A comparison of classical and 21st century genotoxicity tools: A proof of concept study of 18 chemicals comparing in vitro micronucleus, ToxTracker and genomics-based methods (TGx-DDI, whole genome clustering and connectivity mapping)

Ashley Allemang¹ | K. Nadira De Abrew¹ | Yuqing K. Shan¹ | Jesse M. Krailler² | Stefan Pfuhler¹

¹Global Product Stewardship, The Procter & Gamble Company, Cincinnati, Ohio
²Data and Modeling Sciences, The Procter & Gamble Company, Cincinnati, Ohio

Correspondence
Ashley Allemang, M.S. 8700 Mason Montgomery Rd, Mason, OH 45040, USA.
Email: allemang.a@pg.com

Abstract
A key step in the risk assessment process of a substance is the assessment of its genotoxic potential. Irrespective of the industry involved, current approaches rely on combinations of two or three in vitro tests and while highly sensitive, their specificity is thought to be limited. A refined in vitro genotoxicity testing strategy with improved predictive capacity would be beneficial and “3R” friendly as it helps to avoid unnecessary in vivo follow-up testing. Here, we describe a proof of concept study evaluating a balanced set of compounds that have in vivo negative or positive outcomes, but variable in vitro data, to determine if we could differentiate between direct and indirect acting genotoxicants.

Compounds were examined in TK6 cells using an approach in which the same sample was used to evaluate both early genomic markers (Affymetrix analysis 4 hr post treatment), and the genotoxic outcome (micronuclei [MN] after 24 hr). The resulting genomic data was then analyzed using the TGx-DDI biomarker, Connectivity mapping and whole genome clustering. Chemicals were also tested in the ToxTracker assay, which uses six different biomarker genes. None of the methods correctly differentiated all direct from indirect acting genotoxicants when used alone, however, the ToxTracker assay, TGx-DDI biomarker and whole genome approaches provided high predictive capacity when used in combination with the MN assay (1/18, 2/18, 1/18 missed calls). Ultimately, a “fit for purpose” combination will depend on the specific tools available to the end user, as well as considerations of the unique benefits of the individual assays.

Keywords
genotoxicity, in vitro micronucleus assay, mode of action, risk assessment, toxicogenomics

1 | INTRODUCTION

Assessing genotoxicity potential is one of the earliest steps in the risk assessment process of a chemical regardless of its application in a specific industry (chemical, pharmaceutical, etc.). Both internal work processes within industry as well as global regulations recommend a combination of in vitro mutagenicity and clastogenicity assays to fulfill this requirement. Typically a two (Bernauer et al., 2019; COM, 2011;
Kirkland, Reeve, Gatehouse, & Vanparys, 2011; Pfuhler et al., 2007) or three test battery (ECHA, 2017) is used as the first step for this purpose. While highly sensitive, these in vitro strategies have been thought by some to exhibit low specificity, leading to a high percentage of misleading positive results when compared to in vivo data (Kirkland et al., 2007; Kirkland, Kasper, Muller, Corvi, & Speit, 2008). Historically, in vivo follow-up testing was the preferred method to confirm genotoxic potential of chemicals with in vitro positive results. In recent times however, in vivo follow-up has become a last resort due to animal welfare concerns, regulatory restrictions, cost and time constraints. As a result, potentially valuable chemicals are discarded from the innovation pipeline and no longer available for commerce. In order to reduce animal use and protect commercial interest while still protecting public health a refined in vitro genotoxicity testing strategy with improved predictive capacity is needed.

Genotoxicants can be divided into two groups: (a) direct acting and (b) indirect genotoxicants based on their Mode of Action (MoA). Direct acting genotoxicants are usually electrophilic compounds that have the capacity to enter the nucleus of a cell and directly interact with DNA molecules of an organism. Indirect genotoxicants do not directly interact with the DNA molecules, rather they induce genotoxicity via other mechanisms, such as inhibition of topoisomerase II, production of cellular reactive oxygen species, and inhibition of DNA synthesis (Sasaki et al., 2020). This difference in MoA between the two groups has important implications for the application of the current risk assessment paradigm. Direct acting chemicals are assessed using linear extrapolation based on the current (but highly debated) view that risk can be extrapolated linearly to zero and therefore a safe dose does not exist while indirect acting genotoxicants are thought to have a thresholded effect and a safe dose can be established below which genotoxicity is not a concern (Kirsch-Volders, Aardema, & Elhajouji, 2000; Lovell, 2000; Nohmi, 2018). Indirect genotoxicants are considered to act through thresholded mechanisms, in which a cell is able to tolerate or repair certain amounts of damage until the biological systems become overwhelmed resulting in eventual genotoxicity. While this difference in biology is well known, the current in vitro mutagenicity OECD guideline assays such as, for example, the in vitro Chromosomal Aberration Test (OECD, 1997) and the in vitro Mammalian Cell Micronucleus Test (OECD, 2016) are only able to qualitatively identify the mutagenic or clastogenic potential of a chemical but cannot provide insight on the underlying MoA or identify a chemical as a direct or indirect genotoxicant unless protocol modifications are introduced (e.g., addition of reactive oxygen species [ROS] scavengers).

In the work presented here, we report on a proof of concept study to evaluate a variety of in vitro genotoxicity assays/approaches and their ability to distinguish between direct acting vs. indirect acting genotoxicants. Given the early stage of this work, we chose to focus on a small number of chemicals with known in vivo genotoxicity outcomes to first determine the merit of each approach independently, and as a combination. A total of 18 compounds were selected to include a variety of MoAs using the recommended list of genotoxic and non-genotoxic chemicals (Kirkland et al., 2016). A small subset of these compounds was also used in the development of the TGx-DDI and ToxTracker assays, and would thus be expected to be identified correctly. Nevertheless their inclusion in this study is still worthwhile for comparison across the other methods evaluated. Chemical selection was done such that it included a balanced set of compounds that are positive in in vitro and in vivo assays, negative in in vitro and in vivo assays and compounds found positive in vitro but negative in vivo. We investigate whether a suite of in vitro genomics based tools can differentiate between direct and indirect acting genotoxicants as well as possibly reveal MoA insights. The 18 compounds were examined using TK6 human lymphoblastoid cells and an experimental approach in which both early genomic markers (via Affymetrix gene array analysis 4 hr post treatment), as well as the downstream genotoxic outcome (micronuclei after a 20-hr recovery period) from the same cell cultures were considered. The resulting TK6 data were evaluated using three genomic based tools that utilize differing amounts of genomic data. The three tools used to analyze the Affymetrix gene array data were (a) the TGx-DDI biomarker which uses a signature of 64 genes to distinguish DNA damage-inducing (DDI) from non-DDI compounds (Li et al., 2017), (b) CMap (De Abrew et al., 2016; De Abrew et al., 2019; Lamb et al., 2006), and (c) All Differentially Expressed Genes (DEGs) via a hierarchical clustering approach. In addition, the same chemicals were separately tested and analyzed using the ToxTracker assay (Hendriks et al., 2012), which uses six different biomarker genes to identify the induction of DNA damage, oxidative stress, and protein damage. The resulting outcome as well as a discussion of merits and deficiencies of each method as they apply to the analysis of the 18 chemicals is provided. Ultimately, such methods could be used to supplement or replace currently used in vitro genotoxicity assays.

