OXFORD
SOT | Society of Toxicology
www.toxsci.oxfordjournals.org

TOXICOLOGICAL SCIENCES, 157(1), 2017, 20–29

doi: 10.1093/toxsci/kfx014
Advance Access Publication Date: January 14, 2017
Research article

Does the Assessment of Nondisjunction Provide a More Sensitive Assay for the Detection of Aneugens?

Joanne M. Elloway,*[,1,4] Alexandra K. Davies,*[,1,2,3] Julie E. Hayes,[†] and Ann T. Doherty*

*AstraZeneca, Innovative Medicines and Early Development, Drug Safety and Metabolism (Discovery Safety), Unit 310—Darwin Building, Cambridge CB4 0WG, UK; and [†]AstraZeneca, Innovative Medicines and Early Development, Drug Safety and Metabolism (Discovery Safety), AstraZeneca R&D, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

[1]Joint first Authors (in this order in publication).

[2]Present address: Department of Clinical Biochemistry, Cambridge Institute for Medical Research, University of Cambridge, Hills Road, Cambridge, UK.

[3]Work conducted during industrial placement year at AstraZeneca.

[4]To whom correspondence should be addressed at AstraZeneca, Innovative Medicines and Early Development, Drug Safety and Metabolism (Discovery Safety), Unit 310—Darwin Building, Cambridge Science Park Milton Road, Cambridge CB4 0WG, UK. Fax: +44-1223223429.
E-mail: joanne.elloway@astrazeneca.com.

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

ABSTRACT

The detection of aneugenic chemicals is important due to the implications of aneuploidy for human health. Aneuploidy can result from chromosome loss or nondisjunction due to chromosome mis-segregation at anaphase. Frequently, aneugens are detected using the *in vitro* micronucleus assay (IVM), with either centromere or kinetochore labeling. However, this method does not consider nondisjunction, the suggested predominant mechanism of spindle poison induced aneugenicity in primary human lymphocytes. Therefore, the IVM may be relatively insensitive in detecting aneuploidy. To investigate whether chromosome distribution analysis, specifically of nondisjunction, using chromosome-specific centromeric probes provides a more sensitive assay for aneugen detection, six reference aneugens with differing modes of action were tested on human lymphoblastoid TK6 cells. The results show that chromosome loss is a substantial part of the process leading to aneuploidy in TK6 cells. This differs from previous studies on human lymphocytes where nondisjunction has been described as the major mechanism of aneugenicity. However, in the current study more cells and types of aneugenic damage were analyzed. Although compound specific effects on nondisjunction were identified, chromosome distribution analysis did not provide increased sensitivity for the detection of aneugens: For the six reference aneugens examined, chromosome loss was shown at the same concentrations or lower than nondisjunction, even when nondisjunction levels were comparatively high. Therefore, in TK6 cells methods that detect chromosome loss, eg, the IVM, provide a more sensitive technique for the detection of aneugens than the measurement of nondisjunction.

Key words: aneugen; nondisjunction; micronuclei.

Aneugenicity is an umbrella term for multiple molecular mechanisms that involve compounds that act on any component of the cell division apparatus. Therefore, aneugens are a diverse class of chemicals. The ability to detect aneugenic chemicals is important due to the negative implications of aneuploidy on human health including congenital abnormalities and somatic cell disorders. Aneuploidy is also strongly correlated with tumor malignancy via the induction of chromosomal and genomic instability. A recent publication has shown the aneuploidy may also have a role in the initiation of carcinogenesis by the altering the expression of proteins related to DNA replication, in particular the reduction in expression of helicase MCM2-7(Passerini *et al.*, 2016).

© The Author 2017. Published by Oxford University Press on behalf of the Society of Toxicology.
All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com

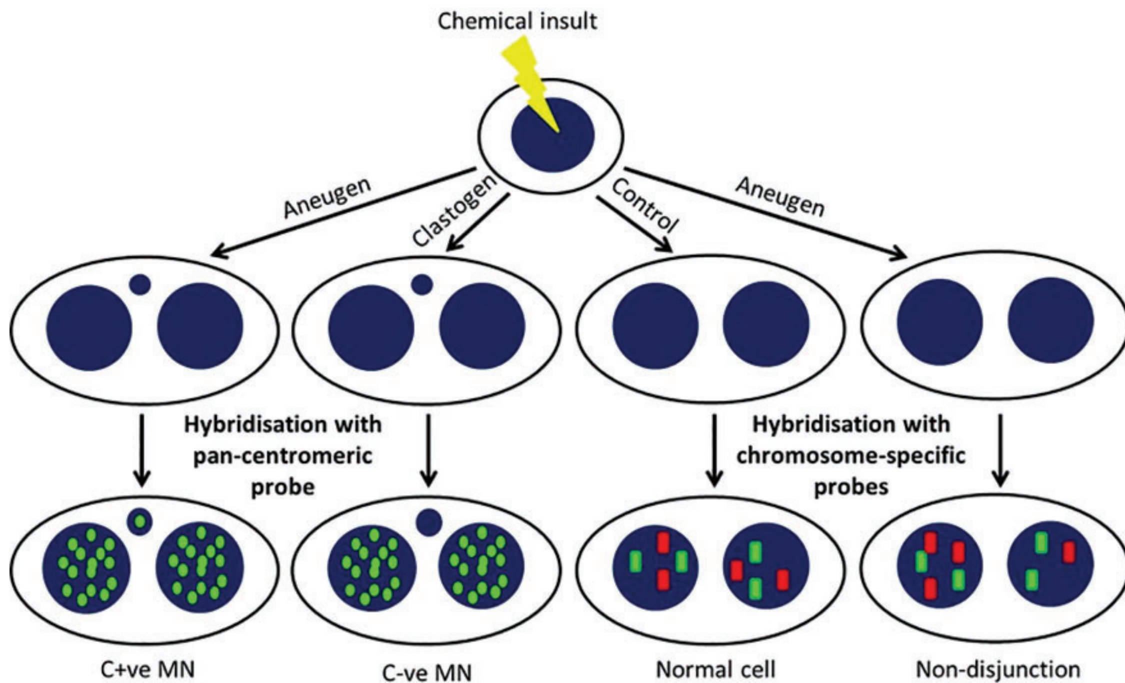

FIG. 1. Diagrammatic representation of the use of FISH techniques for distinguishing aneugenic and clastogenic events in binucleates. A pan-centromeric probe (denoted by green circles) can be used to identify micronuclei containing whole chromosomes. Chromosome-specific probes (red and green rectangles) can be used analyze chromosome distribution and identify nondisjunction.

