 2-fold change in the expression of at least one of the three stress response genes (ATF3, GADD45A, and CDKN1A) at the highest concentration selected, and many at the medium concentration as well. For analysis by DNA microarrays, the three replicates for each exposure were pooled and hybridized against pooled matched controls. The five mutagenic carcinogens (2-aminoanthracene, cyclophosphamide, dibenz[a,h]anthracene, dimethylnitrosamine, and 2-nitrofluorene) were classified as genotoxic using TGx-28.65 at the mid and high concentrations (Fig. 3). All concentrations of 2-aminoanthracene and cyclophosphamide, including the lowest concentrations that did not induce MN or significant effects on apoptosis/necrosis or RS, were classified as >0.9 probability of being genotoxic (i.e., predicting that the chemical causes DNA damage). The low concentrations of dibenz[a,h]anthracene, dimethylnitrosamine, and 2-nitrofluorene did not induce a genotoxic signature, and these concentrations were classified as non-genotoxic. We note that dimethylnitrosamine induced the TGx-28.65 genotoxic signature in the absence of a robust effect on the stress response genes (Supporting Information Fig. 2). In contrast, the two non-genotoxicants (phenanthrene and sucrose) were classified as >0.9 probability of being non-genotoxic. Finally, acetaminophen and furan were classified as genotoxic using TGx-28.65 at all concentrations. We also examined classification by PCA and hierarchical cluster analysis using the TGx-28.65 biomarker genes (Supporting Information Figs. 3a-3i). This analysis was very consistent with classification based on NSC probability. Noted discrepancies included: classification of the lowest concentration of cyclophosphamide as non-genotoxic by PCA; classification of the lowest concentration of dibenz[a,h]anthracene as genotoxic by hierarchical cluster analysis; and classification of all concentrations of 2-nitrofluorene as non-genotoxic by hierarchical cluster analysis.
DISCUSSION

Genotoxicity testing is used to predict potential carcinogenicity or risk of inducing heritable genetic effects. There is increasing emphasis on the use of high-throughput in vitro screening in human cells in culture as the first tier of chemical screening [Kavlock et al., 2012; Thomas et al., 2013b; Kligerman et al., 2014], with growing interest in using toxicogenomics signatures to predict adverse effects and mode of action, and for prioritizing further chemical testing [Thomas et al., 2013b; Zhu et al., 2014]. Shortcomings of existing in vitro high-throughput tests include the lack of: (a) metabolic capacity of many of the cell models used; (b) validated toxicogenomics signatures to predict toxicity; and (c) sensitive biomarkers of genotoxicity for high-throughput screening [Kligerman et al., 2014]. Our previous work established a protocol for assessment of genotoxicity using the TGx-28.65 biomarker [Li et al., 2015], and confirmed that the biomarker accurately classified two established mutagens (benzo[a]pyrene and aflatoxin B1) and two non-mutagens.

Fig. 3. Heat maps of the TGx-28.65 biomarker genes following exposure to the nine test chemicals (a)–(i). The heat map on the left hand side shows the 28 reference compounds used to generate the biomarker [Li et al., 2015], and the heatmap for five benzo[a]pyrene (BaP) positive control pooled replicates run during the experiment. The gene symbols for the classifier genes in the TGx-28.65 biomarker are shown on the right hand-side of the heat maps. Upregulated genes are shown in red and downregulated genes are shown in green. Genes that were similar to solvent controls are in black. The results of classification using the TGx-28.65 biomarker are shown in red (genotoxic) and blue (non-genotoxic) bars above each heat map. Genotoxicity based on the literature review is shown as ‘Expectation based on literature’ (red = genotoxic; blue = non-genotoxic) above the heat maps. The test chemicals are shown in increasing doses with: (a) 2-AA, 2-aminoanthracene; (b) APAP, acetaminophen; (c) CP, cyclophosphamide; (d) D[a,h]A, dibenz[a,h]anthracene; (e) DMN, dimethylnitrosamine; (f) FUR, furan; (g) 2NF, 2-nitrofluorene; (h) PHN, phenanthrene; and (i) SUC, sucrose.
(dexamethasone and phenobarbital) in TK6 cells in the presence of 1% BF/PB-induced rat liver S9 [Buick et al., 2015]. Although BF/PB-induced rat liver S9 is considered an acceptable choice for genetic toxicology analyses, it is recognized that different agents will require different metabolic activation systems [OECD 2014a,b]. In this work, we demonstrate that the biomarker can be used in combination with other S9s, and verified its functionality for nine additional chemicals in the presence of either 2% Aroclor-induced or 5% ethanol-induced S9.