2 | MATERIALS AND METHODS

2.1 | Cell culture and chemical treatment

TK6 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, CRL-8015). Cells were cultured at 37°C with 5% CO₂ and 95% humidity in RPMI 1640 with 10% heat-inactivated FBS and 1% pen-strep. Cells were maintained at or below 1 × 10⁶ cells/ml.

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO). For all experiments, stock solutions of each chemical were made fresh immediately prior to use. The compounds examined, along with their abbreviations, corresponding mode of action, CAS# and solvent are indicated in Table 1. The same lot of each compound was used for all experiments performed in house, as well as externally in the ToxTracker assay. For the TK6 micronucleus/Affymetrix experiments, all chemicals were tested to a top concentration of 10 mM, or the limit of solubility or cytotoxicity. The final concentration of the solvent in these studies was limited to 0.5% (vol/vol) DMSO or water, although in a few isolated cases 1% DMSO or 10% water was used at a top concentration, in accordance with the OECD guidelines.
Between 5 and 6 concentrations of each chemical were tested in triplicate in TK6 cells ($1.5 \times 10^5$ cells/ml) for 4 hr, after which a portion of the sample was removed for lysate preparation using buffer RLT (QIAGEN, Germantown, MD) according to the manufacturer’s recommendations. Resulting cell lysates were stored at $-80^\circ C$ for subsequent genomic analysis, detailed below. The remaining portion of cell suspension was then centrifuged and the compound containing media was aspirated. Cell pellets were then resuspended in fresh media and transferred to new plates for a 20-hr recovery period. After 20 hr, samples were analyzed for micronucleus induction using the Litron (Rochester, NY) in vitro Microflow method, which has been well described previously (Avlasevich, Bryce, Cairns, & Dertinger, 2006). Briefly, cell suspensions were centrifuged and after the supernatant was aspirated, placed on ice for 20 min. Ethidium monoazide staining solution was then added and photoactivated on ice for 30 min to label necrotic and apoptotic cells. A buffer solution containing latex counting beads for relative viability determination was added and the cell suspensions were again centrifuged, and the supernatants aspirated. Cell pellets were resuspended in Lysis solution containing SYTOX green and RNase. After incubating in the dark for 1 hr, Lysis solution 2 was added and cells were incubated for an additional 30 min. Samples were then analyzed using FACS Diva v8.0.1 on a BD Biosciences FACSCanto II (San Jose, CA) equipped with a loader carousel. Per the Litron manual recommendation, 10,000 healthy nuclei were acquired. Micronuclei, nuclei and bead counts were compiled in Microsoft Excel so that “percent micronuclei” and “relative viability” calculations could be performed. Graphpad Prism v6.07 (GraphPad Software, Inc., La Jolla, CA) was then used to compare individual doses to vehicle controls with the Dunnett’s Multiple Comparison test. A $p$ value < .05 was considered a significant difference.

For Affymetrix microarray analysis, total RNA was extracted using Agencourt RNAdvance Tissue XP beads (Beckman Coulter Inc., Danvers, MA) according to the manufacturer’s protocol on the cell lysates that were prepared and frozen as previously described. RNA integrity was validated using a NanoDrop 8000 Spectrophotometer (Wilmington, DE). Labeled cRNA was synthesized from 250 ng of total RNA using the Affymetrix (Santa Clara, CA) IVT-Express labeling kit according to manufacturer’s instructions. Seven and a half microgram of labeled cRNA was fragmented manually and hybridized to Affymetrix Human Genome U219-96 arrays for 16 hr, washed, stained and scanned using an Affymetrix GeneTitan (Santa Clara, CA).

### 2.3 | TGx-DDI

Affymetrix data were analyzed by Health Canada using the TGx-DDI biomarker analysis that has been described previously (Li et al., 2015).
A three-pronged method is used in which a positive call is based on the agent being classified as positive for DDI in any one of three analytical interpretations of the data: (a) a probability analysis based on the nearest shrunk centroid, (b) a principal components analysis, and (c) 2-dimensional clustering (Li et al., 2017). The two aneugenic compounds examined (VB and COL) were included in the analysis, although the TGx-DDI biomarker is reported to not detect this mode of action (Cho et al., 2019).

2.4 | CMap analysis

We have previously shown the applicability of CMap (Lamb et al., 2006) to find connections between chemicals with similar MoA (De Abrew et al., 2016; De Abrew et al., 2019). The CMap analysis was performed using the methods described previously (De Abrew et al., 2016; De Abrew et al., 2019) by creating a database for all dose combinations of the 18 chemicals tested, which resulted in a database consisting of 145 chemical/dose combinations. Briefly, a CMap signature was created for a representative chemical from the true positive or true negative class of compounds at the appropriate concentration as determined by the results obtained in the in vitro micronucleus test performed on the same samples. Each signature was searched against the whole database and barview plots were created for the resulting output. The barview plot uses the average CMap score across all batches for a given chemical/concentration combination. Given a specific signature, a bar is drawn with segments proportionally colored for comparison, chemicals are placed into negative (red), zero (gray) and positive (green) zones based on their CMap scores using the entire score database (145 entries). The selected chemicals of interest are then highlighted to show where they fall against all CMap scores present in the database. Chemicals at the top of the bar are most strongly correlated to the query signature, and those at the bottom are most strongly anticorrelated, there is no standard statistical method to establish statistical significance for the connections observed (Lamb et al., 2006).

2.5 | Hierarchical clustering of differentially expressed genes (DEGs)

Data was visualized by Dendrogram using the R program (R Core Team, 2013) A Bottom-up (complete) Dendrogram was created for DEGs (False discovery rate [FDR] <0.05 FC < −1.2 or >1.2) of all concentrations or a select concentration per chemical using maximum or complete linkage clustering. Select concentrations were determined using a number of criteria including MN induction, cytotoxicity and a moderate number of significant Affy genes induced. This method computes all pairwise dissimilarities between the elements in cluster 1 and the elements in cluster 2, and considers the largest value (i.e., maximum value) of these dissimilarities as the distance between the two clusters. This method tends to produce more compact clusters.

