Genotoxicity Evaluation of Fragrances and Botanicals using ToxTracker® assay.

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BACKGROUND AND INTRODUCTION

The ToxTracker® assay has gained attention as a second tier non-animal test method to the standard battery of in vitro genotoxicity assays by providing mechanistic insights into the modes of action including DNA damage, oxidative stress, cellular stress, protein damage, and the validated method is currently undergoing review at the OECD level for acceptance within international regulatory frameworks. As such, ToxTracker® is designed to identify both genotoxic and non-genotoxic carcinogens, thus providing industry and regulatory toxicologists with highly useful information for hazard and risk assessment purposes. Whereas ToxTracker® can be used as a confirmatory assay when chemicals show positive or equivocal results in Ames and/or micronucleus tests, the test method is also used for rapid and cost-effective screening of chemicals to identify potential DNA mutagens. The test method is reported to have a wide chemical applicability domain; accordingly, this study was focused on evaluating chemicals found to have genotoxic potential either from classic genetox test methods or have compelling evidence of in vivo carcinogenic activity.

METHODS

We tested four chemicals with existing genotoxicity data from Ames and/or in vitro Micronucleus (IVMN) tests: Aristolochic Acid (AA; CAS #313-67-7), a botanical used historically as an herbal medicine which was positive in the Ames and IVMN tests and which has been associated with liver carcinogenesis, nephropathy and urothelial cancers in humans; ; and Pyrrolizidine (CAS: 643-20-9), a botanical toxin used in medicine and insect repellant, who's metabolic conversion into pyrroles that act as alkylating agents is reflected in positive Ames and IVMN tests; 2-octen-4-one (CAS #4643-27-0), a fragrance ingredient which was positive in the IVMN test but negative in the reconstructed skin micronucleus (RSMN) and in vivo tests; and Veratraldehyde (CAS: 120-14-9), a fragrance ingredient which was negative in Ames, Reconstructed Skin Micronucleus (RSMN), and in vivo micronucleus assays, but positive in an IVMN. The ToxTracker® assay was used to assess the chemicals for the underlying mechanisms of genotoxicity. All chemicals were tested in a dose range finding experiment in the presence and absence of Phenobarbital/5,6-Benzoflavone-induced male Sprague Dawley rat liver S9 fraction to determine doses for the definitive trials. The maximum concentration for each chemical was selected based on cell survival ranging between 50% and 25%. In the definitive trials, five doses of the chemicals were treated in each of the six reporter cell lines for 24 hours, followed by measurement of the induction of the GFP-labeled reporter genes by flow cytometry. AA was tested at doses ranging from 6.25 to 100 µM, 2-octen-4-one was tested at doses ranging from 4.37 to 70 µM, Veratraldehyde was tested at doses ranging from 93.75 to 1,500 µM, and Pyrrolizidine was tested at doses ranging from 625 to 10,000 µM. Data analyses were conducted using Microsoft Excel to determine the dose-related induction of the reporter genes relative to vehicle controls.

Evaluation of Results		
–	Negative	< 1.5-fold induction
(+)	Weak Positive	≥ 1.5- to < 2-fold induction
+	Positive	≥ 2-fold

Table 1. Evaluation of results. A negative result is assigned to a reporter if GPF induction fold change is less than 1.5, a weak positive result is assigned to a reporter if GPF induction fold change is between 1.5 and 2, and a positive result is assigned to a reporter if the GFP induction fold change is 2 or more.

Call in 3 Experiments	Overall Call
+ + +	Positive
+ + (+)	Positive
+ (+) (+)	Positive
(+) (+) (+)	Equivocal
+ (+) –	Equivocal
(+) (+) –	Negative
(+) – –	Negative
– – –	Negative

Table 2. Determination of genotoxicity call. Two or three independent runs of the ToxTracker assay were performed. Based on the fold change of GFP induction, a symbol is assigned. Based on the results of all three runs, an overall call is assigned. If the overall call is positive, the results are considered highly reliable and reproducible. If the overall call is equivocal, further testing is required to determine significance. If the overall call is negative, the reporter cell line was not activated, therefore no significant damage/stress was observed.

