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γ H2AX and p53 Responses in TK6 Cells Discriminate Promutagens and Non-genotoxicants in the Presence of Rat Liver S9

Derek T. Bernacki^{#1}, Steven M. Bryce^{#1}, Jeffrey C. Bemis¹, David Kirkland², and Stephen D. Dertinger^{1,†}

¹Litron Laboratories, 3500 Winton Place, Rochester, New York, 14623, USA

²Kirkland Consulting, Tadcaster, UK

[#] These authors contributed equally to this work.

Abstract

Previous work with a diverse set of reference chemicals suggests that an *in vitro* multiplexed flow cytometry-based assay (MultiFlow™ DNA Damage Kit—p53, γ H2AX, Phospho-Histone H3) can distinguish direct-acting clastogens and aneugens from non-genotoxicants [*Environ Mol Mutagen* 57(2016)171-189]. The current work extends this line of investigation to include compounds that require metabolic activation to form reactive electrophiles. For these experiments TK6 cells were exposed to 11 promutagens and 37 presumed non-genotoxicants in 96 well plates. Unless precipitation or foreknowledge about cytotoxicity suggested otherwise, the highest concentration was 1 mM. Exposure occurred for 4 hrs after which time cells were washed to remove S9 and test article. Immediately following the wash and again at 24 hrs, cell aliquots were added to wells of a microtiter plate containing the working detergent/stain/antibody cocktail. After a brief incubation robotic sampling was employed for walk-away flow cytometric data acquisition. Univariate logistic regression analyses indicated that γ H2AX induction and p53 activation provide the greatest degree of discrimination between clastogens and non-genotoxicants. Multivariate prediction algorithms that incorporated both of these endpoints, in each combination of time points, were evaluated. The best performing models correctly predicted 9 clastogens out of 11 and 36 non-genotoxicants out of 37. These results are encouraging as they suggest that an efficient and highly scalable multiplexed assay can effectively identify clastogenic chemicals that require bioactivation. More work is planned with a broader range of chemicals, additional cell lines, and other laboratories in order to further evaluate the merits and limitations of this approach.

[†]corresponding author: S.D.D., 585-442-0930, sdertinger@litronlabs.com.

AUTHOR CONTRIBUTIONS

All authors contributed to experimental design. DTB and SMB executed various aspects of the experiments. An outline of the paper was developed by SDD, and all authors contributed to the writing of the final version.

CONFLICT OF INTEREST STATEMENT

Several of the authors are employed by Litron Laboratories. Litron has filed a patent covering the flow cytometry-based assay described in this manuscript and plans to sell a commercial kit based on these procedures (i.e., MultiFlow™ DNA Damage Kit—p53, γ H2AX, Phospho-Histone H3).

Keywords

genotoxicity; γ H2AX; p53; metabolic activation; flow cytometry

Introduction

A large portion of work directed at improving *in vitro* genotoxicity assays relates to the low throughput and assay specificity of current methods, lack of information relating to mode of action (MoA), and/or their competence to activate promutagens to *in vivo* relevant DNA-reactive metabolites [Audebert *et al.*, 2010; Smart *et al.*, 2011; Hendriks *et al.*, 2012; Tsamou *et al.*, 2012; Garcia-Canton *et al.*, 2013; Nikolova *et al.*, 2014; Bryce *et al.*, 2008, 2014; Nagel *et al.*, 2014; Khoury *et al.*, 2015; Cheung *et al.*, 2015; Yauk *et al.*, 2016]. A previous report by this laboratory addressed several of these issues through the use of a multiplexed p53, γ H2AX, and phospho-histone H3 (p-H3) assay that uses flow cytometry as the analytical platform [Bryce *et al.*, 2016]. That work described experiments with TK6 cells, and the resulting multiplexed measurements were converted into a prediction of group membership (clastogen, aneugen, or non-genotoxicant) based on a four-factor multinomial logistic regression model.

While initial work with this multiplexed assay generated promising results—assay sensitivity and specificity values >90%—only 4 of the 84 chemicals studied required metabolic activation to form DNA-reactive electrophiles. It was therefore of interest to more extensively evaluate the utility of the assay to detect genotoxicants that require metabolic activation using an exogenous activation system. We utilized Aroclor-induced rat liver S9 given its widespread use in the field of genetic toxicology. Furthermore, in contrast to our previous report in which we sought to discriminate clastogens, aneugens and non-genotoxicants, we limited this investigation to discrimination of clastogens and non-genotoxicants. This decision was based on current literature that to the best of our knowledge reports few, if any, compelling examples of metabolically activated aneugens.