Aneuploidy can result from any compound that disrupts the cell division apparatus and manifests via two main processes. The first process is nondisjunction of chromosomes at anaphase, eg, through inappropriate attachment of the mitotic spindle resulting in the production of a 3:1 or 4:0 distribution of the chromosomes between the two daughter nuclei. The second process is chromosome loss, occurring if a chromosome lags at the metaphase plate due to misattachment of the spindle and is not incorporated into either daughter nuclei. The lost chromosome may be retained as a micronucleus, reincorporated into either daughter nuclei or lost from the cell (Parry *et al.*, 2002).

The most commonly used technique to investigate the aneugenic potential of compounds is the *in vitro* micronucleus assay (IVM) in combination with either centromere or kinetochore labeling. Micronuclei form in dividing cells from either acentric chromosome fragments (clastogenic events) or from whole chromosomes lost during telophase (aneugenic events), which are then enveloped in a nuclear membrane to give rise to an extranuclear body (Fenech, 2000). The expression of micronuclei is dependent upon cell division. Therefore, the identification of cells that have divided at least once during or after treatment is important. These can be identified through the use of a modification of the IVM which utilizes cytochalasin-B, an inhibitor of cytokinesis, leading to the formation of binucleated cells post mitosis (Fenech and Morley, 1985). There are several methods available to discriminate micronuclei induced by clastogenic and aneugenic events. These include the use of autoantibodies against kinetochores from patients with CREST syndrome (Moroi *et al.*, 1980), antibodies against human centromeric proteins, and fluorescence *in situ* hybridization (FISH) using an alphoid DNA probe p82H specific to a centromeric sequence found on all human chromosomes (Mitchell *et al.*, 1985). These methods discriminate clastogens and aneugens based on the proportion of induced micronuclei containing centromeric DNA or kinetochore protein; aneugens produce mainly centromere/kinetochore-positive micronuclei (C+ve MN) and clastogens mainly centromere/kinetochore-negative C− ve MN (Becker *et al.*, 1990). Fluorescence *in situ* hybridization has been developed extensively over the last 20 years and is currently the most frequently used technique (Cavallo *et al.*, 2007; Melo *et al.*, 2014). Where commercial pan-centromeric FISH probes are unavailable for the species in question, eg, the rat, kinetochore labeling is required (Doherty, 2012). Fluorescence *in situ* hybridization has the advantage that it can be used to examine chromosome segregation in human and mouse cells, specifically nondisjunction events, using chromosome-specific centromeric probes in binucleate cells (Figure 1). Alternative approaches to examine micronuclei by flow cytometry also can discriminate between aneugenic and clastogen micronuclei (Bryce *et al.*, 2011; Muehlbauer and Schuler, 2005).

Studies in binucleated human lymphocytes using chromosome-specific probes to analyze nondisjunction and chromosome loss have suggested that nondisjunction is the predominant type of damage induced by spindle poisons including colchicine, vinblastine, carbendazim, and nocodazole (Bentley *et al.*, 2000; Elhajouji *et al.*, 1997; Marshall *et al.*, 1996; Sgura *et al.*, 1997; Zijno *et al.*, 1994). If nondisjunction is the driving the mechanism of aneuploidy induction for spindle poisons, the analysis of chromosome segregation may provide a more sensitive assessment of aneugenic risk and the calculation of thresholds based on no observed effect level or lowest observed effect level (Kirsch-Volders *et al.*, 2002). However, many interstudy differences could affect the interpretation of results from chromosome segregation analysis. These include differences in the chromosomes chosen for analysis, the method used to analyze chromosome loss, whether the analysis was restricted to binucleates with the expected number of hybridization signals and whether the frequency of nondisjunction was

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

TABLE 1. Description of the Mechanism of Action of the Reference Compounds

Compound	Description	Uses	Mechanism of Action
Colchicine	Plant alkaloid (*Colchicine autonmale*)	Gout suppressant. Antimitotic properties exploited for making metaphase spreads.	Binds tubulin (Bryan, 1972): Inhibits microtubule (MT) polymerization. Does not disrupt preformed MTs. Also shown to alter structure of kinetochores and to effect elongation of daughter centrioles (Parry and Sors, 1993).
Vinblastine	Plant alkaloid (Vinca alkaloid: *Catharanthus roseus*)	Chemotherapeutic agent (antimitotic properties).	Binds tubulin, distinct site to colchicine (Bryan, 1972): Inhibits MT polymerization, causes preformed MTs to disassemble and crystallization of tubulin. Also shown to cause the formation of multipolar spindles, anaphase dislocations and lagging (Parry and Sors, 1993).
Taxol (Paclitaxel)	Plant alkaloid (*Taxus brevifolia*)	Chemotherapeutic agent (antimitotic properties).	Binds tubulin (distinct site): Acts as a MT stabiliser and enhances polymerization of MTs. Inhibits cell replication by preventing spindle dynamics (Nogales *et al.*, 1995)
Noscapine	Plant alkaloid (*Papaver somniferum*)	Chemotherapeutic agent (antimitotic properties) and cough suppressant.	Binds tubulin (unknown site): Suppresses MT dynamics by increasing the time spent in an attenuated state. Interferes with chromosome attachment to kinetochore MTs and suppresses tension across paired kinetochores (Zhou and Giannakakou, 2005).
Chloral hydrate	Metabolite of trichloro-ethylene and tetrachloro-ethylene	Hypnotic and sedative. General anesthetic in veterinary medicine.	Tubulin binding has not been shown. May act by inhibiting tubulin or MT-associated proteins. Inhibits elongation of the spindle by affecting pole to pole MT formation (Parry and Sors, 1993). Action has been suggested to be a result of alterations in the level of cytosolic free Ca^{2+} (Lee et al., 1987).
Diethyl-stilbestrol	Synthetic estrogen	Therapeutic drug for high-risk pregnancies to prevent spontaneous abortions. Growth promoter for cattle.	Binds tubulin (two binding sites, one at or near to that of colchicine): Destabilizes conformation of tubulin and inhibits MT assembly (Metzler and Pfeiffer, 1995). Induces exposure of hydrophobic areas and sulfhydryl groups and inhibits intrachain cross-linking of tubulin (Prasad *et al.*, 1999).
Mitomycin C (clastogen)	DNA alkylator isolated from bacteria	Therapeutic drug used as antitumor agent.	Specifically alkylates and cross-links guanines in CpG sequences, causing covalent links between the complementary strands of DNA, and inhibition of DNA synthesis and replication (Gargiulo *et al.*, 1994).

extrapolated from the chromosomes analyzed to the whole chromosome set to give "total nondisjunction".

The aim of the present work is to determine if assessment of nondisjunction provides a more sensitive assay for aneugen detection. Due to the limited data available on the effects of spindle poisons on nondisjunction and chromosome loss in different cells types, the transformed human B lymphoblastoid TK6 cell line was selected for use in this investigation based on this cell lines' human origin, the availability of chromosome specific centromeric probes and its routine for genotoxicity studies in this laboratory.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich (UK) unless otherwise stated.

