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Evaluation of γH2AX Assay for Screening Chromosomal Aberrations

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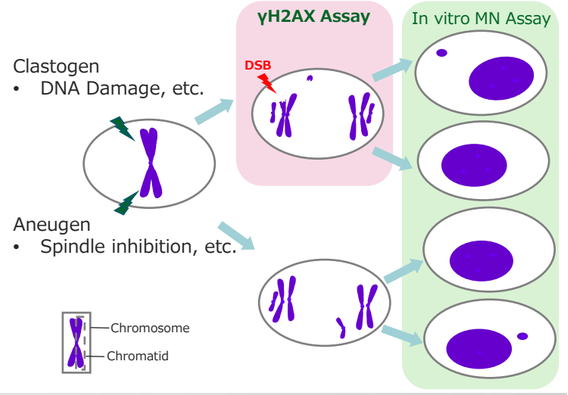
Background and Objective

Recently, nucleic acid-binding small molecules (NBSMs) have raised concerns about chromosomal aberrations caused by DNA damage, highlighting the need for faster and more sensitive evaluation methods that can be applied at the discovery stage.
The MicroFlow assay is widely used as an exploratory in vitro micronucleus test for detecting chromosomal aberrations. However, it requires 6 mg of compound and takes over 10 days to complete. Therefore, higher-throughput methods are needed for the discovery stage.

To address this issue, we focused on γH2AX, a marker of DNA double-strand breaks (DSBs). DSBs are early events that cause chromosomal structural aberrations and can be detected by increased levels of γH2AX. γH2AX is an early and sensitive indicator of DNA damage. In contrast, chromosomal numerical aberrations result from spindle dysfunction and occur independently of DNA damage. Therefore, we assessed the applicability of a γH2AX-based assay capable of sensitively detecting DNA strand breaks for screening chromosomal structural abnormalities.



Materials and Methods

Cells: human lymphoblastoid TK6 cells (ECACC)
Medium: 10% Horse serum supplemented RPMI 1640 Medium (Thermo)
Plate: 96-Well, Non-Treated Microplate (Thermo)
Measurement: NovoCyte Quanteon (Agilent)

Methods:
TK6 cells were seeded onto 96-well plates. The cells were treated with the test compounds at 5 concentrations (up to 100 µM) with a 3.3-fold dilution. After a 3-hour incubation at 37°C with 5% CO_2, the cells were stained using the MultiFlow-γH2AX Kit (Litron Laboratories, USA) and then analyzed by flow cytometry. The γH2AX response was expressed as the fold increase over the solvent control, and values ≥1.51 were considered positive.

In Experiment 1, we tested 20 compounds: 12 known clastogens and 8 non-genotoxic compounds. We compared the γH2AX assay results with the MicroFlow assay results and the compounds' mechanisms of action (MOAs).

In Experiment 2, we evaluated 8 NBSMs to compare the results across the following methods.
- γH2AX assay
- in vitro chromosomal aberration test for pentamidine
- in vitro micronucleus test for branaplam
- MicroFlow assay for other test compounds

Results 1

For the 20 compounds tested, the γH2AX assay showed a predictive accuracy for chromosomal aberrations of 90% (18/20), sensitivity of 83.3% (10/12), and specificity of 100% (8/8).