As with the previous report, the work described herein was conducted with TK6 cells. However, for the present experiments exposure was for 4 hrs in the presence of S9 as opposed to 24 continuous hrs without a metabolic activation system. After washing cells at 4 hrs to remove S9 and test article, sample processing and flow cytometric analysis occurred at one early (4 hr) and one late (24 hr) time point, consistent with the work that was done previously. Univariate logistic regression (L-R) was used to identify promising endpoints. Subsequently several candidate multifactorial computation models were used to predict group membership. The resulting data are discussed in terms of the sensitivity and specificity of the assay to discriminate metabolically activated clastogens from non-genotoxicants.

Materials and Methods

Chemicals

The identity of the 48 chemicals is provided in Table I, along with our *a priori* predictions of genotoxic potential, based on published literature. Eleven clastogens that require metabolic activation were selected to represent a broad range of genotoxic activities, most of which have been studied in multiple laboratories and in both *in vitro* and *in vivo* systems. Thirty-seven nongenotoxicants were selected for their diverse range of activities/toxic mechanisms, and many are part of an ECVAM list of agents that should be negative in genotoxicity testing [Kirkland *et al.*, 2008, 2015]. Collectively these chemicals represented reference agents that were useful for identifying the time points and biomarkers that can best discriminate clastogens and nongenotoxicants and for developing machine learning-based prediction algorithms.

DNA Damage Assay

Reagents necessary to prepare nuclei for flow cytometric analysis were part of a prototype kit, *i.e.*, MultiFlow™ DNA Damage Kit—p53, γ H2AX, Phospho-Histone H3 (Litron Laboratories, Rochester, NY). The proprietary working solution was used to simultaneously digest cytoplasmic membranes, stain chromatin with a fluorescent nucleic acid dye, and label several epitopes with fluorescent antibodies. With this kit, anti- γ H2AX-Alexa Fluor® 647 is used as a DNA double strand break marker, anti-phospho-histone H3-PE serves as a mitotic cell marker, and in the context of the detergent-mediated lysis anti-p53-FITC recognizes nuclear accumulation of p53. RNase and propidium iodide provide cell cycle and polyploidization information, and a known concentration of latex microspheres (“counting beads”; *i.e.*, Sphero™ Multi-Fluorophore Particles, cat. no. FP-3057-2; Spherotech, Inc., Lake Forest, IL) is used to calculate nuclei density.

Cell culture and treatments

TK6 cells were obtained from ATCC® (cat. no. CRL-8015), and grown in a humid atmosphere at 37°C with 5% CO₂. For routine culturing, the cells were maintained at or below 1×10^6 cells/mL. The culture medium consisted of RPMI 1640 with 200 μ g/mL sodium pyruvate, 200 μ M L-glutamine, 50 units/mL penicillin, 50 μ g/mL streptomycin, and 10% v/v heat-inactivated horse serum.

On the day of treatment, logarithmically growing cells were transferred to round-bottom 96-well plate(s) where they were exposed to 20 concentrations of test chemical. Each concentration was evaluated in one well except for the solvent that was evaluated in 4 replicate wells. The solvent was DMSO except in several instances noted in Table I. As per ICH recommendations [2011], top test chemical concentration was 1000 μ M unless precipitation was observed in the 100 $\times$ stock solution or upon addition to culture medium; in these cases the highest non-precipitating concentration was evaluated. Table I notes instances when less than 1000 μ M was used as the top concentration based on precipitation or cytotoxicity considerations. Regardless of the top starting concentration, every successively lower concentration differed by a factor of square root 2, for example 1000, 707, 500 μ M, etc.

All data reported herein are based on cell treatments in the presence of an exogenously supplied metabolic activation system *via* inclusion of Aroclor-induced rat liver S9, final concentration 2% (Mutazyme Lyophilized Rat Liver S9, Molecular Toxicology, Inc., Boone, NC). The S9 mix and cells (2×10^5 cells/mL) were transferred as 198 μ L/well. Solvent and test chemicals were added in a volume of 2 μ L per well at which time the treated cells were re-incubated in a humid atmosphere at 37°C with 5% CO₂. After 4 hrs cells were washed out of exposure medium by two successive centrifugation ($340 \times g$, 5 min) and resuspension steps (200 μ L/well). The cells were resuspended with 200 μ L/well fresh growth medium and at this point 25 μ L of washed cells were removed from each well for the 4 hr time point. The remaining cells were returned to the incubator for an additional 20 hrs when 25 μ L of cells were once again removed from each well for flow cytometric analysis.