Cell line and cell culture conditions. The transformed human B lymphoblastoid TK6 cell line was purchased from the European Collection of Cell Cultures (ECACC; cell line number 95111735). It has a modal chromosome number of 47 and a composite karyotype of 47 XY, +der13t(13;22), der14t(14;20), der21t(21,3). The composite karyotype was achieved from 20 metaphases by G banding and confirmed by FISH (Fellows *et al.*, 2014; Molloy *et al.*, 2010). The average doubling time of the cells was approximately 16–17 h (Doherty *et al.*, 2014). Cells were confirmed as mycoplasma free and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated donor horse serum, 2 mmol/l L-glutamine, 2 mmol/l sodium pyruvate, 200 IU/ml penicillin and 200 μg/ml streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Reference aneugen selection. The present work considered reference aneugens suitable for method validation. All selected reference aneugens act via differing modes of action (see Table 1). Colchicine and vinblastine were selected as they are two of the most potent and extensively studied aneugens for their effects on chromosome loss and nondisjunction (Elhajouji *et al.*, 1997; Marshall *et al.*, 1996; Zijno *et al.*, 1996). Taxol, chloral hydrate, and diethylstilbestrol were selected based on their inclusion in the European Union Project: The detection and evaluation of aneugenic chemicals (Parry *et al.*, 1996). Noscapine was included based on its use as a positive control in the FDA drug approval package for crizotinib (FDA, 2011), in which 98% of micronucleated cells were kinetochore positive. This is an unusually high proportion as potent aneugens typically only produce about 70%–80% kinetochore positive micronuclei (ICH, 2011). A

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

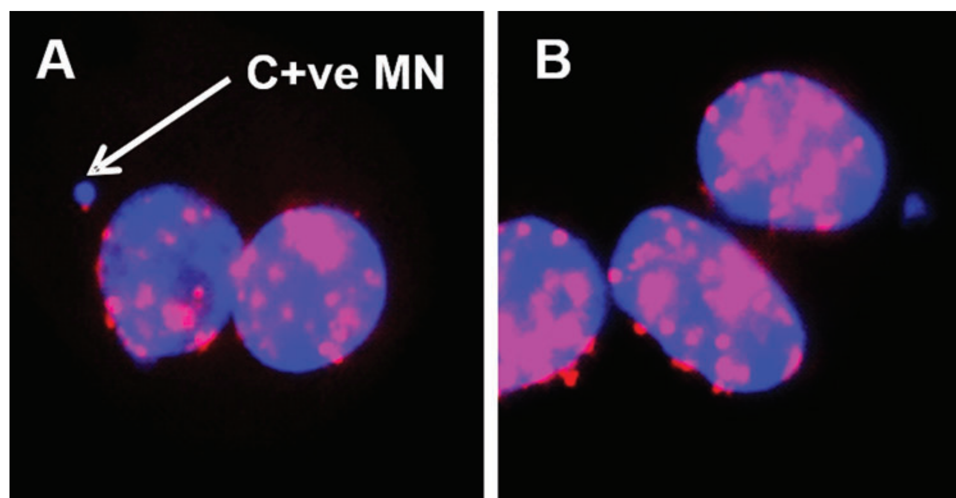

FIG. 2. TK6 cells hybridized with Cy3 pan-centromeric paint. A, Binucleate with a centromere-positive (C + ve) MN. B, Binucleate with a centromere negative (C− ve) MN.

clastogen, mitomycin C (MMC), was also included in this investigation as a negative control.

In vitro cytokinesis-block micronucleus (CBMN) assay. The treatment regime was based on the *in vitro* cytokinesis-block micronucleus assay as described by Doherty (2012). Replicate flasks of TK6 cells were dosed with compounds for 24 h, after which the cells were centrifuged, washed one time with culture media. One replicate flask was treated with cytochalasin-B (5 µg/ml) to produce binucleates and the other replicate flask was untreated to produce mononucleates for cytotoxicity measurements. All flasks were returned to the incubator to recover for 24 h. This treatment schedule aligns with the OECD test guideline 487 (OECD, 2011), with sampling times of one to two cell cycles from the beginning of test treatment. Slides were prepared using a cytocentrifuge (Shandon Cytospin 3) by addition of 850 µl of cell suspension into a Shandon Megafunnel, followed by centrifugation at 1000 rpm for 8 min for centromeric labeling or 800 rpm for 8 min for chromosome distribution analysis. Slides were fixed in 100% methanol for 10–15 min and then air-dried at room temperature.

Cytotoxicity measure. Cytotoxicity was measured by relative population doubling (RPD), calculated in the mononucleate cultures from the start of the treatment period until slide preparation, 48 h later.

Population doubling (PD) was calculated as:

$$PD = \frac{[\log(N_{48}/ \div N_0)]}{\log_2}$$

where N_0 is the initial cell number and N_{48} is the post-treatment cell number at 48 h.

RPD was calculated as:

$$RPD = \left(\frac{\text{PD in treated cultures}}{\text{PD in control cultures}}\right) \times 100$$

Fluorescence in situ *hybridization*. Three or more concentrations were selected for analysis by FISH, based on a combination of the micronucleus and cytotoxicity data. Slides were dehydrated for at least 24 h prior to centromeric labeling. Labeling was carried out using a HYBrite programmable hotplate and following labeling slides were mounted with Vectashield antifading agent containing 4',6-diamidino-2-phenylindole (DAPI). Fluorescence *in situ* hybridization was performed as previously described (Doherty, 2012).

Pan-centromeric labeling. For each slide, 1 µl of human chromosome pan-centromeric paint (Cambio, UK) was mixed with 10 µl of hybridization buffer (Cambio, UK) on a 22 × 22 mm coverslip. Slides were prewarmed to 42 °C for 10 min prior to labeling. The following program was utilized for centromeric labeling: Denaturation at 69 °C for 5 min and hybridization at 42 °C for >16 h. On completion, the coverslip was removed and the slide was washed for 1 min in 0.4 × saline-sodium citrate (SSC) buffer with 0.06% v/v of Tween 20 at 73.5 °C, followed by 2 min in 2× SSC plus one drop of Tween 20 at room temperature.

Slides were scored manually using a fluorescent microscope with a triple band–pass filter. Micronucleus scoring was terminated after 100 micronuclei per slide had been analyzed for the presence of a centromeric signal (Figure 2) or if a total of 5000 binucleates had been examined.

Chromosome-specific centromeric labeling. Chromosomes 7 and 8 were selected for analysis in this investigation because TK6 cells possess two undamaged copies (Fellows *et al*., 2014; Molloy *et al*., 2010). For each slide, 10 µl of premixed centromere-specific probe (Qbiogene) for chromosomes 7 (FITC, green) and 8 (Cy3, red) were placed on a small round coverslip. Chromosomes were labeled using the following program: Denaturation at 76 °C for 5 min and hybridization at 37 °C for >16 h. On completion, the coverslip was removed and the slide was washed for 10 s in 0.4× SSC with 0.06% v/v of Tween 20 at 73.5 °C, followed by 1.5 min in 2× SSC at room temperature.