Experiments were first undertaken to establish whether three different types of S9 (Aroclor-, BF/PF-, or ethanol-induced) cause cytotoxicity and clastogenicity in our experimental model. We found that the different S9s caused declines in RS in the absence of chemical treatment, with ethanol-induced S9 having the greatest effect. All S9s, even at low concentrations, impaired cell survival; this is consistent with published work (e.g., [Li, 1984]). Although none of the S9s caused MN induction on their own, they differed in their ability to bioactivate chemicals to induce MN as expected. We found that 10% Aroclor-induced S9 was the most effective in inducing MN for both dimethylnitrosamine and benzo[a]pyrene in TK6 cells. We also noted that 5% ethanol-induced S9 was not effective in metabolically activating benzo[a]pyrene, highlighting the importance of using different S9s. This was not surprising as benzo[a]pyrene metabolism generally requires high EROD activities that are associated with CYP1A; the specifications sheets from the S9 manufacturer indicated over 200-fold EROD induction (and over 100-fold induction of MROD) in the Aroclor-induced S9, but only 2-fold EROD induction (1.7-fold MROD) in the ethanol-induced S9 (data not shown). It has previously been proposed that ‘supermixes’ of S9 could effectively be used to reduce the need to test chemicals with several different types of S9 [Paolini et al., 1991]. Therefore, we also analyzed various mixes of S9 to assess their baseline toxicity and ability to bioactivate chemicals to cause MN. We found that these mixtures of S9 had similar impacts on RS and were effective in activating dimethylnitrosamine and benzo[a]pyrene to increase MN frequency in TK6 cells, and thus could be used in our model. Although using mixtures of S9 is a promising approach for chemical screening, we proceeded with the next phases of our project using standard S9s on their own to be consistent with current practice.

Because some chemicals require high concentrations of S9 to be metabolically activated, we explored whether the use of a high % S9 impairs the ability to predict genotoxicity using the TGx-28.65 biomarker. Analysis of DMSO in the presence of either 10% Aroclor- or 5% ethanol-induced S9 versus non-S9 DMSO treated control cells did not trigger the TGx-28.65 biomarker to suggest genotoxicity. NSC classification probabilities, PCA, and hierarchical clustering for cells exposed to dimethylnitrosamine (1, 5, or 10 mM), benzo[a]pyrene, or dexamethasone, alongside concurrent DMSO controls, all in the presence of 10% Aroclor-induced S9, were determined using the TGx-28.65 biomarker. We used 10% Aroclor-induced S9 because it was the most effective in bioactivating dimethylnitrosamine. All concentrations of dimethylnitrosamine and the benzo[a]pyrene positive control were classified as genotoxic by NSC. This was consistent with analysis of the TGx-28.65 biomarker by PCA and hierarchical cluster analysis, with the exception that the lowest concentration of dimethylnitrosamine was on the same branch as non-genotoxic agents. Thus, this concentration likely does not induce these genes to the same extent as the higher concentrations. In contrast, two out of three replicates of dexamethasone-treated samples were classified as non-genotoxic. The dendogram revealed that the signature was most similar to non-genotoxic agents for all three replicates of dexamethasone, but the PCA placed the outlier sample on the borderline between genotoxic and non-genotoxic agents. Given that one of the three replicates of dexamethasone was classified as genotoxic, and based on the observed cytotoxic effects of S9 and recommendations in the literature for TK6 cells [Rees et al., 1989; Xiao and Fontanie, 1995; OECD, 2014a], we advise the use of lower concentrations of S9 when possible to avoid false positives. We thus performed our subsequent experiments using 2% Aroclor-induced S9.