Flow cytometric analysis

As noted above, cells at 4 and 24 hr time points were added to a new 96-well plate containing 50 μ L/well of pre-aliquoted working MultiFlow Kit reagent. Mixing occurred by pipetting the contents of each well several times. After a 30 min room temperature incubation period, flow cytometric analysis was performed using a FACSCanto™ II flow cytometer equipped with a BD™ High Throughput Sampler. The user-defined mixing and fluidics parameters were programmed as follows: 40 μ L of sample were mixed 4 times at a mixing speed of 180 μ L/sec, and then 20 μ L were analyzed at a flow rate of 1 μ L/sec until the entire volume was exhausted. Between samples the High Throughput Sampler was programmed to wash the probe with 400 μ L of sheath fluid. Each 4 hr sample provided on average approximately 1,750 nuclei with 2n or greater DNA content analysis, and each 24 hr sample provided approximately 4,820 such nuclei.

Two endpoints, γ H2AX and p53, were based on median channel fluorescence intensity, and for all graphical representation and statistical analyses these values were expressed as fold-change relative to a plate-specific solvent control arithmetic mean. Gating logic required these events to exhibit propidium iodide-associated fluorescence corresponding to 2n - 4n DNA content. In order to limit the influence that mitotic and apoptotic cells might have on γ H2AX measurements, p-H3 positive cells and highly fluorescent γ H2AX-positive events were excluded from analysis [McManus and Hendzel, 2005; Huang *et al.*, 2006; Rogakou *et al.*, 2000].

The p-H3 measurements were the proportion of p-H3-positive events that exhibited propidium iodide-associated fluorescence of 4n and greater DNA content relative to the number of total events with 2n and greater DNA content. Polyploidy was quantified as the proportion of 8n-positive events relative to the number of total events with 2n and greater DNA content. For all graphical representations and statistical analyses presented herein the p-H3 and polyploidy data were converted to fold-change relative to a plate-specific solvent control arithmetic mean value.

Latex microspheres were included in the working dye/antibody solution at a known concentration (between 9.5 and 10.0×10^4 /mL) and this allowed these particles to serve as counting beads. Nuclei to counting bead ratios were calculated for each specimen, and this was used to determine nuclei densities. These values were used to derive 4 and 24 hr

cytotoxicity values relative to plate-specific mean solvent control wells. That is relative nuclei counts (RNC) underwent linear transformations to provide simple cytotoxicity indices, i.e., 100% minus 4 hr RNC and also 100% minus 24 hr RNC.

Electronic compensation was used to eliminate fluorescence spillover of propidium iodide into the adjacent PE channel. A more detailed description of compensation settings can be found in Bryce *et al.*, 2016.

Supervised machine learning

L-R was used to identify predictive biomarkers and to evaluate multifactorial models for categorizing chemicals according to group membership—clastogen versus non-genotoxicant (JMP® software, v12.0.1, SAS Institute Inc., Cary, NC). Initially univariate analyses were performed to identify the most promising biomarkers and time points. Statistics that were useful for this purpose included McFadden's pseudo R^2 , receiver operator characteristic (ROC) curves, and percentage of correctly categorized chemicals. To evaluate whether multiple biomarker data would improve the model, an iterative forward-stepping approach was used. As covariates identified for inclusion were added to the multivariate model, the importance of each endpoint was assessed in a manner similar to the univariate analyses described above. Then, a range of cytotoxicity limits were considered and an optimal value was chosen in the same way—by empirically studying their impact on model performance. Particularly high performing L-R models included two-factors: 24 hr p53 and either 4 or 24 hr γ H2AX in the context of a 70% 24 hr cytotoxicity limit. As described in more detail below, weighting these models on 24 hr cytotoxicity values led to further improvements to R^2 and ROC values.

As with our previous report, the current machine learning approach utilized data from every concentration [Bryce *et al.*, 2016]. A consequence of building a L-R model with data from multiple concentrations is that it calculates numerous group membership probabilities. This explains why multiple probability scores are reported for each chemical. Since a prediction as to group membership occurred at every concentration, this also explains the need to synthesize the model output (probability scores) into a final judgment as to group membership, and this is described below.