Slides were scanned and imaged (Figure 3) using a fluorescent microscope with a triple band–pass filter and the automated image-analysis program Metacyte (MetaSystems GmbH, Germany). Following this, the slides were scored manually and aberrant events for each chromosome were categorized as shown in Table 2 (based on categories defined by Marshall *et al*., 1996). The number and distribution of red and green signals were recorded in at least 200 binucleates per slide, unless there were insufficient analyzable cells.

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

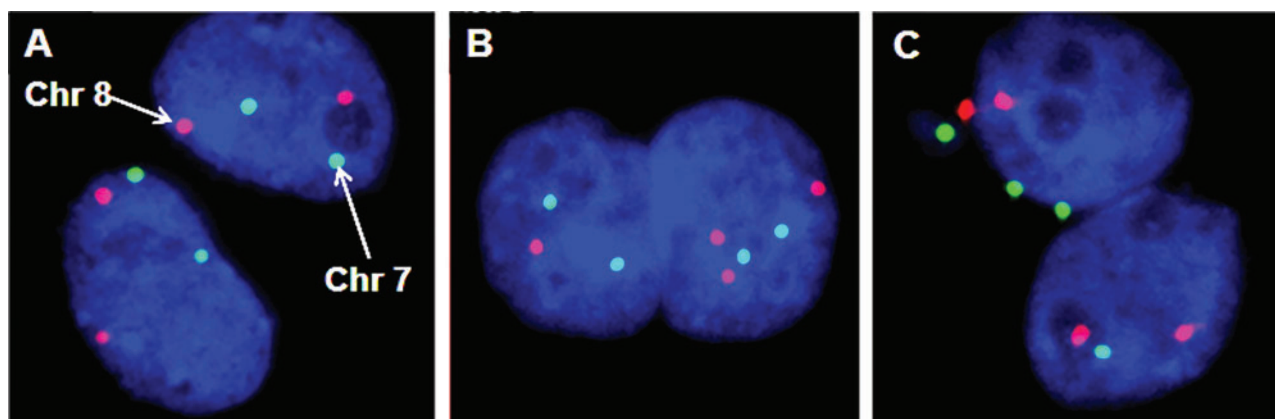

FIG. 3. Images from the MetaCyte image gallery produced on the Metafer 4 master station showing chromosome 7 (green) and 8 (red) distributions in binucleate TK6 cells. A, Normal distribution; B, 3:1 NDJ of chromosome 8; C, loss of chromosome 7 in a MN.

TABLE 2. Categorization of Aberrant Events Used in the Analysis of the Distribution of Chromosomes 7 and 8 in Binucleates

NDJ	Loss	Addition	Hypodiploidy	Hyperdiploidy	Tetraploid	C+ ve MN
3:1	0:1	2:3	1:1	3:3	4:4 + 4:4	2:1 + 1
4:0	0:2	2:4	0:0	4:4		2:0 + 2
	0:3	3:4				
	1:2					

Shown as the number of signals in nucleus 1:nucleus 2.
NDJ, nondisjunction; MN, micronucleus; C+ ve, centromere positive.

Statistical analysis. For the assays, a χ^2 test with one-tailed *P*-values was used to determine the statistical significance of differences between controls and treated samples for each assay individually.

RESULTS

TK6 cells were dosed with the six reference aneugens, colchicine, vinblastine, taxol, diethylstilbestrol, noscapine, and chloral hydrate, and the clastogen mitomycin C. Following treatment binucleated cells were analyzed using two separate assays: The IVM with pan-centromeric labeling and a FISH-based chromosome distribution assay for chromosomes 7 and 8. The ranges and means of aberrant events observed in solvent control containing cultures (DMSO or water) are shown in Table 3. The results from the centromeric labeling and chromosome distribution analyses for each reference aneugen and mitomycin C are shown in Table 4. Statistical significance is calculated relative to concurrent solvent controls and results are considered as biologically relevant when they are outside of the solvent control range (Table 3). The data presented represent a single assay run for each compound. Multiple experiments were conducted and overall results obtained were the same but it was not possible to combine experiments due to shifts in toxicity between repeat experiments.

The results for all the reference aneugens tested show that chromosome loss (as measured by pan-centromeric labeling or with chromosome-specific probes) was detected at the same or lower concentration as any statistically significant aberrant chromosome distribution event, including nondisjunction (Table 4).

Colchicine was the strongest inducer of nondisjunction with 11% of chromosome sets affected at the highest concentration

TABLE 3. Summary of Baseline Frequencies of Aberrant Events Occurring in Solvent Control Cultures Detected in the Centromeric Labeling and Chromosome Distribution Studies

Assay	Aberrant Event	Range (%)	Mean (%)
Centromeric labeling (35 000 BN cells scored)	MNBN	0.2–1.2	1.0
	C+ ve MNBN	0.0–0.4	0.2
	C− ve MNBN	0.2–1.0	0.8
Chromosome distribution (2800 chromosome sets analyzed)	Total aberrant	2.0–4.9	3.5
	Nondisjunction	0.0–0.3	0.1
	Loss	0.3–3.3	2.1
	Addition	0.3–1.8	0.8
	Hypodiploidy	0.0–0.8	0.3
	Hyperdiploidy	0.0–0.5	0.2
	C+ ve MN	0.0–0.3	0.04

BN, binucleate; MNBN, micronucleated binucleate; C+ ve, centromere-positive; C− ve, centromere-negative; MN, micronuclei.

tested (58% RPD), and the only compound for which the contribution of nondisjunction to the total increase in aberrant chromosome sets appeared to be greater than that of chromosome loss. This effect of colchicine on nondisjunction is in line with results from published investigations, ie, nondisjunction and chromosome loss frequencies were similar (Elhajouji *et al.*, 1997; Marshall *et al.*, 1996; Zijno *et al.*, 1996). However, in these studies nondisjunction was detected with statistical significance at lower concentrations than chromosome loss. In contrast, in the chromosome distribution analysis in the current study colchicine induced a clear increase (statistically and biologically relevant) in nondisjunction and chromosome loss at the same concentration, and in the IVM significant increase in induced C+ ve micronuclei was detected at a lower concentration. Therefore, the nondisjunction analysis was no more sensitive than chromosome loss analysis in detecting aneugenicity.