Table 1 Validation results comparing γH2AX, MicroFlow, and MOA for 20 compounds

#	Chemical	γH2AX (LEC µM)	MicroFlow (LEC µM)	MOA[a)]	#	Chemical	γH2AX	MicroFlow (LEC µM)	MOA[a)]
1	Camptothecin	Pos (0.003)	Pos (0.004)	Clastogen (topoisomerase I inhibitor)	11	5-Fluorouracil	Neg	Pos (7)	Clastogen (antimetabolite)
2	Chlorambucil	Pos (9)	Pos (1.5)	Clastogen (nitrogen mustard-type alkylator)	12	Hydroxyurea	Neg	Neg	Clastogen (antimetabolite)
3	Cisplatin	Pos (3)	Pos (0.9)	Clastogen (atypical alkylator)	13	Brefeldin A	Neg	Neg	Non-genotoxicant (ER-golgi transporter inhibitor)
4	Cytosine arabinoside	Pos (1)	Pos (0.037)	Clastogen (antimetabolite)	14	Dexamethasone	Neg	Neg	Non-genotoxicant (glucocorticoid receptor agonist)
5	Etoposide	Pos (1)	Pos (0.029)	Clastogen (topoisomerase II inhibitor)	15	Erythromycin	Neg	Neg	Non-genotoxicant (antibiotic)
6	Methyl methanesulfonate	Pos (44)	Pos (26)	Clastogen (alkylator)	16	Lidocaine	Neg	Neg	Non-genotoxicant (amide local anesthetic)
7	Mitomycin C	Pos (0.1)	Pos (0.04)	Clastogen (DNA cross-linker)	17	Nalidixic acid	Neg	Neg	Non-genotoxicant[b)]
8	Oraparib	Pos (26)	Pos (3.1)	Clastogen (PARP inhibitor)	18	Progesterone	Neg	Neg	Non-genotoxicant (steroid hormone)
9	Thiotepa	Pos (100)	Pos (1)	Clastogen (alkylator)	19	Thapsigargin	Neg	Pos (0.04)	Non-genotoxicant (ER stress-induced apoptosis)
10	Topotecan	Pos (0.006)	Pos (0.006)	Clastogen (topoisomerase I inhibitor)	20	Tunicamycin	Neg	Pos (0.31)	Non-genotoxicant (ER stress-mediated apoptosis)

a) Environ. Mol. Mutagen., 2017, 58:146-161, b) Environ. Mol. Mutagen., 2013, 54:180-194., LEC: Lowest effective concentration

Two compounds with clastogenic MOA yielded negative results in the γH2AX assay.
5-Fluorouracil and hydroxyurea are antimetabolites that induce genotoxicity primarily through inhibition of DNA replication. Because these compounds do not directly cause DNA strand breaks, the resulting damage is unlikely to be detected by the γH2AX assay. In contrast, non-genotoxic apoptosis inducers such as thapsigargin and tunicamycin are sometimes misclassified as positive in the MicroFlow test. However, they were correctly identified as negative in the γH2AX assay.

Table 2 Clastogen predictive accuracy for 20 compounds

		γH2AX assay		
		+	-	Total
MOA	Clastogen	10	2	12
	Non-genotoxicant	0	8	8
	Total	10	10	20

Results 2

For the 8 NBSMs tested, validation comparing the γH2AX assay with the in vitro micronucleus test (including MicroFlow) and the chromosomal aberration test demonstrated a predictive accuracy for chromosomal aberrations of 87.5% (7/8), sensitivity of 80% (4/5), and specificity of 100% (3/3). The detailed results are presented in Table 3 and Fig 1.

Table 3 Validation results comparing γH2AX, in vitro Micronucleus test for 8 NBSMs

#	NBSMs	Molecular Targets	γH2AX (LEC µM)	in vitro MN/CA(LEC µM)
1	CX-5461	G-quadruplex (G4) structures	Pos (1)	Pos (0.078)
2	RHPS4	G-quadruplex (G4) structures	Neg	Neg
3	Pyridostatin	G-quadruplex (G4) structures	Pos (1)	Pos (0.26)
4	TMPyP4	G-quadruplex (G4) structures	Pos (1)	Pos (7.3)
5	Berberine	G-quadruplex (G4) structure	Neg	Pos (64.5)
6	Ridinilazole	DNA minor groove binder	Neg	Neg
7	Pentamidine	DNA minor groove binder	Neg	Neg[a)]
8	Branaplam	SMN2 (Survival Motor Neuron 2) pre-mRNA	Pos (100)	Pos (0.76)

MN: Micronucleus test, CA: Chromosomal aberration test, LEC: Lowest effective concentration
a) FDA SUPPLEMENT APPROVAL, NDA 19-887/S-014, Reference ID: 2901832
b) Biology Open (2021) 10, bio058551.