All genotoxic chemicals except furan were metabolically activated to cause a decline in RS and a concomitant increase in MN in the presence of 2% Aroclor-induced S9 in the TK6 cells (Fig. 2). Thus, the other chemicals that are metabolically activated by CYPE2E1 (i.e., acetaminophen and dimethylnitrosamine) are clearly also metabolized by enzymes present in Aroclor-induced S9. Subsequent concentration-response experiments were performed using ethanol-induced S9 for furan, and the appropriate response was achieved (Fig. 2f).

Classification by TGx-28.65 for our nine test compounds was consistent with expectations (Table I and Fig. 3). The TGx-28.65 biomarker classified the mid and the high concentrations of all established genotoxic agents as genotoxic, and all of the concentrations of non-genotoxic chemicals as non-genotoxic. Thus, despite the increase in cytotoxicity, the use of a higher concentration S9 did not impair the ability to classify chemicals using the TGx-28.65 biomarker.

To explore the performance of the biomarker in predicting genotoxicity, the data were combined with results from our previous study to examine the relationship between measures of cytotoxicity, apoptosis/necrosis, and induction of MN with gene expression changes and classification with the TGx-28.65 biomarker using all three methods (NSC probability, PCA, and hierarchical cluster analysis). This analysis included a total of 13 chemicals for which we had each of the measures (Table III) (note:
### TABLE III. Summary of All Data in TK6 Cells Treated with Chemicals in the Presence of S9 and Analyzed by Flow Cytometry (% Apoptosis, % Relative Survival, % MN), RT-qPCR (ATF3, CDKN1A, GADD45A), and DNA Microarray (TGx-28.65)