Vinblastine induced a concentration-dependent increase in chromosome loss and induced the highest frequency of chromosome loss of all the reference aneugens tested (29.3% of chromosome sets affected at the highest concentration). However, it should be noted that this concentration was highly cytotoxic (16% RPD) and a statistically significant increase in nondisjunction was only observed at the highest concentration tested. Therefore, chromosome loss was the more sensitive measure of the aneugenicity of vinblastine. This result is in

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

TABLE 4. Comparison of the Frequency of Chromosome Loss (Centromeric Signal in Micronuclei) and Nondisjunction Events in TK6 Cells

Concentration	RPD (%)	Pan-centromeric Labeling (Number/% of Cells)						Chromosome-specific Centromeric Labeling (% of Chromosome Sets)						
		BN cells scored	C+ ve MNBN	C− ve MNBN	Total % MNBN	% C+ ve MNBN	% C− ve MNBN	Aberrant	NDJ	Loss	Addition	Hypo-diploidy	Hyper-diploidy	Probe in MN
Colchicine (ng/ml)[a]														
0	100	5000	18	39	1.1	0.4	0.8	4.9	0.0	3.3	0.8	0.5	0.3	0.0
6.0	101	5000	17	35	1.0	0.3	0.7	3.3	0.0	1.8	1.0	0.5	0.0	0.0
7.5	85	2225	76	24	4.5***	3.4***	1.1	4.8	0.5	3.3	0.3	0.5	0.0	0.3
9.0	58	1015	88	12	9.9***	8.7***	1.2	21.0***	11.0***	6.3*	0.3	2.3*	0.8	0.5
Vinblastine (ng/ml)[b]														
0	100	5000	16	44	1.2	0.3	0.9	2.9	0.0	1.5	0.3	0.8	0.3	0.0
0.5	81	5000	20	41	1.2	0.4	0.8	6.1*	0.0	4.3*	1.0	0.8	0.0	0.0
1.0	42	2954	60	40	3.4***	2.0***	1.4*	9.2***	0.5	6.3***	0.8	0.5	0.8	0.3
2.0[c]	16	639	85	15	15.6***	13.3***	2.3**	54.2***	4.5***	29.3***	1.4	12.2***	3.2**	3.6***
Taxol (ng/ml)[a]														
0	100	5000	17	41	1.2	0.3	0.8	4.4	0.0	3.3	0.3	0.3	0.5	0.0
2.0	95	5000	16	47	1.3	0.3	0.9	3.6	0.8	2.0	0.0	0.5	0.3	0.0
3.0	69	2941	66	34	3.4***	2.2***	1.2	14.4***	0.3	6.3*	1.3	3.5***	3.0**	0.0
4.0	51	1004	79	21	10.0***	7.9***	2.1***	34.9***	0.5	15.8***	1.5	11.3***	5.0***	0.8
5.0	35	–	–	–	–	–	–	47.6***	2.5***	20.0***	1.5	14.3***	7.3***	2.0
Diethylstilbestrol (μg/ml)[b]														
0	100	5000	12	50	1.2	0.2	1.0	2.5	0.3	1.3	1.0	0.0	0.0	0.0
2.0	89	5000	12	39	1.0	0.2	0.8	4.5	0.0	3.5*	0.5	0.5	0.0	0.0
3.0	56	5000	31	54	1.7*	0.6**	1.1	7.3**	0.5	3.5*	1.5	1.0	0.5	0.3
3.5	43	4424	53	47	2.3***	1.2***	1.1	6.9**	0.3	3.5*	1.0	0.8	1.0	0.3
4.0	33	3744	53	47	2.7***	1.4***	1.3	12.4***	0.8	6.6***	1.6	1.9**	1.1*	0.4
5.0	27	2326	66	34	4.3***	2.8***	1.5	8.6***	0.3	3.0	2.8	1.0	1.5*	0.0
Noscapine (μg/ml)[b]														
0	100	5000	12	45	1.1	0.2	0.9	3.9	0.3	2.0	0.8	0.5	0.0	0.3
5.0	85	5000	22	44	1.3	0.4	0.9	7.1	0.3	5.8**	0.5	0.5	0.0	0.0
7.5	54	3931	56	44	2.5***	1.4***	1.1	8.3**	0.5	4.3	1.0	2.5*	0.0	0.0
10.0	33	2312	68	32	4.3***	2.9***	1.4*	15.9***	0.3	8.3***	2.5*	3.0**	1.3*	0.5
15.0	27	–	–	–	–	–	–	15.5***	0.8	7.3***	0.8	5.0***	0.8	0.8
Chloral hydrate (μg/ml)[b]														
0	100	5000	16	46	1.2	0.3	0.9	4.1	0.0	3.0	0.5	0.3	0.3	0.0
180	67	–	–	–	–	–	–	5.4	0.8	3.0	1.3	0.3	0.0	0.0
210	60	5000	29	43	1.4	0.6*	0.9	7.4*	0.8	5.3	0.5	0.3	0.5	0.0
240	47	5000	39	57	1.9**	0.8**	1.1	8.3**	0.5	5.3	1.0	1.5	0.0	0.0
270	31	3147	45	55	3.2***	1.4***	1.7***	7.1*	0.5	4.0	1.8	0.5	0.0	0.3

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

TABLE 4. (continued)

Concentration	RPD (%)	Pan-centromeric Labeling (Number/% of Cells)						Chromosome-specific Centromeric Labeling (% of Chromosome Sets)						
		BN cells scored	C+ ve MNBN	C− ve MNBN	Total % MNBN	% C+ ve MNBN	% C− ve MNBN	Aberrant	NDJ	Loss	Addition	Hypo-diploidy	Hyper-diploidy	Probe in MN
Mitomycin C (ng/ml)[a]														
0	100	5000	1	9	0.2	0.02	0.18	2.0	0.0	0.3	1.8	0.0	0.0	0.0
10	98	5000	20	29	1.0***	0.4***	0.6**	1.5	0.0	0.5	0.3	0.3	0.3	0.0
12.5	99	5000	23	47	1.4***	0.5***	0.9***	1.3	0.0	0.5	0.5	0.0	0.3	0.5
20	85	5000	21	49	1.4***	0.4***	1.0***	0.5	0.0	0.0	0.0	0.0	0.5	0.3
35	80	5000	21	45	1.3***	0.4***	0.9***	0.3	0.3	0.0	0.0	0.0	0.0	0.0
50	65	4953	59	89	3.0***	1.2***	1.8***	2.5	0.3	0.5	1.3	0.5	0.0	0.3
75	40	1874	37	63	5.3***	2.0***	3.4***	2.8	0.5	1.5	0.5	0.3	0.0	0.3
100	32	906	44	56	11.0***	4.9***	6.2***	3.5	0.0	2.3*	0.3	0.8	0.0	0.8

For chromosome-specific centromeric labeling 200 cells, and therefore, 400 chromosome sets (chromosomes 7 and 8) were scored per concentration.

[a]Solvent is water.

[b]Solvent is DMSO.

[c]Toxic concentration, only 111 BN cells scored (222 chromosome sets).

