Identifying the Mode of Action of Potential Genotoxicants Using Nuclear Biomarkers

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1 ABSTRACT

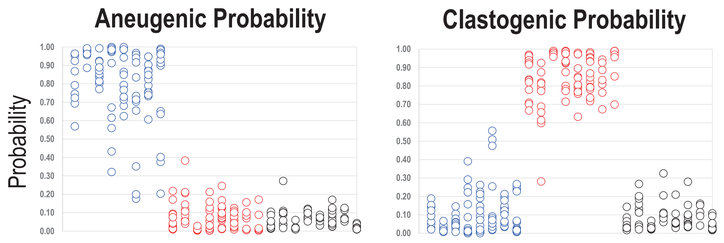
A key component in product development is identifying genotoxic hazards. This includes direct DNA-damaging agents as well as those with alternate modes of action (MOA). The micronucleus (MN) test is routinely used to screen compounds for induction of chromosome damage in vitro as it's high throughput and amenable to automated data collection. However, MN induction is not always indicative of a DNA reactive MOA, and determining the causal molecular pathway is more informative when managing potential risk. Here we describe the installation and qualification of a flow cytometric MOA panel (MultiFlow®) consisting of: γH2AX, p53, and H3PS10. When analyzed at 4 and 24 hours post treatment initiation, multiplexed machine learning models can classify each compound as clastogenic, aneugenic or non-genotoxic. The assays were conducted using TK6 cells in 96-well plates. After collecting data from an initial 12-chemical training set, a separate 12-chemical set (containing DNA reactive/unreactive clastogens, tubulin poisons, kinase inhibitors, and non-genotoxicants) was analyzed to verify assay performance. An additional set of 8 compounds (4 requiring metabolic activation for MN induction) were tested to confirm assay functionality using a 3-4 hour treatment +S9. Overall, this work demonstrates how to properly install and qualify the MultiFlow® assay, and illustrates that these nuclear biomarkers can be used to discriminate MOA. The next steps to advancing our understanding of MOA as related to risk assessment will be implementing high-throughput assays that detect key and molecular initiating events within non-DNA reactive pathways.

2 BACKGROUND

The micronucleus assay detects potential clastogens and aneugens, but provides limited information on the biological mechanism of micronucleus formation.[1] Alternately, the nuclei-based MOA screening assay presented herein evaluates upstream biological effects of a test article that are necessary and essential for micronucleus formation by either a clastogenic or aneugenic mechanism. Nuclear localization of p53 protein has been used as a surrogate marker of genotoxicity for both direct DNA damaging agents, pro-mutagens, and non-DNA reactive carcinogens.[2] Hence, when utilizing a p53 proficient cell line, non-genotoxicants that may induce MN via a cytotoxic mechanism can be identified, as nuclear translocation and activation of p53 is directly related to genotoxicity.[3] Double strand DNA breaks result in γH2AX foci, which recruit DNA repair enzymes to resolve the lesion.[4] However, when repair is insufficient or saturated, this results in formation of acentric DNA fragments that can form micronuclei in daughter cells. Hence, a concentration-dependent increase in nuclear γH2AX content indicates a clastogenic MOA. When enzymes and proteins involved in cellular division are inhibited or perturbed, entire chromosomes may remain at the metaphase plate after anaphase, and these lagging chromosomes form micronuclei in daughter cells.[5] These perturbations cause mitotic checkpoint delays, which causes persistent chromosomal condensation that are dependent upon phosphorylation of H3.[6] Hence, a concentration dependent increase in nuclear H3PS10 content indicates an aneugenic MOA.