2.6 | ToxTracker

The ToxTracker assay was performed by Toxys (Leiden, The Netherlands). The assay is a mouse embryonic stem cell-based reporter assay and has been well described in the literature (Hendriks et al., 2016). Briefly, six different reporter cell lines were created that are GFP-tagged biomarkers that respond specifically to different types of damage/cellular responses including DNA damage (Bsci2, Rtkn), oxidative stress (Srxn1, Blvrb), protein damage (Ddit3) and p53 activation (Btg2). Biomarker activation is then measured by high throughput flow cytometry. To independently evaluate ToxTracker performance, aliquots of the same lot of chemicals tested at P&G were blinded and shipped to Toxys for testing per their standard fee-for-service procedures. Biomarker performance was confirmed by the inclusion of the standard ToxTracker controls: Cisplatin (DNA damage), Aflatoxin B1 (metabolism), diethyl maleate (oxidative stress) and Tunicamycin (protein misfolding). All P&G compounds were tested to a top concentration of 1 mM or the limit of solubility as is common practice for this assay, except for O-TOL which was tested to 10 mM in a follow-up study.

3 | RESULTS

The purpose of this study was to determine how well direct and indirect acting genotoxicants can be distinguished using currently available and novel genomics/biomarker-based methods. We identified 3 groups of chemicals for this purpose: 8 known in vitro and in vivo genotoxic compounds, 5 known in vitro and in vivo non-genotoxic compounds and 5 in vitro positive but in vivo negative compounds ultimately resulting in a balanced set of 18 chemicals in which 8 are expected to have positive outcomes and 10 are expected to have negative outcomes.

3.1 | In vitro and in vivo negative results

As expected for in vitro and in vivo non-genotoxic compounds, 2DG, AMTR, CH and MAN showed no increases in the TK6 MN assay (Table 2, Supplemental Figure 1a). These compounds were also considered non-DDI in the TGx-DDI analysis (Figure 1, Table 2). However, significant increases in MN were observed for DICLO at the top two concentrations, the highest of which exceeded the cytotoxicity cutoff recommended for the assay (Table 2, Supplemental Figure 1a). When the top three concentrations of DICLO were evaluated using the TGx-DDI biomarker, they were considered DDI (Figure 1, Table 2). DICLO was also considered as weakly genotoxic in the ToxTracker assay, as it was found to induce DNA damage, oxidative stress and p53 damage (Figure 2, Supplemental Figure 2a). 2DG gave a positive response similar to DICLO in the ToxTracker, however it was negative in the TK6 MN assay and was considered non-DDI using the TGx-DDI biomarker approach (Figures 1 and 2, Table 2, Supplemental Figures 1a and 2a).
| Class                              | Compound          | Dose (μM) | %MN | Stdev | %relative survival | Stdev | # Affy genes FDR < 0.05 | TGx-DDI call |
|-----------------------------------|-------------------|-----------|-----|-------|--------------------|-------|------------------------|--------------|
| In vitro and in vivo negative     | 2DG               | 0         | 0.38| 0.09  | 99.73              | 7.58  |                        |              |
|                                   |                   | 2.5       | 0.39| 0.08  | 93.77              | 11.16 |                        | Te                |
|                                   |                   | 5         | 0.49| 0.06  | 81.44              | 11.72 |                        | Te                |
|                                   |                   | 10        | 0.38| 0.02  | 86.49              | 5.01  |                        | Te                |
|                                   |                   | 20        | 0.47| 0.10  | 92.41              | 2.80  | 430                    | Non-DDI       |
|                                   |                   | 40        | 0.38| 0.08  | 89.47              | 14.47 | 522                    | Non-DDI       |
| Amitrol                           |                   | 0         | 1.86| 0.22  | 100.00             | 6.59  |                        |              |
|                                   |                   | 625       | 1.36| 0.17  | 101.68             | 9.23  | 51                     | Non-DDI       |
|                                   |                   | 1,250     | 1.43| 0.19  | 87.08              | 11.3  |                        |              |
|                                   |                   | 2,500     | 1.47| 0.07  | 86.79              | 9.65  | 51                     | Non-DDI       |
|                                   |                   | 5,000     | 1.52| 0.08  | 80.67              | 1.84  | 987                    | Non-DDI       |
|                                   |                   | 10,000    | 1.54| 0.05  | 71.37              | 6.49  | 1,438                  | Non-DDI       |
| Cyclohexanone                     |                   | 0         | 1.59| 0.29  | 100.00             | 6.79  |                        |              |
|                                   |                   | 625       | 1.10| 0.24  | 87.55              | 15.08 | 0                      | Non-DDI       |
|                                   |                   | 1,250     | 1.44| 0.39  | 103.25             | 21.72 |                        |              |
|                                   |                   | 2,500     | 1.59| 0.08  | 88.12              | 18.76 | 7                      | Non-DDI       |
|                                   |                   | 5,000     | 1.49| 0.23  | 88.37              | 1.82  |                        |              |
|                                   |                   | 10,000    | 1.26| 0.16  | 84.24              | 11.94 | 2,193                  | Non-DDI       |
| Mannitol                          |                   | 0         | 1.59| 0.29  | 100.00             | 6.79  |                        |              |
|                                   |                   | 625       | 1.42| 0.20  | 91.50              | 9.71  |                        |              |
|                                   |                   | 1,250     | 1.70| 0.11  | 88.77              | 4.39  |                        |              |
|                                   |                   | 2,500     | 1.54| 0.28  | 76.84              | 13.87 | 7                      | Non-DDI       |
|                                   |                   | 5,000     | 1.44| 0.18  | 76.18              | 2.57  |                        |              |
|                                   |                   | 10,000    | 1.40| 0.15  | 97.88              | 27.54 | 6                      | Non-DDI       |
| Sodium diclofenac                 |                   | 0         | 1.64| 0.14  | 100.00             | 9.30  |                        |              |
|                                   |                   | 30        | 1.84| 0.16  | 89.05              | 9.53  | 11                     | Non-DDI       |
|                                   |                   | 50        | 1.80| 0.18  | 83.20              | 9.43  | 1,285                  | Non-DDI       |