RESULTS

Significant dose-related inductions of the Rtkn gene above 2-fold were determined for AA at doses ranging from 25 to 100 µM in the presence and absence of S9, showing evidence of DNA double-strand breaks. Additionally, with AA, the Srxn1 and Blvrb genes as well as the Btg2 gene were induced above 2-fold at the higher doses, showing evidence of ROS and p53 activities, respectively. For Pyrrolizidine, only the Srxn1 gene was induced >1.5-fold in absence of S9 and above 2-fold at 10,000 µM, showing evidence of ROS activity following metabolic activation. A >1.5-fold increase in was also observed in absence of S9. For the fragrance chemical, 2-octen-4-one, significant induction above 2-fold of the genes Srxn1 and Blvrb were determined at the doses of 35 µM and 70 µM, respectively, in the absence of S9, showing evidence of ROS activity. For Veratraldehyde at 1500 µM, the Rtkn was induced above 2-fold in the presence and absence of S9, the Srxn1 was induced >1.5-fold in absence of S9 and above 2-fold in the presence of S9, showing DNA damage associated with oxidative stress. However, no notable changes in Bscl2 and Rtkn gene expression were determined for either 2-Octen-4-one or Pyrrolizidine showing an absence of DNA mutations or damage. The results demonstrated that the AA and Veratraldehyde were genotoxic while 2-octen-4-one and Pyrrolizidine were identified as non-genotoxic, under the test conditions of the experiment. Although Pyrrolizidine was negative or equivocal for the DNA damage marker induction, it did show dose-dependent increasing trend.

Marker Induction and Cell Survival

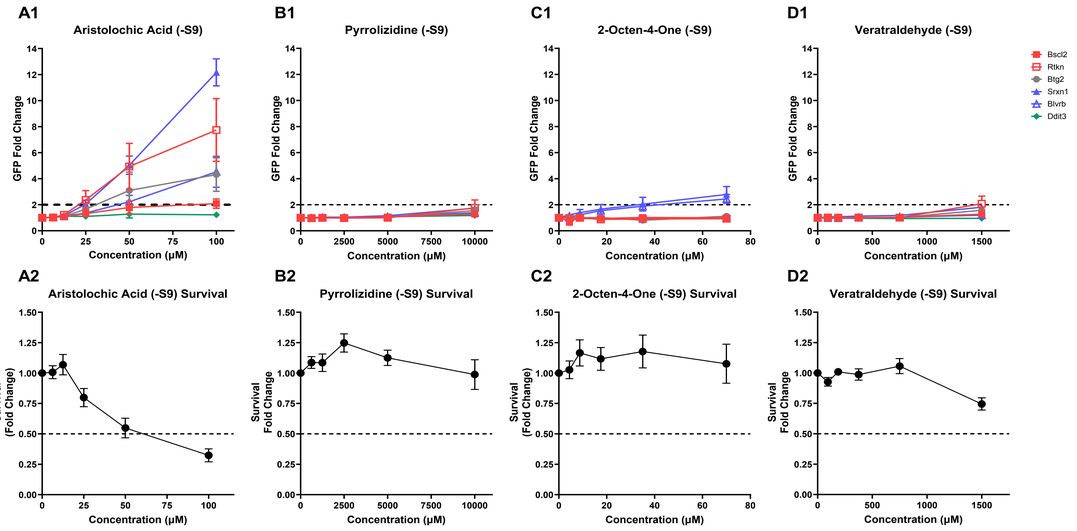

Figure 1. Biomarker induction and corresponding cell survival after exposure to chemicals. The four chemicals (**A**: Aristolochic Acid, **B**: Pyrrolizidine, **C**: 2-Octen-4-one, and **D**: Veratraldehyde) were tested in the ToxTracker® assay in three independent runs to determine genotoxicity potential of each chemical. All four chemicals were dosed at 5 concentrations (selected based on the dose-finding data) to assess DNA damage, cellular stress, oxidative stress, and protein damage using six reporter gene cell lines (Bscl2, Rtkn, Btg2, Srxn1, Blvrb, and Ddit3) without a metabolic activation system. The mean green fluorescence protein (GFP) induction in each reporter cell line was measured after a 24 h exposure using flow cytometry (**A1, B1, C1, and D1**). The mean cell survival (all reporter cell lines) was determined using the viable cell counts from the flow cytometer at each concentration of the chemical (**A2, B2, C2, and D2**).