Call criteria, definitions

For the proof-of-principle work described herein clastogen and non-genotoxicant call criteria were selected to maximize assay sensitivity. With this goal in mind, we provisionally required a clastogen call to exhibit at least one concentration with a clastogen probability score $\geq 80\%$. A non-genotoxicant call was defined as no concentration exhibiting a clastogen probability score $\geq 80\%$. Assay sensitivity was calculated by determining the percentage of clastogens that were identified as such. Assay specificity was calculated by determining the percentage of non-genotoxicants that were identified as such. Assay concordance was calculated by determining the percentage of correct calls compared to total calls.

Results and Discussion

Identifying candidate biomarkers

MultiFlow kit reagents were successfully applied to TK6 cells exposed to chemicals in the presence of a rat liver S9 activation system. We found that centrifugation and subsequent resuspension of cells with fresh growth medium effectively reduced the fine microsome particulate matter and resulted in cells that were compatible with the kit's detergent/antibody cocktail. See Figure 1 for flow cytometry-generated p53, γ H2aX, and p-H3 profiles.

Figure 2 shows representative biomarker responses for a metabolically-activated genotoxicant, specifically the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) at both early (4 hr) and late (24 hr) time points. PhIP serves as a useful example for several reasons. First, it shows concentration-related increases in γ H2AX-associated fluorescence at both time points, a phenomenon that was typical for the genotoxicants that were effectively bioactivated. Exceptions were 2-nitrofluorene and 3,4-dimethylimidazo[4,5-f]quinolin-2-amine, both of which showed robust responses at 4 hrs that were considerably reduced at the later time point. PhIP-induced p53 responses were also prototypical. Whereas γ H2AX responses tended to increase or plateau with increasing concentration and cytotoxicity, p53 induction tended to plateau or even downturn with increasing cytotoxicity. Second, PhIP, like most clastogens, induced higher p53 responses at 24 hr relative to the 4 hr time point. This was particularly true for cyclophosphamide, where no p53 response was observed at 4 hr but at 24 hr a peak increase of 3.3-fold occurred. Not surprisingly, some chemicals affected the proportion of mitotic (p-H3 positive) cells, and PhIP is representative of agents that caused concentration-dependent reductions to these events. In this case the p-H3 effect was particularly evident at 24 hrs, and this manifestation of cytotoxicity may provide some insight into why certain endpoints, such as p53, were observed to saturate or even exhibit a downturn at the highest concentrations tested. As seen in Figure 2, polyploid cells were not induced by PhIP. As described below polyploidization was observed for certain compounds, but in all cases it was in the context of high cytotoxicity. Finally, the PhIP data shown in Figure 2 are illustrative of another consistent finding. That is, when reduction to RNC is used to assess treatment-induced cytotoxicity, 24 hr measurements were always more responsive/sensitive than measurements that occurred at the earlier time point.

In an effort to begin evaluating candidate biomarkers for their ability to discriminate clastogens from non-genotoxicants, fold-change (relative to mean solvent control) was graphed against cytotoxicity (Figure 3). This is an aggregate view, where every chemical and every concentration was included, irrespective of cytotoxicity level. These data suggest that γ H2AX and p53 responses are promising predictive biomarkers. While the PhIP data described above suggest that a cytotoxicity limit may be useful for maximizing the sensitivity of the p53 endpoint, the aggregate data shown in Figure 3 suggest another advantage. Thus, it appears that a cytotoxicity limit may also be useful for maintaining the specificity of certain biomarkers, for example γ H2AX.

Univariate logistic regression analyses were used to investigate biomarkers and cytotoxicity limits in a more quantitative and systematic manner. R^2 and ROC values were calculated for

each biomarker/time point combination (no cytotoxicity limit) and are presented in Table II. This table also summarizes logistic regression predictions. These analyses suggest that γ H2AX and p53, at both 4 and 24 hrs, are the most promising biomarkers in terms of predicting metabolite clastogenicity.

Additional analyses considered whether different cytotoxicity limits improved R^2 and ROC values, and whether applying a weight function based on cytotoxicity would be beneficial. These results are shown in Figure 4. In general, applying a cytotoxicity limit was useful, and a cytotoxicity cutoff value that worked well for both γ H2AX and p53 endpoints was 70% (based on 24 hr RNC). These data also indicate that giving more weight to concentrations that exhibited greater cytotoxicity markedly improved statistics associated with model performance.