RPD, relative population doubling; BN, binucleate; MNBN, micronucleated binucleate; C+ ve, centromere-positive; C− ve, centromere-negative; NDJ, nondisjunction; MN, micronucleus. Statistical significance over the concurrent control value as determined by χ^2 test is shown as: *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$.

disagreement with data collected in previous studies of human lymphocytes (Marshall *et al.*, 1996; Zijno *et al.*, 1996).

Taxol behaved similarly to vinblastine, inducing higher levels of chromosome loss than nondisjunction. Diethylstilbestrol and noscapine had more subtle effects on chromosome distribution. No significant increases in nondisjunction were seen at any concentrations tested and although the data suggest that chromosome loss is the more important process, this was not as evident as for vinblastine and taxol. Chloral hydrate appeared to be the weakest of the six reference aneugens tested and did not significantly increase either nondisjunction or chromosome loss in the chromosome distribution analysis. However, it did cause a significant increase in centromere-positive micronuclei and in total aberrant chromosome distribution events when all endpoints were analyzed.

In the chromosome specific assay mitomycin C, a reference clastogen, did not have a significant effect on any category of aneugenic event, except for a single dose at high cytotoxicity (32% RPD) which showed a significant increase in chromosome loss (Table 4). This result confirms that chromosome distribution analysis using chromosome-specific probes can be used to distinguish clastogens and aneugens.

DISCUSSION

The results obtained with the reference aneugens tested show that chromosome loss, rather than nondisjunction, is a substantial part of the process leading to aneuploidy in TK6 cells. Responses varied between these aneugens, but all induced statistically and biologically relevant increases in the frequency of chromosome loss at a concentration the same as or lower than nondisjunction. Only colchicine showed an increase in nondisjunction that was greater than the increase in chromosome loss at the same concentration in the chromosome distribution assay. Nevertheless, in the IVM colchicine an increase in C+ ve MNBN was observed at lower concentrations. These investigations demonstrate that chromosome loss in the IVM is at least an equal, if not a more sensitive, end-point for the detection of aneuploidy as compared to nondisjunction measured using a chromosome distribution assay, in the TK6 cell line in this laboratory.

The results of this study show that not all aneugens not all aneugens result or cause NDJ. Therefore, an assay that relies solely on nondisjunction will miss aneugenic compounds. This result is unsurprising because "aneugen" is an umbrella term for the many mechanisms that result in the loss or gain of a chromosome (Aardema *et al.*, 1998) and although the reference aneugens tested were all spindle poisons which bind tubulin, they do so through different modes of action and affinities (see Table 1). Consequently, some may affect the spindle apparatus resulting into NDJ and others in chromosome loss.

The results from the current investigation using TK6 cell line differs from the results obtained in previous studies in primary human lymphocytes, in which nondisjunction is reported as the main process that leads to aneuploidy (Bentley *et al.*, 2000; Elhajouji *et al.*, 1997; Marshall *et al.*, 1996; Sgura *et al.*, 1997; Zijno *et al.*, 1996). Possible reasons for these differences are discussed below.

Firstly, it is difficult to directly compare frequencies of micronuclei with those of aberrant chromosome events because the IVM detects micronucleated cells produced as a consequence of chromosome loss events involving all chromosomes, while the chromosome distribution assay involves the analysis of only two chromosomes. Therefore, the latter one can only

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

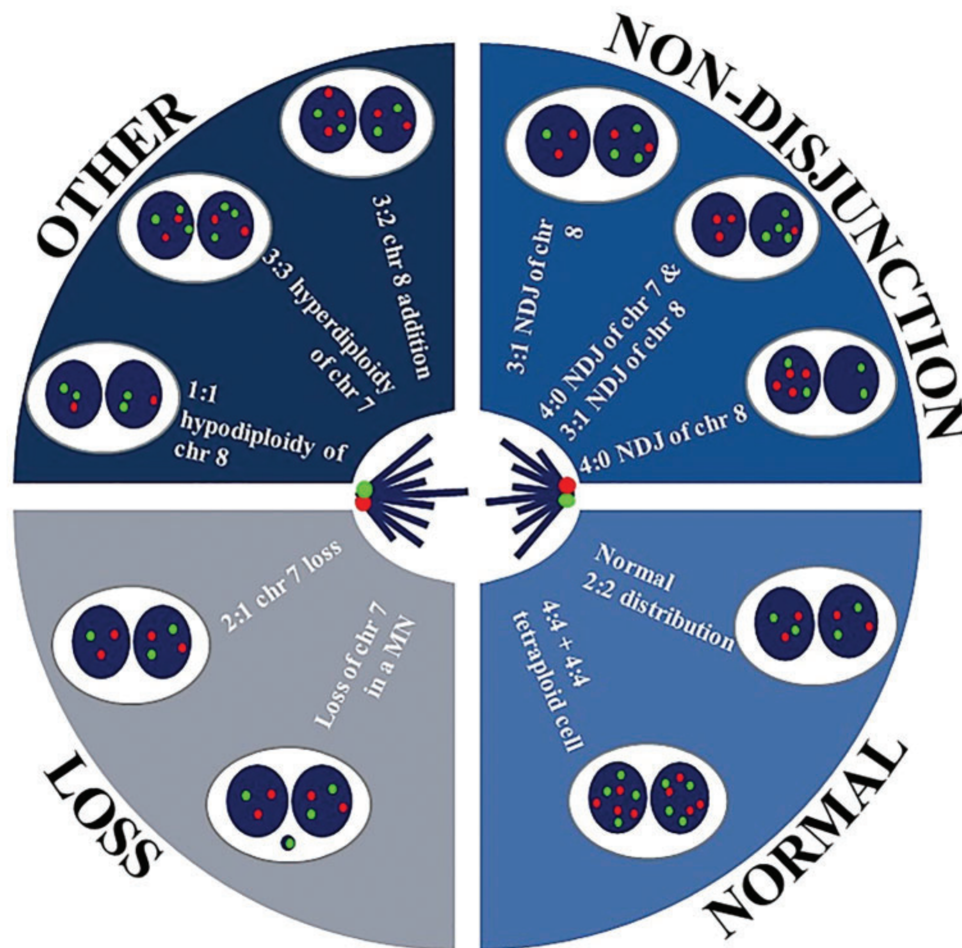

FIG. 4. A diagrammatic representation of some of the potential aberrant distributions involving chromosomes 7 (green) and 8 (red).

provide an estimate of the percentage of cells affected by aberrant events involving all chromosomes. Elhajouji *et al.* (1997) and Sgura *et al.* (1997) extrapolated data from two chromosome-specific probes to estimate total nondisjunction for all chromosomes—they multiplied the frequency of cells with nondisjunction for the two chromosomes analyzed by 23/2. This type of analysis relies on the assumption that nondisjunction occurs randomly and independently for all chromosomes. This contrasts previous reports which have shown that different chromosomes occur at different frequencies in micronuclei following treatment due to differences in spindle alignment sequence or the relative number of microtubules attached to the kinetochore (Caria *et al.*, 1996; Fauth *et al.*, 2000; Norppa and Falck, 2003; Wuttke *et al.*, 1997). This calculation will also overestimate total nondisjunction as it does not take into account the probability of multiple events occurring to different chromosomes within the same cell. The in vitro micronucleus showing chromosome loss is powered differently to the nondisjunction assay looking at segregation of chromosomes, which may contribute to the relative sensitivities of these assays when directly compared.