| Genotoxic agents | % Apoptotic cells | % Relative Survival | % MN | ATF3 | CDKN1A | GADD45A | NSC | PCA | TGx-28.65 | TGx-28.65 |
|------------------|-------------------|---------------------|------|------|--------|---------|-----|-----|-----------|-----------|
| 2-Aminooanthracene | DMSO | 4.84 | 100.00 | 0.68 |        |         |      |     |           |           |
| 2% Aroclor S9 | (µg/ml) | 0.75 | 6.81 | 87.44 | 0.94 | 0.9 | 1.0 | 0.8 | + | + | + | + |
| 1.5 | 14.20 | 59.61 | 1.04 | 1.6 | 2.2 | 1.5 | + | + | + | + |
| 2 | 18.84 | 46.78 | 1.22 | 2.3 | 4.6 | 1.7 | + | + | + | + |
| Acetaminophen (mM) | DMSO | 7.52 | 100.00 | 0.94 |        |         |      |     |           |           |
| 2% Aroclor S9 | 1 | 9.64 | 89.12 | 1.75 | 1.9 | 1.8 | 1.2 | + | + | + | + |
| 3.2 | 18.64 | 61.81 | 4.30 | 6.1 | 7.5 | 3.8 | + | + | NC | NC |
| 10 | 28.43 | 42.44 | 14.36 | 12.9 | 15.5 | 7.2 | + | + | NC | NC |
| Aflatoxin B1 (µM)c | DMSO | 2.8 | 100 | 0.70 |        |         |      |     |           |           |
| 1% BF/PB S9 | 0.025 | 4.9 | 79.3 | 1.42 | 2.0 | 1.7 | 1.3 | – | – | – | – |
| 0.075 | 10.9 | 48.1 | 5.72 | 4.6 | 2.6 | 1.9 | + | + | + | + |
| 0.1 | 14.1 | 40.2 | 9.65 | 4.7 | 3.0 | 2.3 | + | + | + | + |
| Benzo[a]pyrene (µg/ml)d | DMSO | 3.7 | 100 | 0.73 |        |         |      |     |           |           |
| 1% BF/PB S9 | 0.45 | 7.7 | 79.9 | 1.18 | 1.1 | 1.6 | 1.0 | – | + | + | – |
| 1.4 | 10.4 | 56.5 | 2.57 | 1.5 | 3.0 | 1.3 | + | + | + | + |
| 10 | 11.5 | 47.4 | 4.45 | 2.1 | 5.8 | 1.6 | + | + | + | + |
| Cyclophosphamide (µM) | DMSO | 10.02 | 100.00 | 0.94 |        |         |      |     |           |           |
| 2% Aroclor S9 | 1 | 11.11 | 89.11 | 1.25 | 1.3 | 1.1 | 0.5 | + | + | + | + |
| 5 | 14.51 | 67.10 | 2.38 | 1.6 | 1.8 | 0.7 | + | + | + | + |
| 20 | 28.43 | 42.00 | 7.78 | 1.9 | 2.6 | 0.9 | + | + | + | + |
| Dibenz[a,h]anthracene (µg/ml) | DMSO | 10.36 | 100 | 1.89 |        |         |      |     |           |           |
| 2% Aroclor S9 | 1 | 7.94 | 97.79 | 2.16 | 1.1 | 1.2 | 1.2 | – | – | + | – |
| 20 | 16.82 | 64.12 | 3.42 | 1.6 | 2.4 | 1.7 | + | + | + | + |
| 280 | 31.79 | 53.91 | 3.80 | 2.3 | 4.2 | 2.0 | + | + | + | + |
| Dimethylnitrosamine (mM) | DMSO | 6.36 | 100.00 | 0.71 |        |         |      |     |           |           |
| 2% Aroclor S9 | 1 | 8.47 | 76.40 | 1.15 | 0.8 | 1.0 | 1.0 | – | – | – | – |
| 5 | 12.27 | 72.92 | 2.29 | 0.8 | 1.3 | 1.2 | – | – | – | – |
| 10 | 30.63 | 45.65 | 2.80 | 0.8 | 1.2 | 1.5 | + | + | + | + |
| 2-Nitrofluorene (µM) | DMSO | 7.52 | 100.00 | 0.94 |        |         |      |     |           |           |
| 2% Aroclor S9 | 10 | 9.84 | 91.69 | 1.48 | 1.3 | 1.0 | 0.8 | – | – | – | – |
| 50 | 12.62 | 75.78 | 2.23 | 1.2 | 1.4 | 0.8 | + | + | + | + |
| 300 | 20.43 | 55.43 | 3.13 | 2.0 | 2.5 | 1.4 | + | + | + | + |
| Non-genotoxic agents | Phenanthrene (µg/ml) | 10.36 | 100.00 | 1.89 |        |         |      |     |           |           |
| 2% Aroclor S9 | 8 | 10.60 | 94.13 | 2.15 | 0.8 | 1.0 | 1.2 | – | – | – | – |
| 40 | 14.71 | 65.32 | 2.07 | 0.7 | 0.9 | 1.0 | – | – | – | – |
| 100 | 26.73 | 38.91 | 2.31 | 0.9 | 1.3 | 1.2 | – | – | – | – |
| Phenobarbital (mM)e | DMSO | 10.02 | 100.00 | 0.94 |        |         |      |     |           |           |
| 1% BF/PB S9 | 1 | 9.18 | 101.14 | 1.03 | 1.4 | 1.1 | 0.6 | – | – | – | – |
| 3.2 | 12.11 | 85.06 | 1.00 | 2.7 | 2.2 | 0.9 | – | – | – | – |
| 10 | 23.42 | 53.76 | 1.46 | 3.7 | 2.2 | 0.7 | – | – | – | – |
| Sucrose (mM) | Water | 8.87 | 100.00 | 0.76 |        |         |      |     |           |           |
| 2% Aroclor S9 | 1 | 8.98 | 95.04 | 0.94 | 0.6 | 0.4 | 0.8 | – | – | – | – |
| 3.2 | 8.95 | 92.09 | 0.97 | 0.8 | 0.4 | 0.8 | – | – | – | – |
| 10 | 9.75 | 90.93 | 0.97 | 0.8 | 0.4 | 0.8 | – | – | – | – |
| Dexamethasone (µM)f | DMSO | 3.8 | 100 | 0.64 |        |         |      |     |           |           |
| 1% BF/PB S9 | 0.63 | 5.5 | 82.4 | 0.81 | 0.8 | 1.3 | 1.1 | – | – | – | – |
| 1 | 12.2 | 71.3 | 1.07 | 0.7 | 1.0 | 1.0 | – | – | – | – |
| 7.5 | 22 | 66.4 | 0.45 | ND | ND | ND | – | – | – | – |
| Test agent | Furan (mM) | DMSO | 3.89 | 100.00 | 1.22 |        |         |      |     |           |           |
| 5% ethanol S9 | 2 | 7.07 | 80.06 | 1.57 | 2.3 | 3.2 | 1.4 | + | + | + | + |
| 3 | 8.40 | 64.52 | 1.77 | 3.1 | 5.3 | 1.4 | + | + | + | + |
| 5 | 15.26 | 48.45 | 2.78 | 5.4 | 9.8 | 2.4 | + | + | + | + |