|                                   |                   | 90        | 2.00| 0.06  | 73.69              | 1.05  | 7,739                  | DDI*          |
|                                   |                   | 160       | 3.18| 0.44  | 61.65              | 4.73  | 11,244                 | DDI*          |
|                                   |                   | 280       | 3.77| 0.29  | 34.73c             | 8.17  | 12,289                 | DDI*          |
| In vitro and in vivo positive     | 5-fluorouracil    | 0         | 0.71| 0.21  | 100.00             | 8.35  |                        |              |
|                                   | 48.1              | 1.05      | 0.19| 61.18 | 1.94  | 809                    | DDI          |
|                                   | 96.1              | 1.63      | 0.76| 48.33 | 2.71  | 1,373                  | DDI          |
|                                   | 192.2             | 2.01      | 0.82| 46.44 | 6.71  | 2,417                  | DDI          |
|                                   | 384.4             | 4.76**    | 0.65| 45.71 | 3.76  |                        |              |
|                                   | 768.8             | 5.46**    | 3.33| 39.84c | 3.08  |                        |              |
| Camptothecin                      |                   | 0         | 2.26| 0.30  | 100.00             | 3.79  |                        |              |
|                                   | 0.00195           | 4.72***   | 0.92| 106.33| 1.37  | 282                    | DDI          |
|                                   | 0.00391           | 7.65***   | 0.40| 66.73 | 8.78  | 1,513                  | DDI          |
|                                   | 0.00781           | 8.45***   | 0.80| 33.21c| 1.86  | 4,084                  | DDI          |
|                                   | 0.0156            | 9.16****  | 0.60| 24.37c| 3.04  |                        |              |
|                                   | 0.03125           | 9.47****  | 0.99| 16.75c| 1.69  |                        |              |
| Colchicine                        |                   | 0         | 1.97| 0.21  | 100.00             | 6.64  |                        |              |
|                                   | 0.00469           | 1.43      | 0.12| 102.56| 6.54  | 0                      | Non-DDI*      |
| Class     | Compound                        | Dose (uM) | %MN | Stdev | %relative survival | Stdev | # Affy genes FDR < 0.05 | TGx-DDI call |
|-----------|---------------------------------|-----------|-----|-------|--------------------|-------|------------------------|--------------|
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
| ENU       | 0.01878                         | 1.84      | 0.02|       | 95.50              | 6.25  | 2                      | Non-DDI      |
| ENU       | 0.03755                         | 5.02***   | 0.38|       | 67.44              | 3.08  | 22                     | Non-DDI      |
| ENU       | 0.07511                         | 16.85**** | 0.63|       | 27.47 c            | 2.94  | 534                    | Non-DDI      |
| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
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| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
| ENU       | 0.01878                         | 1.84      | 0.02|       | 95.50              | 6.25  | 2                      | Non-DDI      |
| ENU       | 0.03755                         | 5.02***   | 0.38|       | 67.44              | 3.08  | 22                     | Non-DDI      |
| ENU       | 0.07511                         | 16.85**** | 0.63|       | 27.47 c            | 2.94  | 534                    | Non-DDI      |
| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
| ENU       | 0.01878                         | 1.84      | 0.02|       | 95.50              | 6.25  | 2                      | Non-DDI      |
| ENU       | 0.03755                         | 5.02***   | 0.38|       | 67.44              | 3.08  | 22                     | Non-DDI      |
| ENU       | 0.07511                         | 16.85**** | 0.63|       | 27.47 c            | 2.94  | 534                    | Non-DDI      |
| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
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| ENU       | 0.03755                         | 5.02***   | 0.38|       | 67.44              | 3.08  | 22                     | Non-DDI      |
| ENU       | 0.07511                         | 16.85**** | 0.63|       | 27.47 c            | 2.94  | 534                    | Non-DDI      |
| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
| ENU       | 0.01878                         | 1.84      | 0.02|       | 95.50              | 6.25  | 2                      | Non-DDI      |
| ENU       | 0.03755                         | 5.02***   | 0.38|       | 67.44              | 3.08  | 22                     | Non-DDI      |
| ENU       | 0.07511                         | 16.85**** | 0.63|       | 27.47 c            | 2.94  | 534                    | Non-DDI      |
| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
| ENU       | 0.01878                         | 1.84      | 0.02|       | 95.50              | 6.25  | 2                      | Non-DDI      |
| ENU       | 0.03755                         | 5.02***   | 0.38|       | 67.44              | 3.08  | 22                     | Non-DDI      |
| ENU       | 0.07511                         | 16.85**** | 0.63|       | 27.47 c            | 2.94  | 534                    | Non-DDI      |
| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
| ENU       | 0.00939                         | 1.64      | 0.48|       | 106.93             | 16.13 | 0                      | Non-DDI      |
| ENU       | 0.01878                         | 1.84      | 0.02|       | 95.50              | 6.25  | 2                      | Non-DDI      |
| ENU       | 0.03755                         | 5.02***   | 0.38|       | 67.44              | 3.08  | 22                     | Non-DDI      |
| ENU       | 0.07511                         | 16.85**** | 0.63|       | 27.47 c            | 2.94  | 534                    | Non-DDI      |
| ENU       | 0                               | 1.59      | 0.24|       | 100.00             |       | 16.78                  |              |
CMap uses a unique genomic signature for a given compound concentration combination, this signature is then searched against the whole database of compound concentration combinations (145 entries in this study). Similar signatures show positive connections (cluster to the top of the green portion of the barview plot (Figure 3). When a signature was created for a representative in vitro and in vivo negative compound (e.g., MAN 2500 μM, Supplemental Table 1), there was no clear positive connections between the CMap signature for MAN and the other negative compounds nor the in vitro positive, but in vivo negative compounds (Figure 3a). Using CMap for assessing direct vs. indirect acting genotoxicants is a new application. The pros and cons of using CMap for a proof of concept study with a limited database (18 compounds/145 instances) are laid out in detail in the discussion section.