Marker Induction and Cell Survival with Metabolic Activation

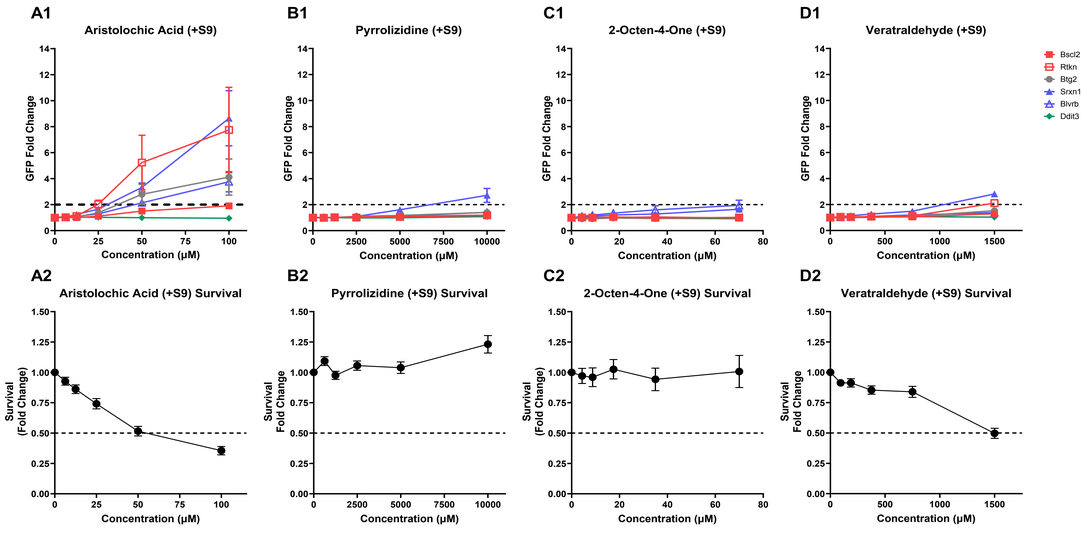

Figure 2. Biomarker induction and corresponding cell survival after exposure to chemicals with S9. The four chemicals (**A**: Aristolochic Acid, **B**: Pyrrolizidine, **C**: 2-Octen-4-one, and **D**: Veratraldehyde) were tested in the ToxTracker® assay in presence of Phenobarbital/5,6-Benzoflavone-induced male Sprague Dawley rat liver S9 fraction, in three independent runs to determine genotoxicity potential of each chemical. All four chemicals were dosed at 5 concentrations (selected based on the dose-finding data) to assess DNA damage, cellular stress, oxidative stress, and protein damage using six reporter gene cell lines (Bscl2, Rtkn, Btg2, Srxn1, Blvrb, and Ddit3) without a metabolic activation system. The mean green fluorescence protein (GFP) induction in each reporter cell line was measured after a 24 h exposure using flow cytometry (**A1, B1, C1, and D1**). The mean cell survival (all reporter cell lines) was determined using the viable cell counts from the flow cytometer at each concentration of the chemical (**A2, B2, C2, and D2**).

DOSE-FINDING

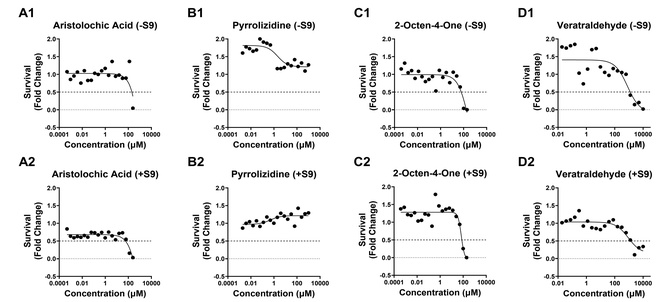

Figure 3. Cell survival in dose-finding step to determine appropriate chemical concentrations for the ToxTracker® assay. The four chemicals (**A**: Aristolochic Acid, **B**: Pyrrolizidine, **C**: 2-Octen-4-one, and **D**: Veratraldehyde) were tested at 20 concentrations in wild type mouse embryonic stem cells to determine a 50% to 25% cell survival concentration to use as the maximum concentration in the definitive ToxTracker® assay. All concentrations of test substances were tested in absence (**A1, B1, C1, and D1**) or presence (**A2, B2, C2, and D2**) of Phenobarbital/5,6-Benzoflavone-induced male Sprague Dawley rat liver S9 fraction. The cell survival was determined using the viable cell counts from the flow cytometer at each concentration of the chemical.