aThe numbers in these columns represent mean fold change relative to vehicle control (+S9), measured by RT-qPCR.
b represents classification as genotoxic; - represents classification as non-genotoxic. NSC = Nearest shrunk centroids using TGx-28.65; PCA = Principal Component Analysis of TGx-28.65 gene set with classification of + to the left of the red line demarking 0 in PC1 and – to the right of the red line; Dendogram created using TGx-28.65 gene set with classification based on branch position.
cValues are from our previous publication [Buick et al., 2015].
dAbbreviations: NC, non-classifiable; ND, analysis not done; BF/PB, benzo[a]pyrene/phenobarbital.
our work on agents not requiring metabolic activation did not measure RS, apoptosis/necrosis, or MN, and thus was not included). Because the derivation of expression changes in the TGx-28.65 biomarker in this study was done on pooled samples, a quantitative association analysis between TGx-28.65 biomarker genotoxicity calls and the other endpoints could not be undertaken. However, we visually inspected these data for qualitative trends.

There was no apparent relationship between the probability of a genotoxic or a non-genotoxic call by NSC analysis of TGx-28.65 biomarker genes relative to the other endpoints. Both genotoxic and non-genotoxic chemicals (classified as such, and based on the literature) induced significant decreases in RS and increases in apoptosis/necrosis as expected. Probability of genotoxicity by TGx-28.65 was consistent with measured % MN, although concentrations that did not induce MN, and chemicals that did not induce MN (e.g., 2-aminoanthracene), were classified as genotoxic. Thus, this signature is not simply an indicator of clastogenicity.

We also explored the relationship between induction of the three prototypical stress response genes (ATF3, CDKN1A, and GADD45) used for concentration selection [Li et al., 2015] and other measures in the experiment. Induction of the stress response marker genes was somewhat consistent with probability of genotoxicity by TGx-28.65. Chemicals that were classified as non-genotoxic tended not to induce these genes or induced them to a lesser extent. Because both CDKN1A and GADD45A are part of the biomarker, this was as expected. However, we note that dimethylnitrosamine did not induce any of the genes in the stress response panel and yet was classified as genotoxic, and concentrations of genotoxic chemicals that did not induce the stress response genes (e.g., the low concentrations of 2-aminoanthracene, cyclophosphamide, dibenz[a,h]anthracene, and 2-nitrofluorene) were classified as genotoxic by the TGx-28.65 biomarker.

Analysis across the alternative methods of classification (PCA and hierarchical clustering) using the biomarker was also consistent with the above with a few noted exceptions. In particular, classification by hierarchical cluster analysis using the genes in the TGx-28.65 biomarker was inconsistent with NSC classification for four datapoints, including all doses of 2-nitrofluorene. However, these additional methodologies broadly supported the NSC classification. Overall, it appears that TGx-28.65 is a sensitive biomarker of genotoxicity, as probabilities of genotoxicity were found even at low concentrations for genotoxic chemicals, and non-genotoxic chemicals were classified accurately.