The 70% cytotoxicity limit is consistent with an observation made in our previous report that noted γ H2AX and p53 responses either saturated or exhibited a downturn by 80% cytotoxicity [Bryce *et al.*, 2016]. In any event, when considering the appropriateness of the 70% cytotoxicity limit for short-term treatment in the presence of an S9 system, several points should be kept in mind. First, as described above, it was not simply adopted from other assays/endpoints, but rather was empirically determined by an objective and systematic investigation of data from the endpoints under consideration. Second, the cytotoxicity limit was based on RNC at the 24 hr time point. It is not accurate to think of RNC being equivalent to cell death. Rather, these measurements integrate cell division, loss of cells due to necrosis and/or apoptosis, and also cytostasis. Finally, at this point we consider the 70% cutoff value provisional in nature. While it was effective for the experiments described herein, it is important to recognize that the analyses considered a single cell line, one treatment/harvest schedule, and only 48 chemicals.

Model building, optimization

L-R models were built by combining γ H2AX and p53 data associated with concentrations that exhibited 70% cytotoxicity, and applying a weight function based on %cytotoxicity. For this exercise, both endpoints were considered at each combination of time points. A supplementary file shows fold-increase values for these two endpoints at both time points and for every chemical studied. Figures 5-8 displays the resulting L-R probabilities for the four models: 4hr γ H2AX and p53; 24 hr γ H2AX and p53; 4 hr γ H2AX and 24 hr p53; and 24 hr γ H2AX and 4 hr p53, respectively.

The two highest performing models consisted of (i) 4 hr γ H2AX and 24 hr p53 data, and (ii) 24 hr γ H2AX and 24 hr p53 data. In both cases, 9 out of 11 total clastogens were correctly identified, and 36 out of 37 total non-genotoxicants were correctly classified. Assay performance values were 82% for sensitivity, 97% for specificity, and 94% for concordance with *a priori* classifications.

The clastogens that were not predicted correctly were *N*-nitrosodiethylamine (DEN) and hexamethylphosphoramide (HMPA). In both cases no evidence of cytotoxicity was observed, either in terms of reductions to RNC, or to p-H3 positive events. This suggests that the S9 metabolic activation system was not effective at biotransforming these agents to

reactive metabolites. In the case of DEN, this is not surprising. DEN is chiefly activated by CYP2E1, an isoform that does not exhibit high levels of activity in Aroclor-treated rat livers [Burke *et al.*, 1994]. The literature surrounding HMPA is considerably sparser relative to the other progenotoxicants studied herein. Reports by Sarraf *et al.* [1997] and Doherty and colleagues [2012], suggest that formaldehyde is the proximate genotoxicant. However, the known appreciable cytotoxicity of formaldehyde, and the lack of cytotoxicity observed in our experiments, supports our premise that HMPA was not effectively bioactivated by the S9 reagent that we used, thus the lack of γ H2AX and p53 responses was not surprising.

Conclusions

A method that reports on γ H2AX, p-H3, p53, polyploidization, as well as cytotoxicity, was successfully applied to TK6 cells treated with chemicals in the presence of an exogenous metabolic activation system. γ H2AX induction and p53 activation were found to provide the greatest degree of discrimination between clastogens and non-genotoxicants. Multivariate prediction algorithms based on these endpoints were evaluated and the best performing models correctly predicted 9 clastogens out of 11 total and 36 non-genotoxicants out of 37 total. The misclassification of two clastogens can likely be attributed to deficiencies with the rat liver S9-based metabolic activation system as opposed to issues with the endpoints or time points *per se*.

These results are encouraging as they suggest data from an efficient and highly scalable multiplexed assay can be used to make predictions about chemicals' potential to cause S9-mediated clastogenicity. Overall, the characteristics of this assay make it well suited for early screening programs, including high throughput environments that utilize robotic liquid handling instruments. Further work is planned with different treatment/harvest schedules, a broader range of chemicals, additional cell lines, and other laboratories in order to further evaluate the merits and limitations of this approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Flow cytometry bivariate plots for TK6 nuclei from solvent (left) or benzo(a)pyrene (right) treated cultures prepared with MultiFlow kit reagents as described herein. Top panel: p53 fluorescence versus nucleic acid dye fluorescence at the 24 hr time point. Note the increased p53-associated fluorescence associated with clastogen exposure. Middle panel: γ H2AX fluorescence versus nucleic acid dye fluorescence at the 4 hr time point. Note the increased γ H2AX fluorescence associated with clastogen exposure. Bottom panel: Phospho-histone H3 fluorescence versus nucleic acid dye fluorescence at the 4 hr time point. Whereas the proportion of phospho-histone H3 events was not found to be highly informative in terms of genotoxic versus non-genotoxic predictions, it does convey information about cell proliferation.