The utilization of a different cell type, ie, in this investigation the TK6 cell line, may affect comparative analysis between studies. TK6 cells have been shown to produce good concordance with results produced in human lymphocytes for chromosome aberration and micronucleus assays (Pfuhler *et al.*, 2011) and they have been shown to have a relatively stable karyotype with few changes observed for up to 4 weeks of continuous culture (Molloy *et al.*, 2010). However, TK6 cells do differ karyotypically from normal human cells. They have a modal chromosome number of 47 and 3 characteristic abnormalities: 47, XY + 13, 14q+, 21p+ (Grosovsky *et al.*, 1996). Therefore, in this study chromosomes 7 and 8 were analyzed due to an unaltered chromosome number and no visible chromosomal abnormalities.

When comparing the relative sensitivity of the IVM centromeric labeling and chromosome distribution analyses it is important to consider the differences in sample sizes between the two methods. For centromeric labeling up to 5000 cells were analyzed per concentration for the presence of a centromere-positive micronucleus. As the analysis of chromosome distribution is more time consuming than micronuclei scoring, only 200 cells (400 chromosome sets) were analyzed per concentration. This difference in statistical power could explain why the detection of aneugenic events via chromosome distribution analysis did not offer greater sensitivity than the IVM, even though more aberrant events were detected by this method.

When comparing the results of the present investigation with those of earlier investigations, differences in study design that may affect the analysis and interpretation of results should be considered. For example, Zijno *et al.* (1996) and Sgura *et al.* (1997) analyzed nondisjunction and chromosome loss using

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

pairs of chromosome-specific probes and restricted their analysis to only cells containing the expected number of hybridization signals (ie, four signals for each chromosome per binucleate cell), so as to exclude technical artefacts caused by signal overlap, poor probe penetration or nonspecific binding. Using this analysis, cells displaying chromosome loss (eg, 2:1, 2:0, and 1:0 distributions) were disregarded and only chromosome loss where the missing probe was retained in the cell in a micronucleus were recorded. Therefore, the frequency of chromosome loss may be underestimated and that of nondisjunction artificially elevated. In fact, our results using chromosome-specific FISH probes suggest that chromosome loss in a micronucleus is a rare event compared to the complete loss of a chromosome.

The use of chromosome-specific probes in binucleates allows tracking and analysis of chromosomes through mitosis into the daughter cells to be analyzed. From this, a prediction of what aneugenic events may have occurred can be made. Both chromosome loss and nondisjunction have the potential to generate a large variety of aberrant chromosome distributions (Figure 4). As the spindle microtubules are attached to the chromosomes in a nondisjunction event, the assumption is that the most likely outcome will be a 3:1 or 4:0 distribution and that it is unlikely that a chromosome would then be lost to give 2:1 or 3:0. However, when a chromosome lags at the metaphase plate it may be lost, perhaps forming a micronucleus, or it may be reincorporated into either daughter nuclei resulting in a 3:1 distribution, so appearing as nondisjunction, or a 2:2 distribution, so appearing normal (Parry *et al.*, 2002).

In summary, the results from this investigation show that IVM and thus, chromosome loss, was a more sensitive assay for aneugenicity than NDJ in this laboratory in the TK6 human lymphoblastoid cell line. For all six reference aneugens examined, chromosome loss was shown at the same or lower concentrations than nondisjunction, even when nondisjunction levels were comparatively high (eg, colchicine).

REFERENCES

Aardema, M. J., Albertini, S., Arni, P., Henderson, L. M., Kirsch-Volders, M., Mackay, J. M., Sarrif, A. M., Stringer, D. A., and Taalman, R. D. (1998). Aneuploidy: A report of an ECETOC task force. *Mutat. Res.* **410**, 3–79.

Becker, P., Scherthan, H., and Zankl, H. (1990). Use of a centromere-specific DNA probe (p82H) in nonisotopic in situ hybridization for classification of micronuclei. *Genes, Chromosomes Cancer* **2**, 59–62.

Bentley, K. S., Kirkland, D., Murphy, M., and Marshall, R. (2000). Evaluation of thresholds for benomyl- and carbendazim-induced aneuploidy in cultured human lymphocytes using fluorescence in situ hybridization. *Mutation Research* **464**, 41–51.

Bryan, J. (1972). Definition of three classes of binding sites in isolated microtubule crystals. *Biochemistry* **11**, 2611–2616.

Bryce, S. M., Avlasevich, S. L., Bemis, J. C., and Dertinger, S. D. (2011). Miniaturized flow cytometry-based CHO-K1 micronucleus assay discriminates aneugenic and clastogenic modes of action. *Environ. Mol. Mutagen.* **52**, 280–286.

Caria, H., Chaveca, T., and Rueff, J. (1996). Preferential sensitivity of acrocentric chromosomes to the aneugenic effect of colchicine. *Teratog. Carcinog. Mutagen.* **16**, 243–252.

Cavallo, D., Ursini, C. L., Omodeo-Sale, E., and Iavicoli, S. (2007). Micronucleus induction and FISH analysis in buccal cells and lymphocytes of nurses administering antineoplastic drugs. *Mutat. Res.* **628**, 11–18.

Doherty, A. T. (2012). The in vitro micronucleus assay. In *Genetic Toxicology: Principles and Methods* (J. M. P. E. M. Parry, Ed.). Humana Press, New York.

Doherty, A. T., Wilson, A., Chocian, K., Molloy, J., Clatworthy, M., Doak, S., Jenkins, G., and O'Donovan, M. (2014). Genotoxins induce binucleation in L5178Y and TK6 cells. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **770**, 29–34.

Elhajouji, A., Tibaldi, F., and Kirsch-Volders, M. (1997). Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors in vitro in human lymphocytes. *Mutagenesis* **12**, 133–140.

Fauth, E., Scherthan, H., and Zankl, H. (2000). Chromosome painting reveals specific patterns of chromosome occurrence in mitomycin C- and diethylstilboestrol-induced micronuclei. *Mutagenesis* **15**, 459–467.

FDA. (2011). *XALKORI Capsules: Pharmacology Review*. Available at: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/202570Orig1s000TOC.cfm. Accessed April 1, 2016.