Given that the TGx-28.65 biomarker was developed using a training set of chemicals causing genotoxicity through diverse modes of action (i.e., chemicals causing DNA alkylation, bulky DNA adducts, topoisomerase inhibition, X-rays, etc.), part of the present work involved exploring its application to testing a chemical that may be genotoxic through an indirect mechanism. Furthermore, we sought to determine what insight TGx-28.65 analysis may provide for a chemical yielding equivocal findings in standard genetic toxicity tests. Furan is a contaminant in heat-treated foods that causes liver cancer in both rats and mice [NTP, 1993]. There is some debate over the role of genotoxicity in the carcinogenicity of furan because of inconsistent findings in standard genotoxicity tests (Table I). Furan is negative in the Ames test, but causes chromosomal aberrations in Chinese hamster ovary cells and B6C3F1 mouse bone marrow cells [NTP, 1993]. Analyses of MN in vivo in mice and rats have yielded mixed findings, but are typically negative [Durling et al., 2007; Leopardi et al., 2010; McDaniel et al., 2012]. Assessment of red blood cell MN frequency, PigA mutant frequency, Hprt lymphocyte mutant frequency, and cII liver mutation in Big Blue rats treated for 8 weeks with high doses of furan were negative [McDaniel et al., 2012]. However, assessment of liver DNA strand breaks in this same study using the comet assay revealed DNA damage at high doses. F344 rats exposed to furan at cancer-causing doses exhibited a near-linear dose-dependent increase in DNA strand breaks by the comet assay, in parallel with increases in oxidized purines and pyrimidines [Ding et al., 2012]. The authors of these studies concluded that the genotoxic action of furan is likely a secondary consequence of oxidative stress produced during furan metabolism, which is consistent with increases in 8-oxo-dG adducts found in furan-treated rat livers in another study [Hickling et al., 2010]. Furan is metabolized by cytochrome P450 2E1 (CYP2E1) [Kedderis et al., 1993]; it has been experimentally demonstrated that metabolic activation is necessary for furan to induce cytotoxicity [Fransson-Steen et al., 1997; Kellert et al., 2008; Gates et al., 2012]. Reactive oxygen species are produced during chemical metabolism by CYP2E1 [Gonzalez, 2005; Lu and Cederbaum, 2008], and furan metabolism causes concomitant depletion in cellular glutathione levels (in rat hepatocytes) [Carfagna et al., 1993]. Toxicogenomics analyses in both rat and mouse livers support a role for genotoxicity as a consequence of high levels of reactive oxygen species following furan exposure [Ding et al., 2012; Jackson et al., 2014; Dong et al., 2015]. These experiments support that the mode of action is cytotoxicity, driven primarily through oxidative stress to the cells, leading to regenerative proliferation and cancer.

Our analysis of MN supports that furan is clastogenic at high concentrations and TGx-28.65 classification indicated genotoxicity at all concentrations (2 – 5 mM), in support of the above findings. Moreover, the PCA and dendrogram show that the TGx-28.65 gene expression profile for furan is consistent with other agents that also cause oxidative stress (i.e., hydrogen peroxide, x-rays, and arsenite; Supporting Information Fig. 3f). Thus, a
finding such as this one would suggest that oxidative stress is a component of the chemical’s mechanism of toxicity, but additional experiments (beyond the scope of this paper) would be required to confirm this. Unfortunately, the experimental design of our study is not suitable to explore oxidative stress response genes in the furan experiment (pooled samples run in duplicate at a single time point, and only furan was treated with ethanol-induced S9) to support that the genotoxicity is caused via the generation of oxidative stress. However, future experiments could be designed to provide an opportunity to apply such a post-hoc analysis. Overall, our conclusions are consistent with previous findings that furan induces genotoxicity at high doses. Moreover, our findings support that the TGx-28.65 biomarker can be used to identify chemical agents that cause genotoxicity via the generation of reactive oxygen species as indicated in our previous work [Li et al. 2015].