As described in the materials and methods section, a hierarchical clustering of all doses (Figure 4a) and of one dose selected based on micronucleus assay results (Figure 4b, selected doses indicated in bold in Table 2) using DEGs was performed. While most concentrations of the same compound clustered together not all in vitro and in vivo negative compounds did. Five main clusters (total of 6 clusters) were observed for the all compound/concentration dendrogram (Figure 4a), of these, 3 clusters were composed of in vitro and in vivo negative and in vitro positive but in vivo negative compounds (Figure 4a, clusters (1), (4),(6)). All in vitro and in vivo negative and in vitro positive but in vivo negative compounds clustered within these 3 clusters except for DICLO 30 μM, CH 625 μM and CH 2500 μM. These three compounds formed their own sixth cluster (Figure 4a, cluster [5]) separate from the five other main clusters (Figure 4a). When a single concentration was used for each chemical based on the results of the micronucleus test and hierarchical clustering was performed on the 18 chemicals (Figure 4b), all in vitro and in vivo negative compounds clustered together as 3 separate clusters (Figure 4b, clusters (1), (2), (4)). These three clusters were completely separated from the fourth cluster (Figure 4b, cluster (3)) composed of in vitro and in vivo positive compounds (Figure 4b).

### 3.2 In vitro and in vivo positive results

As expected, all in vitro and in vivo positive compounds examined resulted in significant increases in MN in the TK6 cell line (Table 2, Supplemental Figure 1b). While strong cytotoxicity was observed in some cases (CPT, MMS), positive increases in MN were also observed at non-cytotoxic concentrations. All compounds tested also resulted in positive DDI calls for at least some of the concentrations examined, aside from the aneugenic compounds (COL and VB) which are not

#### Table 2 (Continued)

| Class           | Compound          | Dose (μM) | %MN  | Stdev | %relative survival | Stdev | # Affy genes FDR < 0.05 | TGx-DDI call |
|-----------------|-------------------|-----------|------|-------|--------------------|-------|------------------------|--------------|
| Isobutyraldehyde | 0                 | 0.92      | 0.16 | 100.00| 15.85              | 0     | Non-DDI                |              |
|                 | 625               | 0.81      | 0.13 | 92.49 | 6.02               | 0     | Non-DDI                |              |
|                 | 1,250             | 1.06      | 0.20 | 96.37 | 25.39              | 0     | Non-DDI                |              |
|                 | 2,500             | 2.50      | 0.14 | 82.47 | 8.96               | 0     | Non-DDI                |              |
|                 | 5,000             | 3.73      | 0.56 | 72.45 | 4.30               | 32    | Non-DDI                |              |
|                 | 10,000            | 4.05      | 0.79 | 72.45 | 16.07              | 2,337 | Non-DDI                |              |
| Quercetin       | 0                 | 0.71      | 0.21 | 100.00| 8.35               | 0     | Non-DDI                |              |
|                 | 4.14              | 1.23      | 0.15 | 93.90 | 8.26               | 176   | DDI                    |              |
|                 | 8.27              | 1.35      | 0.31 | 83.40 | 6.98               | 1,595 | DDI                    |              |
|                 | 16.54             | 1.83      | 0.36 | 75.65 | 6.50               | 5,839 | DDI                    |              |
|                 | 33.09             | 2.78      | 0.43 | 51.29 | 4.85               | 11,648| DDI                    |              |
|                 | 66.17             | 4.12      | 0.65 | 22.27 | 0.97               | 0     |                        |              |
| Sodium saccharin| 0                 | 1.86      | 0.22 | 100.00| 6.59               | 0     |                        |              |
|                 | 625               | 1.81      | 0.27 | 113.81| 20.51              | 0     | Non-DDI                |              |
|                 | 1,250             | 2.04      | 0.25 | 102.29| 3.90               | 3     | Non-DDI                |              |
|                 | 2,500             | 1.71      | 0.16 | 93.46 | 4.99               | 0     | Non-DDI                |              |
|                 | 5,000             | 1.79      | 0.44 | 104.98| 4.52               | 2     | Non-DDI                |              |
|                 | 10,000            | 1.93      | 0.10 | 108.92| 2.93               | 3     | Non-DDI                |              |

Note: Portions of the cell suspensions used for Affymetrix analysis were processed immediately post treatment, while the remaining cell suspensions used for MN analysis were allowed 20 hr recovery (n = 4–8 for controls, 3 for treated samples, c indicates overly cytotoxic dose). Concentrations in bold indicate those that were evaluated alone in the whole genome (selected dose only) approach. The number of genes reported via Affymetrix was those with a false discovery rate (FDR) of less than 0.05. Affymetrix results were also analyzed using the TGx-DDI biomarker, incorrect calls are indicated by x. Level of statistical significance (p value < .05) is indicated by *,**,***,****.
detected by the TGx-DDI biomarker as they act via the spindle apparatus rather than inducing direct DNA damage (Figure 1, Table 2). 5FU, CPT, and ENU were considered DDI at all concentrations examined, including concentrations that induced minimal MN induction or cytotoxicity. ETOP, MMS and o-TOL all displayed a concentration dependent shift from non-DDI to DDI. Etoposide was only considered DDI at concentrations where a clear increase in MN also occurred. MMS was non-DDI at the lowest concentration, despite a clear increase in MN. At concentrations with more cytotoxicity (>40% cytotoxicity cutoff) MMS was correctly considered as DDI. o-TOL was only considered DDI at a single concentration, 9,330 μM, but was non-DDI at the next, highest concentration. Although MN induction was similar for the two concentrations, the highest dose would be considered overly cytotoxic. All in vitro and in vivo positive compounds except o-TOL were correctly identified by the ToxTracker assay, but o-TOL tested weakly genotoxic after additional testing to 10 mM in a follow-up experiment (Figure 2, Supplemental Figure 2b). All in vitro and in vivo positive compounds except o-TOL activated the p53 biomarker, as well as one or both markers for DNA damage and oxidative stress. o-TOL was the only compound tested that significantly activated the unfolded protein response biomarker.

For CMap analysis, a signature was created using a representative in vitro and in vivo positive compound (5FU at a concentration of 48 μM, Supplemental Table 2) and was then searched against the CMap database. The top 5 positive connections were chemicals within the same class (Figure 3b). The chemicals o-TOL, COL showed

FIGURE 1 Heatmap of TGx-DDI biomarker genes after TK6 cells were treated with the indicated compounds for 4 hr and analyzed using Affymetrix whole genome analysis. Red indicates an increase in fold change compared to control while green indicates a decrease in fold change. Prediction indicates overall TGx-DDI call while Class indicates known in vivo genotoxic outcome (blue = negative, red = positive). *COL, VB, 2DG, CPT, 5-FU, ETOP, and MMS were used to train the TGx-DDI biomarker (Li et al., 2015)
positive connections with 5FU however they were not as strong as the first 5 compounds (ENU, MMS, ETOP, and CPT) while VB resulted in a negative connection. Outside of the aneugenic compounds (COL and VB), only o-TOL showed a weak correlation with other in vitro and in vivo positive compounds.

Hierarchical clustering of DEGs showed good concordance of all in vitro and in vivo positive compounds except for all concentrations of o-TOL and two concentrations of ETOP (Figure 4a, clusters (2), (3) vs. (4)). When a single concentration was picked based on micronucleus assay data, all in vitro and in vivo positive compounds clustered into a single group, except the two aneugens (COL and VB) and o-TOL which exhibited inconsistent results in most assays used in this study (Figure 4b, cluster (3) vs. (1) and (2)).

### 3.3 | In vitro positive but in vivo negative results

In vitro positive but in vivo negative compounds gave mixed results in the TK6 MN assay (Table 2, Supplemental Figure 1c). ETHIN and SACC did not induce MN or cytotoxicity and these compounds were also considered non-DDI and negative in the ToxTracker (Figures 1 and 2). EUG, IBA and QUE all induced statistically significant increases at MN at multiple concentrations, however only QUE was considered DDI in the TX-DDI assay. QUE was non-DDI at the lowest Affymetrix concentration examined (4.14 μM), while all other doses including a range of MN and cytotoxicity responses were DDI. Of the in vitro positive but in vivo negative compounds that exhibited positive MN responses, only IBA was negative in the ToxTracker when considering

| DNA damage | Oxidative stress | UPR | p53 |
|------------|-----------------|-----|-----|
| Bcl2       | Rlrn            | Srxn1| Blrb |
| -S9        | +S9             | -S9 | +S9 |
| -S9        | +S9             | -S9 | +S9 |
| -S9        | +S9             | -S9 | +S9 |
| -S9        | +S9             | -S9 | +S9 |

*O-toluidine was tested to 10mM (-S9 only)

**Etoposide and MMS were used in the identification of the ToxTracker biomarkers (Hendriks et al. 2011)**

FIGURE 2  ToxTracker Assay results. Overall induction of gene reporters, up to 50% cytotoxicity
the twofold induction rule (Figure 2, Supplemental Figure 2c). QUE was considered genotoxic in the ToxTracker and it significantly activated all biomarkers except Ddit3. Notably, the oxidative stress marker Srxn1 was induced very strongly compared to the other biomarkers, even at low concentrations with minimal cytotoxicity and no involvement of the DNA damage markers (Supplemental Figure 3c).