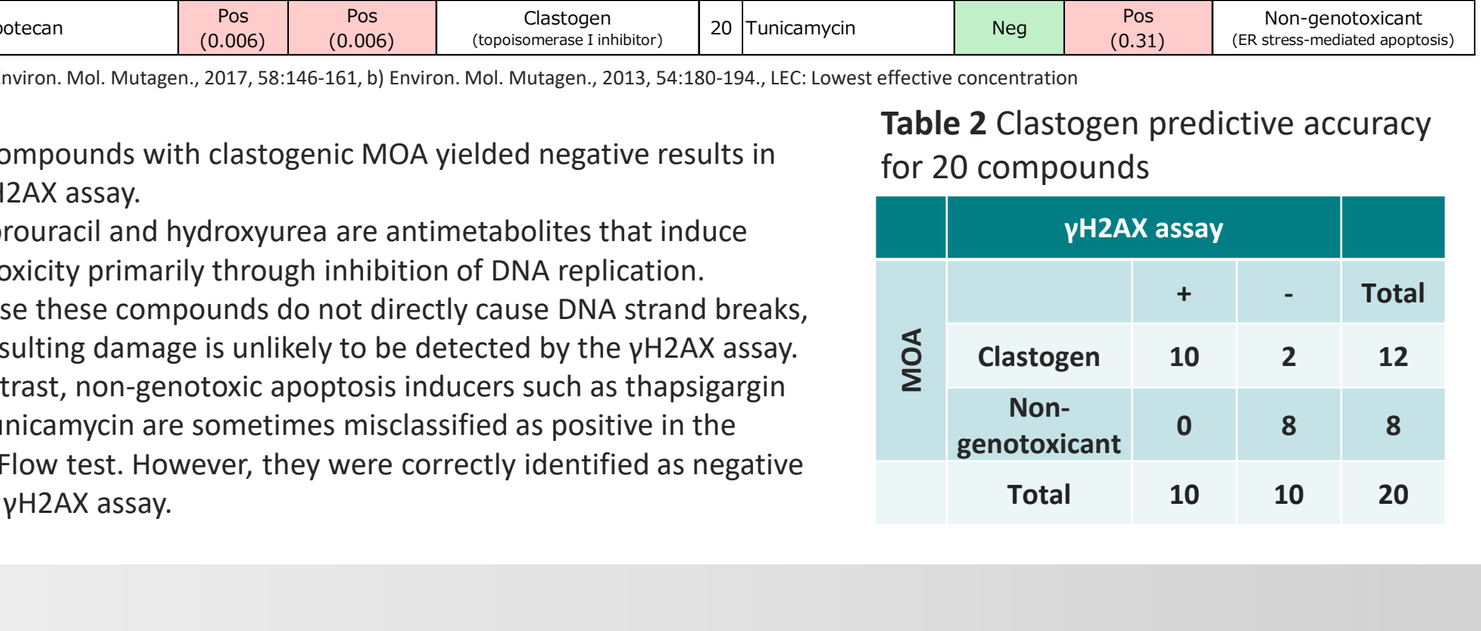


Fig 1 γH2AX assay results for 8 NBSMs

The γH2AX assay result for berberine was inconsistent with the MicroFlow result. Berberine is a plant-derived topoisomerase II inhibitor that generates DNA double-strand breaks. Etoposide has the same mechanism and showed positive γH2AX induction in our assay (Tabel 1). However, berberine's topo II-mediated DNA cleavage activity is weaker than that of etoposide, and induction has been reported only at high concentrations above 1 mM (Food and Chemical Toxicology, 2025, 202:115485). Since this assay used 100 µM as the maximum treatment concentration, it is unlikely to be detected by the γH2AX assay.

Table 4 Chromosomal aberration predictive accuracy for 8 NBSMs

		γH2AX assay		
		+	-	Total
In vitro MN/CA	+	4	1	5
	-	0	3	3
	Total	4	4	8

Conclusion

We evaluated a γH2AX-based assay for screening chromosomal structural abnormalities.
The assay achieved 90% accuracy for known small-molecule chromosomal aberration inducers and 87.5% for NBSMs. Compared to the widely used MicroFlow assay, it requires as less as 0.15 mg of sample (vs. 6 mg) and can be completed within 5 days (vs. 10 days).

These results suggest that the γH2AX assay is a sensitive, cost-effective, and reliable tool for early-stage screening of chromosomal structural abnormalities.

Overview of the In Vitro Chromosome Aberration Assay

	γH2AX Assay	Microflow
Cell	TK6	
Treatment condition	3h, -S9mix	3h, +S9mix, 24h,-S9mix
Required compound amount	0.15 mg	6 mg
Minimum data turnaround time from the start of the study	5 days	10 days