3 METHOD VALIDATION -S9

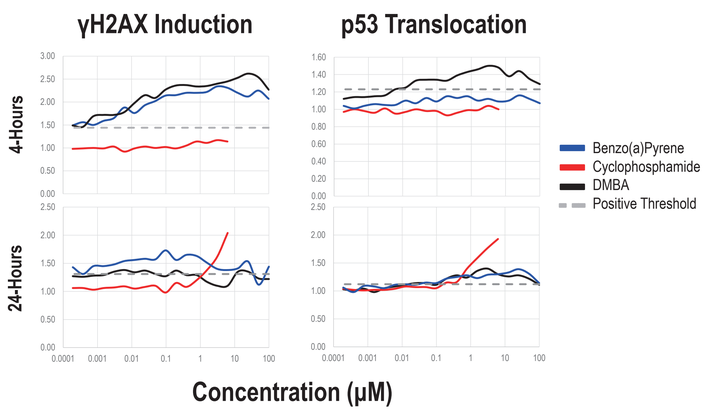
Standard MultiFlow® Assay: TK6 cells were seeded in 96-well plates at ~4x10^4 cells/well in 198 µL complete cell culture medium (CCM), and were dosed with 2 µL of 100X chemical formulations in DMSO. Plates were incubated at ~37C in a humidified atmosphere of 5% CO_2 in air and at 4- and 24-hours after treatment, 25 µL of resuspended cell culture was removed and added forcefully to 50 µL labeling solution, containing lysis reagents, fluorescent beads, and antibodies against p53, γH2AX, and H3PS10. Data were acquired in standard mode on the high throughput sampler of a FACSCanto II flow cytometer using BD FACSDiva software (v8.0). A total of 20 µL was sampled from each well and FCS files (v3.0) were exported and analyzed by Litron Laboratories in FlowJo Software for number of nuclei, number of beads, %polyploidy, %H3PS10^{POS} nuclei, and median channel fluorescent intensities of p53-FITC and γH2AX-AF647. Data were uploaded to JMP software for creation of the machine learning (ML) models to predict MOA as follows (random forest [RF] displayed, though neural net [NN] and linear regression [LR] models were also created):



Each vertical line is dose-response of a color coded chemical listed to the right.

4 METHOD VALIDATION +S9

Standard MultiFlow® Assay: TK6 cells were seeded, dosed, and incubated as described above, with the addition of 1% rat S9 v/v during the treatment period (Mutazyme, Moltox Inc.). Four hours after treatment, 25 µL of resuspended cell culture was processed as described above and the remaining culture volume was washed with 200 µL warm CCM and resuspended in 175 µL warm CCM. The plate was incubated for another 20-hours prior to the final sampling. Three model pro-mutagens were evaluated, as follows:

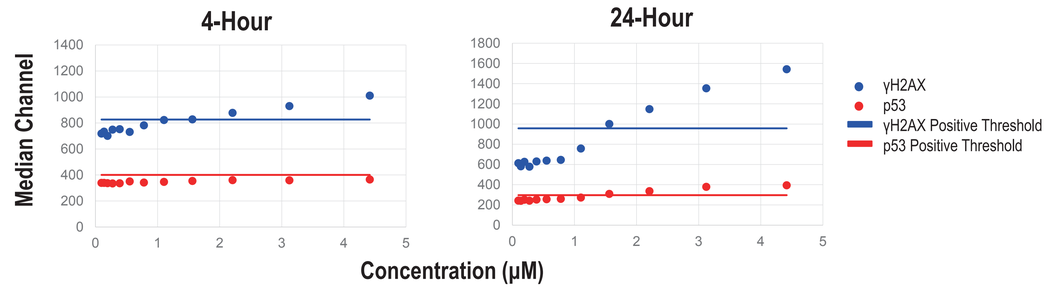


CHEMICALS EVALUATED –S9

MOA	MIE	Name
Aneugens	Kinase Inhibition	AMG900
		Crizotinib
		Tozaserib
		ZM-447439
	Tubulin Poison	Carbendazim
		Nocodzole
		Mebendazole
		Griseofulvin
Clastogens	Alkylation	MMS
		MNNG
	Pool Perturbation	5FU
		Hydroxyurea
		Methotrexate
	ROS induction	Menadione
	TopoII Inhibition	Etoposide
	Nuc Analog	Stavudine
Non-Genotoxicants	UPR	Brefeldin A
	ATP Depletion	CCCP
	Protein Depletion	Cyclohexamide
	PAH	Phenanthrene
	Fibrate	Clofibrate
	PTK Inhibitor	Gleevac
	Haloalkane	Hexachloethane
	Glycan Block	Tunacamycin