EUG was considered weakly genotoxic in the ToxTracker, as it only significantly activated the Rtkn and Srxn1 biomarkers at the top concentration examined (Figure 3, Supplemental Figure 2c).

As mentioned above, the in vitro positive but in vivo negatives compounds did not cluster with the in vitro and in vivo negatives when using CMap (Figure 3b). When hierarchical clustering of DEGs was done on all chemicals, all in vitro positive but in vivo negatives compounds clustered with the in vitro negative, in vivo negative compounds clustered with in vitro negative, in vivo negative compounds as mixed groups (Figure 4b, clusters (1), (2), (4)).

4 | DISCUSSION

The research presented here explores a number of currently available and novel genomic methods and evaluates their ability to distinguish between direct acting vs. indirect acting genotoxicants using a test set of 18 compounds and directly compares the outcome with established genotoxicity assays. The ideal method (or combination of methods) would accurately predict the expected in vivo genotoxicity outcome. Here we provide an overview of where each method stands in achieving this goal based on the assessment of the 18 compounds considered. In addition, we discuss the biological reasoning as to why each method may, or may not, be providing the expected results, and what improvements can be made to ultimately reach the goal of being able to distinguish between direct vs. indirect acting genotoxicants either
by improving the individual assays or by using them in a test battery approach.

The only “true negative” compound incorrectly identified by all methods was DICLO. DICLO was identified as DDI at multiple concentrations, including a concentration that did not display a statistically significant increase in micronuclei or exhibit excessive cytotoxicity. DICLO strongly activated one of two DNA damage markers (Rtkn) and one of two oxidative stress markers (Srxn1). Btg2 was also significantly activated, however to a much lower extent. The activation of the DNA strand break reporter Rtkn, is in line with the observed increase in MN for DICLO, while the strong Srxn1 induction indicates that this response is predominantly driven via oxidative stress. There are other reports of DICLO exhibiting genotoxic characteristics in TK6 cells, for example DICLO increased pH 3, γH2AX and nuclear p53 in the MultiFlow assay after 4 hr (Smart et al., 2020), as well as exhibited significant increases in MN in the reconstructed human skin MN (RSMN) assay (Pfuhler et al., 2020). Reports of DICLO driven oxidative stress are also present in the literature and support the ToxTracker findings, although predominantly in the context of aquatic environmental exposures. For example, carp exposed to DICLO had increases in MN, DNA damage (as measured by the Comet assay), as well as increases in various oxidative stress biomarkers such as lipid peroxidation and superoxide dismutase, catalase and glutathione peroxidase activities (Islas-Flores et al., 2017) Given the above findings for DICLO, it seems unsurprising that all 3 TK6 cell-based methods identify DICLO as genotoxic, with hints from the ToxTracker that it may act through an oxidative stress pathway. The final true negative compound, the glycolysis inhibitor 2DG, was incorrectly considered positive in only the ToxTracker assay, while the other approaches using TK6 cells and subsequent genomic analysis were negative. One hypothesis is that this may be due to cell type specificity.

CMap (Lamb et al., 2006) did not show positive connections among the five in vitro and in vivo negative compounds. This was not unexpected for a limited database containing only 18 compounds. In order to create a genomic signature in CMap, DEGs were listed in

**FIGURE 4** A bottom-up (complete) dendrogram was created for DEGs (FDR <0.05 FC < −1.2 or >1.2) of (a) all chemical/concentrations or (b) one concentration per chemical based on micronucleus assay results, using maximum or complete linkage clustering. Dashed blue lines demarcate clusters, each cluster is numbered from top to bottom for easy reference in the text. Chemicals are color coded as follows: in vitro and in vivo positive: Red, in vitro and in vivo negative: Green, in vitro positive but in vivo negative: Blue
descending order and a cutoff was picked based on a statistical method. The resulting signature is a list of genes unique for the specific chemical/cell line/concentration combination. When this signature is searched against the database of compounds, in order for other compounds to show a positive connection, they too should contain a majority of the same genes. Based on this explanation of the CMap procedure, we can assume that in order to see a majority of the same genes affected between two compounds, the same pathways (MoAs) would need to be activated. This likely explains why these negative compounds are not showing positive connections. The only thing that these in vitro and in vivo negative compounds likely share is the absence of genotoxicity mechanisms which does not necessarily equate to a similarity in other non-genotoxic MoA. Hence, it is not expected that these compounds show positive connections since each of them are most likely activating different biological processes, represented by different genes specific to each compound.

When whole genome clustering was performed on DEGs only DICLO 30 μM, CH 625 μM and CH 2500 μM clustered separately from the rest of the in vitro and in vivo negative compounds and/or in vitro positive in vitro negative compounds. The DICLO observation is consistent with what was observed with the other methods. However, four of the DICLO concentrations were present in cluster 4, containing both in vitro negative and positive compounds which have in vitro negative outcomes. This highlights the complexity and the dose dependent nature of the DICLO response which may hint towards an indirect MoA. It is unclear why CH clustered separately, and this may be an artifact of only using 18 chemicals rather than any specific biological reasoning. Due to the limited number of chemicals used in this exercise, the two concentrations of CH could possibly have more overlapping DEGs with DICLO than any of the other 15 compounds by chance. When a single concentration that was optimized based on the micronucleus response was used for each chemical for the second part of this analysis, all in vitro and in vivo negative compounds and/or in vitro positive in vitro negative compounds clustered together, albeit as three separate clusters. This clustering could be explained with the same logic used for the CMap observation for this same class of compounds. The only thing that was similar among these compounds is an absence of genotoxic MoA. However, an absence of genotoxic MoA does not equate to a common non-genotoxic MoA. Regardless, these three clusters showed a clear separation from the in vitro and in vivo positive compounds, a strong indication that these non-genotoxic MoAs are distinct from the genotoxic MoAs represented in this dataset.