CONCLUSIONS

Table 3. Individual run and overall calls for each reporter gene with and without metabolic activation. Using Table 1, individual calls were determined for each chemical that showed ≥ 25% cell survival for each run. Overall calls were determined using Table 2.

*Only two runs were performed, limiting the overall call of '+ (+)' to equivocal since more data is needed to determine significance. Any run with two of the same calls was determined overall positive, negative, or equivocal based on the call symbol.

		DNA Damage (Genotoxicity)		Cellular Stress	Oxidative Stress		Protein Damage
Chemical	Calls	Bscl2	Rtkn	Btg2	Srxn1	Blvrb	Ddit3
Aristolochic Acid (-S9)	Individual	+ (+)*	+ +*	+ +*	+ +*	+ +*	– –*
	Overall	Equivocal	Positive	Positive	Positive	Positive	Negative
2-Octen-4-one (-S9)	Individual	– – –	– – –	– – –	+ + +	+ + +	– – –
	Overall	Negative	Negative	Negative	Positive	Positive	Negative
Veratraldehyde (-S9)	Individual	– – –	– + +	– (+)(+)	(+)(+)(+)	– – –	– – –
	Overall	Negative	Positive	Negative	Equivocal	Negative	Negative
Pyrrolizidine (-S9)	Individual	– – –	+ – –	+ – –	+ – (+)	– – –	– – –
	Overall	Negative	Negative	Negative	Equivocal	Negative	Negative
Aristolochic Acid (+S9)	Individual	(+) + (+)	+ + +	+ + +	+ + +	+ + +	– – –
	Overall	Positive	Positive	Positive	Positive	Positive	Negative
2-Octen-4-one (+S9)	Individual	– –*	– –*	– –*	(+) (+)*	+ (+)*	– –*
	Overall	Negative	Negative	Negative	Equivocal	Equivocal	Negative
Veratraldehyde (+S9)	Individual	– – –	+ (+) +	– – (+)	+ + +	– – –	– – –
	Overall	Negative	Positive	Negative	Positive	Negative	Negative
Pyrrolizidine (+S9)	Individual	– – –	– – –	– – (+)	(+) + +	– – (+)	– – –
	Overall	Negative	Negative	Negative	Positive	Negative	Negative

Whereas there are numerous citations showing the carcinogenic and nephrotoxic activity of Aristolochic Acid in humans, and several citations showing mutagenic activity in vitro, there are no published reports of AA being tested in the ToxTracker® assay. The testing of AA in the ToxTracker® assay provides further data to support likely modes of action related to the incidence of carcinogenesis. Pyrrolizidine was an outlier of the ToxTracker® assay, as no mutagenic results were determined even though literature states that Pyrrolizidine is a known carcinogen and mutagen when metabolically activated. However, it did show dose-dependent increasing trend in the DNA damage marker. While the literature shows that 2-octen-4-one is positive in standard IVMN tests, our test results in the ToxTracker® assay confirm a non-genotoxic outcome presented in the RSMN and in vivo tests, further substantiating the ability of ToxTracker® to predict in vivo outcomes. In fragrance safety literature, Veratraldehyde has been considered non-mutagenic. However, published ToxTracker results, along with our own ToxTracker data, does show genotoxic potential as signaled by the Rtkn reporter cell line which indicates double-strand DNA damage. The results may indicate a higher sensitivity from the ToxTracker® assay than other in vitro assays such as AMES and in vivo models. These test results provide both evidence of modes of action of these chemicals as well as confidence in the use of the test method for hazard screening purposes. Specifically, our results show that the ToxTracker® assay may be a valuable tool in the genotoxicity assessment of fragrance chemicals and botanicals.