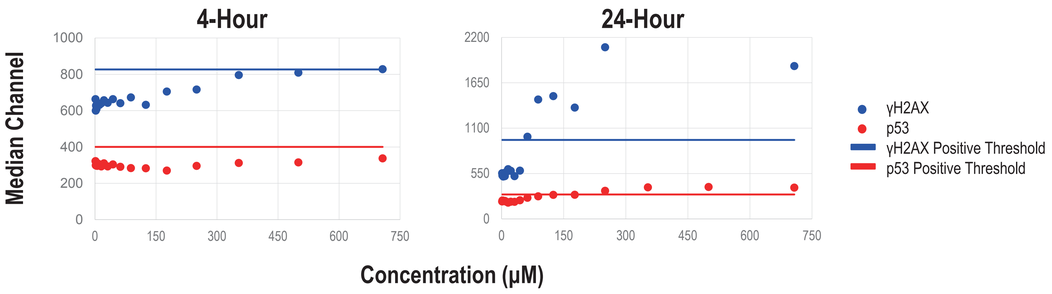
5 TEST CASE A: BLEOMYCIN –S9

Bleomycin uniquely intercalates at G:C rich sites and binds divalent cations (Cu^{2+}/Fe^{2+}), which causes DNA damage via two pathways: generation of reactive oxygen species (ROS, Fenton reaction)[7] and fork collapse (due to the resulting DNA strand breaks).[8] Both of these adverse pathways lead to MN formation via a clastogenic MOA, with clastogenic probabilities up to 97.9% (RF), 100% (NN), and 99.8% (LR) for ≥ 5 consecutive concentrations.



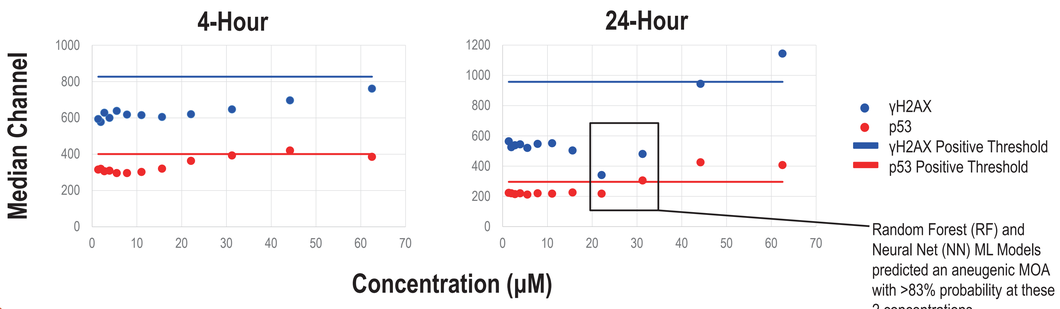
6 TEST CASE B: HOPO –S9

2-hydroxypyridine-N-oxide (HOPO, CAS: 13161-30-3), is a reagent used during chemical manufacturing and it's genotoxicity profile is debated. HOPO has been both negative and positive in bacterial reverse mutation assays; was positive for MN induction in CHO-WBL (but not TK6); and was negative in a rat Pig-a/comet (liver and duodenum)/peripheral blood MN combination study (not shown, Dr.'s Laura Custer and Maik Schuler, personal communications). However, the MultiFlow® assay reveals a clear clastogenic MOA, with clastogenic probabilities up to 97.3% (RF), 100% (NN), and 100% (LR) for 8 consecutive concentrations.



7 TEST CASE C: EDC –S9

N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, CAS 25952-53-8), is another reagent used in chemical manufacturing to synthesize peptide bonds, which also has a debated genotoxicity profile. Depending on formulations, this chemical has been shown to be both negative and positive in bacterial reverse mutation assays; was positive for MN induction in CHO-WBL (with increasing frequencies of hypodiploidy (>10X, 2.5%); and was negative in a rat Pig-a/comet (liver and duodenum)/peripheral blood MN combination study (not shown, Dr. Laura Custer, personal communication, and disclosed in poster and podium formats). However, the MultiFlow® assay reveals a clear genotoxic, and predominantly clastogenic, MOA:



8 CONCLUSIONS

- This work demonstrates the ease of transferability of this high throughput 2-day MOA assay.
- The MultiFlow® assay has been successfully installed in CRL-Skokie, and can be used as a screening assay ±S9, or as a follow up assay to further understand the mechanism of MN induction in TK6 cells.
- Utilizing a 24-compound static model, instead of the standard 12-chemical training set, increases the predictivity of the ML models.[9]
- The test cases demonstrate that while the assay is capable of identifying genotoxic MOA, it does not provide information on key events or the molecular initiating events within those biological pathways (as illustrated by the variation in MIE's across the training set).

9 REFERENCES

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