Figure 2.

Fold-increase responses for each of five biomarkers at each of two time points are graphed against 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) concentration. PhIP is representative of clastogenic-activity response profiles observed for the metabolically activated genotoxigants studied reported herein.

Figure 3.

Fold-increase responses for each of four biomarkers at each of two time points are graphed against a measure of cytotoxicity (*i.e.*, 100% - Relative Nuclei Count at 24 hrs). The graphs are aggregate data for all 48 chemicals studied herein, and are coded according to group membership: clastogens = red circles, non-genotoxicants = green triangles. These graphs provide insight into the most predictive biomarkers, and suggest that testing up to cytotoxic concentrations, to a point, maximizes signal to baseline ratios.

Figure 4.

R^2 and ROC values associated with univariate logistic regression analyses show the influence of using a cytotoxicity limit (0, 80, 70, or 60%), as well as weighting the model on %cytotoxicity. These graphs show that the ability of the endpoints γ H2AX and p53 to discriminate between clastogens and non-genotoxins generally improves when a cytotoxicity limit is used, and when the responses are weighted on cytotoxicity.

Figure 5.

Logistic regression probabilities for a clastogen classification are graphed for each of 48 chemicals. This logistic regression model was based on 4 hr γ H2AX and 4 hr p53 fold-change data. Chemicals are color-coded: clastogens = red circles, non-genotoxicants = green triangles. A series of probabilities are plotted for each chemical, as each represents a different concentration. These data show that 8/11 clastogens were correctly classified by this two-factor model, while 36/37 non-genotoxicants were correctly classified.

Figure 6.

Logistic regression probabilities for a clastogen classification are graphed for each of 48 chemicals. This logistic regression model was based on 24 hr γ H2AX and 24 hr p53 fold-change data. Chemicals are color-coded: clastogens = red circles, non-genotoxicants = green triangles. A series of probabilities are plotted for each chemical, as each represents a different concentration. These data show that 9/11 clastogens were correctly classified by this two-factor model, while 36/37 non-genotoxicants were correctly classified.

Figure 7.

Logistic regression probabilities for a clastogen classification are graphed for each of 48 chemicals. This logistic regression model was based on 4 hr γ H2AX and 24 hr p53 fold-change data. Chemicals are color-coded: clastogens = red circles, non-genotoxicants = green triangles. A series of probabilities are plotted for each chemical, as each represents a different concentration. These data show that 9/11 clastogens were correctly classified by this two-factor model, while 36/37 non-genotoxicants were correctly classified.

Figure 8.

Logistic regression probabilities for a clastogen classification are graphed for each of 48 chemicals. This logistic regression model was based on 24 hr γ H2AX and 4 hr p53 fold-change data. Chemicals are color-coded: clastogens = red circles, non-genotoxicants = green triangles. A series of probabilities are plotted for each chemical, as each represents a different concentration. These data show that 8/11 clastogens were correctly classified by this two-factor model, while 36/37 non-genotoxicants were correctly classified.

Table I

Chemicals and *a priori* Classifications.