Although our findings are consistent with genotoxicity for furan, we speculate that genotoxicity would be reduced in a model where conjugating enzymes are more active (e.g., hepatocytes *in vivo*). Consistent with this, it is of interest to note the strong genotoxicity measured for acetaminophen through both analysis of MN and classification by TGx-28.65 in our model. We note that the concentration range in which we observe the toxic effects of acetaminophen in our study (3 – 10 mM) are consistent with the concentrations that cause liver toxicity in humans based on physiologically based pharmacokinetic modeling (2 – 4 mM) [Diaz Ochoa et al., 2012]. Acetaminophen is also metabolized by CYP2E1 and can cause DNA damage through formation of reactive oxygen species [Jaeschke et al., 2012]. Both acetaminophen and its metabolite N-acetyl-p-benzoquinone imine can bind to DNA and cause DNA damage [Bergman et al., 1996], although acetaminophen is negative in standard Ames (±S9) testing and is not a carcinogen (Table 1). Inter-individual variability in cytotoxic and genotoxic response to acetaminophen is tightly linked to expression of Phase I and II enzymes in humans [Jetten et al., 2014]. Glutathione metabolism is important in mediating these effects. Thus, analysis of acetaminophen using a model that reflects more biologically relevant levels of Phase I enzymes would likely be more appropriate. Our previous study demonstrates that the TGx-28.65 biomarker can be used in human HepaRG cells using an external dataset [Doktorova et al., 2013; Buick et al., 2015]. HepaRG cells are derived from human hepatocellular carcinoma and have inherent Phase I and II metabolic capabilities, closely resembling human primary hepatocytes [Aninat et al., 2006; Guillouzo et al., 2007; Pfuhler et al., 2011]; thus, their use would eliminate the need for external metabolic activation and would be more consistent with primary hepatocytes in culture than TK6 cells. Future work in our laboratory is aiming to expand the dataset of chemicals tested and assayed using TGx-28.65 in HepaRG cells.

In summary, we demonstrate the utility of the TGx-28.65 biomarker in classifying genotoxic and non-genotoxic compounds in human TK6 cells using different metabolic activating systems and nine additional compounds with different modes of genotoxic action. Our analysis revealed that TGx-28.65 is a sensitive biomarker of genotoxicity, revealing effects at low concentrations below those where measurable decreases in RS occur. Our work supports the use of the TGx-28.65 biomarker in the presence of different S9s, although we recommend the use of lower S9 concentrations when possible. This work is consistent with other research in this field demonstrating that transcriptomic analyses can be done *in vitro* in HepG2 cells in the presence of BF/PB-induced rat liver S9 [Boehme et al., 2011], and in mouse fibroblast [Rohrbeck et al., 2010] and lymphoma cells with metabolic activation [Dickinson et al., 2004; Hu et al., 2004].

Currently, the TGx-28.65 biomarker is proposed to complement the genotoxicity test battery through provision of genotoxicity data in a human relevant system. The assay could be used prior to the conduct of the test battery or as a follow-up for positive responses in a regulatory test battery. Furthermore, the regulatory test battery provides little data regarding mode of action; the proposed approach can thus fulfill a regulatory requirement (through integration with the *in vitro* MN assay) and the genomics arm can be used as part of the weight of evidence to assess mode of action information needed in risk assessments. The biomarker could also be used when toxicogenomics data are produced as part of chemical screening to support or refute the genotoxicity of a chemical, and provide a rationale for the follow-up testing required. Additional studies are necessary to more precisely define the biomarker’s specific applications in regulatory toxicology testing and decision-making. Integration of this biomarker with other flow-cytometry, mechanism-based *in vitro* screening approaches (e.g., [Bryce et al., 2016]) will also be a promising avenue for future research.

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**AUTHOR CONTRIBUTIONS**

CY and JB designed the study, in consultation with all authors. CY obtained funding to support the project. LR and CS supervised the cellular exposures, and flow cytometry methods and analysis. JB performed the
microarray experiments. ET designed and supervised the RT-qPCR experiments. AW conducted all of the statistical analyses and prepared the figures, with input from HL, JB, JA, AF, and CY. CY and JB prepared the manuscript with important intellectual input from all authors. All authors read, revised, and approved the final manuscript.

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