Fellows, M. D., McDermott, A., Clare, K. R., Doherty, A., and Aardema, M. J. (2014). The spectral karyotype of L5178Y TK(+)/(-) mouse lymphoma cells clone 3.7.2C and factors affecting mutant frequency at the thymidine kinase (tk) locus in the microtitre mouse lymphoma assay. *Environ. Mol. Mutagen.* **55**, 35–42.

Fenech, M. (2000). The in vitro micronucleus technique. *Mutat. Res.* **455**, 81–95.

Fenech, M., and Morley, A. A. (1985). Measurement of micronuclei in lymphocytes. *Mutat. Res.* **147**, 29–36.

Gargiulo, D., Kumar, G., Musser, S., and Tomasz, M. (1994). Structural and function modification of DNA by mitomycin C. *Mechanism of the DNA sequence specificity of mitomycins*, pp. 169–170.

Grosovsky, A. J., Parks, K. K., Giver, C. R., and Nelson, S. L. (1996). Clonal analysis of delayed karyotypic abnormalities and gene mutations in radiation-induced genetic instability. *Mol. Cell. Biol.* **16**, 6252–6262.

Guideline, I. H. T. (2011). Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2 (R1). pp. 1–25.

Kirsch-Volders, M., Vanhauwaert, A., De Boeck, M., and Decordier, I. (2002). Importance of detecting numerical versus structural chromosome aberrations. *Mutat. Res.* **504**, 137–148.

Lee, G. M., Diguiseppi, J., Gawdi, G. M., and Herman, B. (1987). Chloral hydrate disrupts mitosis by increasing intracellular free calcium. *J. Cell Sci.* **88**, 603–612.

Marshall, R. R., Murphy, M., Kirkland, D. J., and Bentley, K. S. (1996). Fluorescence in situ hybridisation with chromosome-specific centromeric probes: A sensitive method to detect aneuploidy. *Mutat. Res.* **372**, 233–245.

Melo, K. M., Grisolia, C. K., Pieczarka, J. C., de Souza, L. R., Filho Jde, S., and Nagamachi, C. Y. (2014). FISH in micronucleus test demonstrates aneugenic action of rotenone in a common freshwater fish species, *Nile tilapia* (Oreochromis niloticus). *Mutagenesis* **29**, 215–219.

Metzler, M., and Pfeiffer, E. (1995). Effects of estrogens on microtubule polymerization in vitro: Correlation with estrogenicity. *Environ. Health Perspect.* **103(Suppl 7)**, 21.

Mitchell, A. R., Gosden, J. R., and Miller, D. A. (1985). A cloned sequence, p82H, of the alphoid repeated DNA family found at the centromeres of all human chromosomes. *Chromosoma* **92**, 369–377.

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024

Molloy, J., Doherty, A. T., Chocian, K., Hayes, J., and O'Donovan, M. (2010). Karyotypic analysis and stability with time in culture of TK6 and L5178Y cell lines. *Mutagenesis* **25**, 645–645.

Moroi, Y., Peebles, C., Fritzler, M. J., Steigerwald, J., and Tan, E. M. (1980). Autoantibody to centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1627–1631.

Muehlbauer, P., and Schuler, M. (2005). Detection of numerical chromosomal aberrations by flow cytometry: A novel process for identifying aneugenic agents. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **585**, 156–169.

Nogales, E., Wolf, S. G., Khan, I. A., Luduena, R. F., and Downing, K. H. (1995). Structure of tubulin at 6.5 A and location of the taxol-binding site. *Nature* **375**, 424–427.

Norppa, H., and Falck, G. C. (2003). What do human micronuclei contain? *Mutagenesis* **18**, 221–233.

OECD. (2014). *Test No. 487: In Vitro Mammalian Cell Micronucleus Test*. OECD Publishing.

Parry, E. M., Parry, J. M., Corso, C., Doherty, A., Haddad, F., Hermine, T. F., Johnson, G., Kayani, M., Quick, E., Warr, T., *et al*. (2002). Detection and characterization of mechanisms of action of aneugenic chemicals. *Mutagenesis* **17**, 509–521.

Parry, J. M., Parry, E. M., Bourner, R., Doherty, A., Ellard, S., O'Donovan, J., Hoebee, B., de Stoppelaar, J. M., Mohn, G. R., Onfelt, A., *et al*. (1996). The detection and evaluation of aneugenic chemicals. *Mutat. Res.* **353**, 11–46.

Parry, J. M., and Sors, A. (1993). The detection and assessment of the aneugenic potential environmental chemicals: The European Community Aneuploidy Project. *Mutat. Res.* **287**, 3–15.

Passerini, V., Ozeri-Galai, E., de Pagter, M. S., Donnelly, N., Schmalbrock, S., Kloosterman, W. P., Kerem, B., and Storchová, Z. (2016). The presence of extra chromosomes leads to genomic instability. *Nat. Commun.* **7**, 1–12.

Pfuhler, S., Fellows, M., van Benthem, J., Corvi, R., Curren, R., Dearfield, K., Fowler, P., Frotschl, R., Elhajouji, A., Le Hegarat, L., *et al*. (2011). In vitro genotoxicity test approaches with better predictivity: Summary of an IWGT workshop. *Mutat. Res.* **723**, 101–107.

Prasad, V., Garber, S. E., and Ludueña, R. F. (1999). Effect of diethylstilbestrol on the polymerization and alkylation of tubulin. *Drug Dev. Res.* **48**, 104–112.

Sgura, A., Antoccia, A., Ramirez, M. J., Marcos, R., Tanzarella, C., and Degrassi, F. (1997). Micronuclei, centromere-positive micronuclei and chromosome nondisjunction in cytokinesis blocked human lymphocytes following mitomycin C or vincristine treatment. *Mutat. Res.* **392**, 97–107.

Wuttke, K., Streffer, C., and Muller, W. U. (1997). Detection of chromosome 2 and chromosome 7 within X-ray- or colchicine-induced micronuclei by fluorescence in situ hybridization. *Mutagenesis* **12**, 55–59.

Zhou, J., and Giannakakou, P. (2005). Targeting microtubules for cancer chemotherapy. *Curr. Med. Chem. Anti-Cancer Agents* **5**, 65–71.

Zijno, A., Marcon, F., Leopardi, P., and Crebelli, R. (1994). Simultaneous detection of X-chromosome loss and non-disjunction in cytokinesis-blocked human lymphocytes by in situ hybridization with a centromeric DNA probe; implications for the human lymphocyte in vitro micronucleus assay using cytochalasin B. *Mutagenesis* **9**, 225–232.

Zijno, A., Marcon, F., Leopardi, P., and Crebelli, R. (1996). Analysis of chromosome segregation in cytokinesis-blocked human lymphocytes: Non-disjunction is the prevalent damage resulting from low dose exposure to spindle poisons. *Mutagenesis* **11**, 335–340.

Downloaded from https://academic.oup.com/toxsci/article/157/1/20/2903871 by guest on 23 April 2024