Chemical	CAS No.	<i>a priori</i> Classification, Notes about Biotransformation, Misc.	References
2-Acetylaminofluorene	53-96-3	Clastogen, requires metabolic activation (CYP1A2), forms C8 adduct on guanine; top non-precipitating conc. 250 µM	Otteneider <i>et al.</i> , 1999; Kirkland <i>et al.</i> , 2015
2-Aminoanthracene	613-13-8	Clastogen, aromatic amine, requires metabolic activation (CYP1B1, 2A family); top non-precipitating conc. 500 µM	Carrière <i>et al.</i> , 1992
7,12-Dimethylbenzanthracene	57-97-6	Clastogen, requires metabolic activation (CYP1B1), forms bulky adducts	Kirkland <i>et al.</i> , 2015
2-Nitrofluorene	607-57-8	Clastogen, nitrated polycyclic aromatic hydrocarbon, requires metabolic activation; top non-precipitating conc. 177 µM	Wierckx <i>et al.</i> , 1990; Matsuoka <i>et al.</i> , 1991
Benzo[a]pyrene	50-32-8	Clastogen, polycyclic aromatic hydrocarbon, requires metabolic activation (CYP1A1, 1B1, epoxide hydrolase), forms bulky adducts; top non-precipitating conc. 250 µM	Kirkland <i>et al.</i> , 2015
Cyclophosphamide monohydrate	6055-19-2	Clastogen, nitrogen mustard, requires metabolic activation (CYP2B6, CYP2C19, CYP2C9 and CYP3A4/5)	Kirkland <i>et al.</i> , 2015; Rodriguez-Antona and Ingelman-Sundberg, 2006
Dibenzo[a,l]pyrene	191-30-0	Clastogen, polycyclic aromatic hydrocarbon, requires metabolic activation (thought to be primarily activated by CYP1A1); 10 µM highest tested conc. due to cytotoxicity	Arif and Gupta, 1997
Hexamethylphosphoramide	680-31-9	Clastogen, phosphoramidate, requires metabolic activation to form proximate genotoxicant (presumably formaldehyde)	Sarrif <i>et al.</i> , 1997; Doherty <i>et al.</i> , 2012
3,4-Dimethylimidazo[4,5- <i>f</i>]quinolin-2-amine (MeIQ)	77094-11-2	Clastogen, heterocyclic amine, requires metabolic activation (CYP1A2); while clastogenic <i>in vivo</i> , often not clearly or only weakly clastogenic <i>in vitro</i> ; weak inducer of MN in HepG2 cells	Ramsey <i>et al.</i> , 1998; Sasaki <i>et al.</i> , 1992; Aeschbacher <i>et al.</i> , 1989; Thompson <i>et al.</i> , 1987; Rueff <i>et al.</i> , 1996; Knasmüller <i>et al.</i> , 1999; Kim and Guengerich, 2004
N-Nitrosodiethylamine (DEN)	55-18-5	Clastogen, requires metabolic activation to form alkylating agent (likely involves CYP2E1 which is not highly expressed in rat liver S9); often only positive at high concentrations (10 mM)	Ishidate <i>et al.</i> , 1988; Yamazaki <i>et al.</i> , 1992;
2-amino-1-methyl-6-phenylimidazo [4,5- <i>b</i>]pyridine (PhIP)	105650-23-5	Clastogen, heterocyclic amine, requires metabolic activation (CYP1A family); top non-precipitating conc. 250 µM	Kirkland <i>et al.</i> , 2015; Kraus <i>et al.</i> , 2016
Amitrole	61-82-5	Non-genotoxicant	Kirkland <i>et al.</i> , 2015
Anthranilic acid	118-92-3	Non-genotoxicant	Kirkland <i>et al.</i> , 2015
Brefeldin A	20350-15-6	Non-genotoxicant; ER-golgi transporter inhibitor, ER stress-induced apoptosis; top conc. 500 µM due to solubility	Moon <i>et al.</i> , 2012
Caffeine	58-08-2	Non-genotoxicant; mitochondria-dependent apoptosis, ROS involvement likely	Lu <i>et al.</i> , 2008