The in vitro and in vivo positive compound o-TOL, while clearly positive in the TK6 MN assay, was difficult to correctly identify using the different genomic based methods. It is unclear why o-TOL was only identified as DDI at a single dose, despite similar levels of MN induction at the other doses. It seems unlikely that the highest o-TOL dose was not detected as DDI due to cytotoxicity, as other positive compounds were still correctly identified at similar levels of cytotoxicity (CPT, ENU, ETOP, MMS Table 2). Initially, o-TOL was only tested to 1 mM in the ToxTracker assay, however given the observed positive response in the MN assay it was later retested up to 10 mM. It was weakly positive when tested at this higher concentration and the ToxTracker results indicate that the observed genotoxicity is likely driven by oxidative stress. Indeed, o-TOL related oxidative stress has been reported using the yeast DEL assay, in which o-TOL induced genotoxicity was reduced upon the addition of the free radical scavenger N-acetyl cysteine (Brennan & Schiestl, 1999). In addition, cell extracts exposed to o-TOL and the free radical-sensitive reporter compound dichlorofluorescein diacetate showed sharp increases in fluorescence indicating oxidation.

In vitro and in vivo positive compounds with aneugenic MoAs, such as COL and VB that are examined here, are not detected by the TGx-DDI biomarker as they exert their genotoxicity via spindle apparatus interruption rather than inducing DNA damage. In particular, these two compounds both selectively interact with tubulin to inhibit microtubule assembly ultimately resulting in mitotic arrest (Wilson, 1986). Similar results were observed for CMap; the three in vitro and in vivo positive compounds COL, VB and o-TOL while showing positive connections, landed in the bottom end of the positive connections (appeared in the green area of the barview plot, albeit in the latter half). As explained above, the aneugenic compounds have MoAs distinct from the rest of the compounds in this group and would not be expected to show positive connections with direct acting genotoxins via CMap. Similarly, o-TOL only caused weak genotoxic effects in TK6 cells which is contributing to its negative connection. Hierarchical clustering of DEGs for all concentrations showed a similar trend to the rest of the assays and all o-TOL concentrations clustered with non-in vitro and in vivo positive compounds. Likewise, all COL and VB concentrations clustered as a separate group from the rest of the in vitro and in vivo positive compounds. This was also observed in the select concentration dendrogram, where COL, VB and o-TOL did not cluster with the in vitro and in vivo positive compounds. The ToxTracker method, as well as the micronucleus test, is able to detect aneugens as they often selectively activate only the DNA damage marker Rtkn, instead of both Rtkn and Bsc12, as demonstrated here by COL, but not VB. Including kinetics can provide further confirmation of an aneugenic MoA in contrast to direct DNA damaging MoAs (Hendriks et al., 2016), although this parameter was not evaluated here.

As expected for compounds known to be positive in vitro, but not in vivo, a number of the compounds we examined were identified as positive in the TK6 MN assay (IBA, EUG, QUE). IBA however, was correctly identified as negative by the other methods examined. Although the incorrect call for IBA is thought to be driven via oxidative stress (Kirkland et al., 2008), oxidative stress biomarkers were not activated in the ToxTracker assay. EUG was incorrectly identified via ToxTracker, but not the TGx-DDI biomarker. Only two biomarkers were activated in the ToxTracker, Rtkn and Srxn1, indicating DNA breaks and oxidative stress. This result is somewhat surprising because EUG is most often reported to have antioxidant properties (Nagababu, Rikkind, Boindala, & Nakka, 2010; Yasuhisa, Hideki, & Muneyoshi, 1993), it is, however, known that antioxidants often operate as pro-oxidants at high doses. QUE however was incorrectly identified via both ToxTracker and TGx-DDI and was also identified as
non-DDI only at the lowest dose tested. The difficulty of accurately identifying QUE is perhaps unsurprising as literature reports are quite varied. QUE is generally considered to have a mixed MoA, including both oxidative stress and topoisomerase II inhibition, and is also reported to have both pro and anti-mutagenic and pro and anti-oxidant properties (Boots et al., 2007; Ündeğer, Aydin, Başaran, & Başaran, 2004). In addition, reports of QUE instability in cell culture medium add further complexity to accurately examining this compound (Adeleye et al., 2015). As described earlier, compounds known to be positive in vitro were not expected to cluster with in vitro and in vivo negative compounds when using CMap. Hierarchical clustering methods performed as expected for the in vitro positive but in vivo negative compounds. When all concentrations were used, all in vitro positive but in vivo negative compounds clustered with in vitro and in vivo negative compounds or in vitro positive in vivo negative compounds except for two concentrations of EUG. This is most likely a forced clustering in which in vitro and in vivo positive compounds were clustered together based on similarity of gene expression, while whatever was left was clustered based on a lack of similarity to in vitro and in vivo positive compounds. The in vivo positive but in vivo negative chemical group fared even better when a single, optimized concentration was picked, hinting to dose specific genotoxic effects among this class. While this group of compounds is sometimes referred to as “false” or “misleading” positives, the results presented here demonstrate that for the limited set of compounds we examined within this class, when methods are combined all compounds except QUE would be more accurately predicted when compared to their known in vivo genotoxic outcome. While EUG and IBA displayed increases in MN, the addition of the TGx-DDI biomarker and/or ToxTracker would give an overall negative call. Although ETH and SACC did not cause increases in MN in the studies presented here, they are reportedly positive in other in vitro genotoxicity assays (Kirkland et al., 2016), and if tested in conjunction with the TGx-DDI biomarker and/or ToxTracker would also give an overall negative call.

The results from all methods were compared to in vivo outcomes. The TK6 MN assay, incorrectly identified 4/18 compounds (Table 3). The ToxTracker assay incorrectly identified 5/18 compounds, however when the additional MoA information provided by the ToxTracker assay as well as dose–response is considered rather than using only the overall genotoxicity call (BscI2 or Rtkn positive), the number of incorrect calls would be reduced to 2/18 (2DG and EUG). When the TK6 MN assay is then reconsidered in combination with the ToxTracker assay, only EUG was still incorrectly identified as positive. The TGx-DDI biomarker incorrectly identified 4/18 compounds (DICLO, QUE, VB, COL). While it is known that the TGx-DDI biomarker does not detect aneugens, in a screening scenario where

### Table 3: Comparison of resulting genotoxicity call of all methods evaluated

| Class                        | Compound                  | Abbreviation | In Vitro MN | ToxTracker | TGx-DDI | CMap | Whole Genome (doses picked) | Whole Genome (all doses) |
|------------------------------|---------------------------|--------------|-------------|------------|---------|------|----------------------------|--------------------------|
| In vitro and in vivo negative| 2-deoxy-D-glucose **      | 2DG          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Amitrol                   | AMTR         | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Cyclohexanone             | CH           | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | D-Mannitol                | MAN          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Sodium dicyclohexyl maltate | DICTIO     | Red         | Red        | Red     | Gray | Green                     | Green                    |
| In vitro and in vivo positive| 5-fluorouracil **         | SFU          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Camptothecin **           | CPT          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Colchicine **             | COL          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | N-Nitroso-N-ethylurea     | ENU          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Etoposide **              | ETOP         | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Methyl methanesulfonate **| MMS          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | m-Toluidine               | m-TOL        | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Vinblastine               | VB           | Red         | Red        | Red     | Gray | Green                     | Green                    |
| In vitro positive but in vivo negative| Ethionamide              | ETHIN       | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Eugenol                   | EUG          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Isobutyraldehyde          | IBA          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Quercetin                 | QUE          | Red         | Red        | Red     | Gray | Green                     | Green                    |
|                              | Saccharin Sodium          | SACC         | Red         | Red        | Red     | Gray | Green                     | Green                    |

Note: aETOP and MMS were used in the identification of the ToxTracker biomarkers (Hendriks et al., 2011). bCOL, VB, 2DG, CPT, 5-FU, ETOP, and MMS were used to train the TGx-DDI biomarker (Li et al., 2015).
unknown compounds are being tested, we would consider these to be incorrect calls. This inability to detect aneugens would be remedied by combining the TGx-DDI biomarker with the TK6 MN assay, which does detect aneugens, however DICLO and QUE would still be incorrectly identified as positive. The CMap approach, at the current stage of development using a database of limited size, can only be used to evaluate the in vitro and in vivo positive compounds. When accounting for this group of chemicals, CMap correctly identified 7/8 compounds and only missed the one aneugen (VB) which would actually be a correct call if only direct acting genotoxins were in scope. When CMap is combined with the TK6 MN assay, all in vitro and in vivo positive compounds would be correctly identified. Hierarchical clustering using all doses results in 1 incorrect call (o-TOL), however 7 compounds would be considered inconclusive as different concentrations of a single compound were split between two clusters. This method can be improved by using the TK6 MN assay data to pick an ideal concentration for further analysis, resulting in a total of 4/18 incorrect calls (COL, o-TOL, VB, QUE). When these are then considered in conjunction with the overall TK6 MN response, all compounds except QUE were correctly identified (Table 2).

While similar results were obtained by combining the TK6 MN assay with either the ToxTracker, TGx-DDI biomarker or the whole genome approach (selected dose only) (1/18, 2/18, 1/18 missed calls respectively), we recognize that the ideal experimental approach is ultimately a fit for purpose approach. One benefit of using the TGx-DDI biomarker is that it gives the opportunity to analyze the same set of genomic data with other computational methods such as CMap and/or Hierarchical Clustering that use the same genomic input.

The in vitro MN assay remains a useful initial screening tool as it is affordable, easy to perform and accessible to many labs, especially because flow cytometers have become increasingly more affordable. Employing either the ToxTracker, TGx-DDI or the whole genome approach (selected dose only) in combination with the MN assay all have benefits as well as drawbacks in terms of cost—either associated with having compounds analyzed as a service (ToxTracker) or requiring expensive specialty instrumentation for generating genomic data. The TGx-DDI biomarker is becoming more accessible as it has now been successfully extended to multiple more cost effective/accessible approaches such as qRT-PCR and NanoString nCounter technology (Cho et al., 2019; Li et al., 2017), however some methods still require computational biology expertise. Further analysis of the resulting genomic data can present a challenge depending on the route taken; for example a web-based platform has been built for TGx-DDI analysis (https://manticore.niehs.nih.gov/tgxddi) and this platform does thus not require the user to be proficient in computation biology expertise. However, computational biology expertise would be required to interpret/analyze the whole genome approaches such as CMap and Hierarchical Clustering. A unique advantage of the ToxTracker assay is that, at present, it is the only method evaluated here that provides more than a yes/no readout and specifically includes oxidative stress biomarkers giving an indication of an indirect MoA for DNA damage as a standard readout. However, it is likely that the whole genome clustering and CMap methods can be further adapted to fill this need. We and others are exploring the possibility of developing genomic tools that are able to capture the specific MoA underlying the activity of an indirect genotoxicant. Both the TK6 MN and ToxTracker assays can be performed using modulators such as ROS scavengers to provide further confirmation of an indirect oxidative stress MoA. Neither the TGx-DDI nor CMap/Whole genome methods provide a similar indication at this time, although it seems feasible with further development and larger datasets that specific signatures could be identified. A useful tool to achieve this goal would be employing the recently developed adverse outcome pathway (AOP) for oxidative DNA damage leading to mutations and chromosomal aberrations (Cho et al, in submission). The AOP approach can act as a toolbox to identify pathways and key events related to the molecular initiating event, in this case oxidative DNA damage.

CMap has been shown to be a useful method to connect chemicals based on underlying MoA. However, the utility of the method is dependent on the density of the CMap database used. In the current exercise the signatures were compared only among 18 chemicals, this does not demonstrate the full breadth and the depth of the method. The database used here needs to be expanded to include compounds that mediate their effects via various indirect genotoxicity MoAs. These efforts are in progress, with an initial focus on oxidative stress, and will contribute to the utility of CMap as a method to identify direct vs. indirect acting genotoxicants. Hierarchical clustering of DEGs is a common tool used in genomics to identify compounds with similar gene expression profiles and, while useful, it also has drawbacks. The merit of the method depends on the stringency of the statistics used to identify the DEGs. Either too little or too many DEGs may lead to clustering of unrelated chemicals. The criteria for determining DEGs should be established on a case by case basis using pragmatic approaches that are both statistically sound and biologically relevant.

In conclusion, the work presented here provides a comparison of four different in vitro genomics based genotoxicity assays/approaches that may be used to identify direct vs. indirect acting genotoxicants. While none of the methods examined here, as stand-alone approach, correctly identify all direct vs. indirect acting genotoxicants, they can provide a reasonably high predictive capacity when used in combination. There remain still some challenges, for example methods such as CMap need further development to fit genotoxicity specific needs. Developing methods that enable the detection of activity of a substance towards a specific indirect genotoxicity MoA, such as oxidative stress, may provide targeted tools that could be useful also in the context of delineating AOPs. Ultimately, the user will need to pick from a toolbox of such assays and combine their outputs to surpass a weight of evidence threshold that is deemed acceptable for protecting human health.

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CONFLICT OF INTEREST
All authors approved the final manuscript and declare no conflicts of interests.

AUTHOR CONTRIBUTIONS
Ashley Allemang, K. Nadira De Abrew, and Stefan Pfuhler contributed to the overall concept, planning, and drafting of the manuscript. Ashley Allemang also performed the TK6 MN assay and related sample and chemical preparations, along with any associated data analysis, figures or tables. K. Nadira De Abrew performed the whole genome and CMap data analysis and figure generation. Yuqing K. Shan maintained the CMap database and contributed to the whole genome and CMap data analysis and figure generation. Jesse M. Krailler created all of the R tools used in the whole genome and CMap analysis. Stefan Pfuhler provided oversight and leadership for the research activity and its execution.

ORCID
Ashley Allemang https://orcid.org/0000-0002-6799-8675

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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