Chemical	CAS No.	<i>a priori</i> Classification, Notes about Biotransformation, Misc.	References
Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)	555-60-2	Non-genotoxicant; uncoupler of oxidative phosphorylation	de Graaf <i>et al.</i> , 2004
Clofibrate	637-07-0	Non-genotoxicant; antilipidemic agent	IARC monograph
Cyclohexanone	108-94-1	Non-genotoxicant; industrial chemical	Kirkland <i>et al.</i> , 2008
Cycloheximide	66-81-9	Non-genotoxicant; protein synthesis inhibitor	Youngblom <i>et al.</i> , 1989
D-Mannitol	69-65-8	Non-genotoxicant; polyol	Kirkland <i>et al.</i> , 2015
Dexamethasone	50-02-2	Non-genotoxicant; glucocorticoid receptor agonist	Krishna <i>et al.</i> , 1995
Dextrose	50-99-7	Non-genotoxicant; sugar	Lotz <i>et al.</i> , 2009
Di-(2-ethylhexyl)phthalate (DEHP)	117-81-7	Non-genotoxicant; organic plasticizer	Kirkland <i>et al.</i> , 2015
Diethanolamine	111-42-2	Non-genotoxicant; secondary amine	Kirkland <i>et al.</i> , 2015
Erythromycin	114-07-8	Non-genotoxicant; antibiotic	Kirkland <i>et al.</i> , 2015
Pepcid® (Famotidine)	76824-35-6	Non-genotoxicant; histamine H ₂ receptor antagonist	FDA approved label
Gleevec® (imatinib mesylate)	152459-95-5	Non-genotoxicant; protein-tyrosine kinase inhibitor	FDA approved label
Hexachloroethane	67-72-1	Non-genotoxicant; industrial chemical	Kirkland <i>et al.</i> , 2015
Lidoderm® (Lidocaine)	137-58-6	Non-genotoxicant; amide local anesthetic	FDA approved label
Mevacor® (Lovastatin)	75330-75-5	Non-genotoxicant; HMG-CoA reductase inhibitor	FDA approved label
Melamine	108-78-1	Non-genotoxicant; industrial organic base	Kirkland <i>et al.</i> , 2015
Methyl carbamate	598-55-0	Non-genotoxicant; industrial intermediate	Kirkland <i>et al.</i> , 2015
N-Butyl chloride	109-69-3	Non-genotoxicant; fumigant	Kirkland <i>et al.</i> , 2015
Floxin® (Ofloxacin)	82419-36-1	Non-genotoxicant; fluoroquinolone antibiotic; top conc. 500 µM due to solubility	FDA approved label
Paxil® (Paroxetine)	61869-08-7	Non-genotoxicant; SSRI antidepressant	FDA approved label
Phenanthrene	85-01-8	Non-genotoxicant; polycyclic aromatic hydrocarbon	Kirkland <i>et al.</i> , 2008
Phenformin HCl	834-28-6	Non-genotoxicant; biguanide antidiabetic	Kirkland <i>et al.</i> , 2015
Progesterone	57-83-0	Non-genotoxicant; steroid hormone	Kirkland <i>et al.</i> , 2008
Pyridine	110-86-1	Non-genotoxicant; heterocyclic organic compound	Kirkland <i>et al.</i> , 2015
Sodium chloride	7647-14-5	Non-genotoxicant; prepared in RPMI medium	Matsushima <i>et al.</i> , 1999
Sodium dodecyl sulfate	151-21-3	Non-genotoxicant; ionic detergent	NTP website
Sucrose	57-50-1	Non-genotoxicant	Diaz <i>et al.</i> , 2007

Chemical	CAS No.	<i>a priori</i> Classification, Notes about Biotransformation, Misc.	References
Tert-butyl alcohol	75-65-0	Non-genotoxicant	Kirkland <i>et al.</i> , 2015
Thapsigargin	67526-95-8	Non-genotoxicant; ER stress-induced apoptosis	Futami <i>et al.</i> , 2005
Tunicamycin	11089-65-9	Non-genotoxicant; glycosylation inhibitor, ER stress-mediated apoptosis; top conc. 500 μ M due to solubility	Han <i>et al.</i> , 2008
Alosetron HCl	122852-42-0	Non-genotoxicant; 5-HT ₃ antagonist; top conc. 250 μ M due to solubility	Kirkland <i>et al.</i> , 2015
D-Limonene	5989-27-5	Non-genotoxicant; male rat kidney tumors due to α 2 μ -globulin nephropathy	Kirkland <i>et al.</i> , 2015
Tolterodine L-tartrate	124937-52-6	Non-genotoxicant; muscarinic receptor antagonist	Kirkland <i>et al.</i> , 2015
Zonisamide	68291-97-4	Non-genotoxicant; sulfonamide anticonvulsant	Kirkland <i>et al.</i> , 2015

Table II

Univariate Logistic Regression Analyses.

Endpoint	Time	Pseudo R ²	ROC	Correct Clastogen Predictions	Correct Non-Genotoxicant Predictions
γH2aX	4 hrs	0.2264	0.80410	6 / 11	32 / 37
p-H3		0.0048	0.43147	0 / 11	37 / 37
p53		0.0989	0.65948	2 / 11	35 / 37
Polyploidy		0.0090	0.58132	0 / 11	37 / 37
γH2aX	24 hrs	0.1538	0.75507	5 / 11	33 / 37
p-H3		0.0618	0.63035	0 / 11	37 / 37
p53		0.1706	0.73776	6 / 11	34 / 37
Polyploidy		0.0019	0.55785	0 / 11	37 / 37