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Original Article

Validation of retrospective evaluation method for false genotoxic chemicals with strong cytotoxicity: re-evaluation using *in vitro* micronucleus test

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ABSTRACT — To reduce the false positives in *in vitro* mammalian genotoxicity tests for chromosomal damage, which is caused by severe cytotoxicity, OECD test guidelines have adopted new cytotoxicity indices, and we developed a retrospective evaluation workflow that can be used to identify false positives that are ultimately deemed negative when the new indices are used. Overall, 14 chemicals were estimated to have a negative result. The aim of the present study was to validate the strategy used for the identification of false positives via re-evaluation of these 14 chemicals using the *in vitro* micronucleus test to evaluate the clastogenicity and aneugenicity. As a result, 11 chemicals became negatives, whereas the other three chemicals remained positives. In particular, chemicals of high priority for re-evaluation are more likely to become negative. Therefore, we conclude that our developed strategy is useful to find false positives that have specifically shown chromosomal aberrations at doses inducing strong cytotoxicity. These chemicals can be correctly evaluated as negatives using the new cytotoxicity indices.

Key words: *In vitro* micronucleus test, *In vitro* chromosomal aberration test, False positives

INTRODUCTION

In vitro chromosomal aberration and micronucleus tests have been widely used to detect chemicals that induce chromosomal damage, which allow for the rapid prediction of genotoxicity potential with high sensitivity (Morita *et al.*, 2016). Such *in vitro* tests have become particularly important in the cosmetics industry in consideration of animal welfare and regulatory policies (Pfuhler *et al.*, 2014). Several studies have demonstrated that strong cytotoxicity induces chromosomal damage as a secondary genotoxic effect (Galloway *et al.*, 1987; Ishidate *et al.*, 1984), which can result in false positives in *in vitro* mammalian cell genotoxicity tests. For example, Bradley *et al*. (1987) reported that a detergent induced chromosomal damage by lysosome disruption at a strong cytotoxic dose without a direct interaction with DNA. In fact, high population of false positives has been a major concern when interpreting the results of *in vitro* chromosomal aberration and micronucleus tests. Therefore, proper measurement of cytotoxicity is important to determine the appropriate evaluation dose.

Recently, to reduce the false positives of these tests, the OECD updated their test guidelines with new cytotoxicity indices, relative increase in cell count (RICC) or relative population doubling (RPD), to consider the delay of the cell cycle caused by chemical exposure as an alternative to the conventional index of relative cell count (RCC) (OECD, 2014a, 2014b). It is expected that the number of false positives will be reduced under these new test guidelines. In addition, past-positive results might need to be updated by re-evaluation under the new test guidelines. These predictions are important for the overall risk assessment of genotoxicity and improvement of *in silico* evaluation methods.

We developed formulae to estimate the RICC and RPD values from measured RCC values derived from past tests (Fujita *et al.*, 2016a) and established a retrospective evaluation workflow that can be used to quantitatively estimate the likelihood of positive chemicals to be ultimately revealed as negatives in genotoxicity tests. Using the developed formulae, comprehensive retrospective evaluation of existing *in vitro* chromosomal aberration data of 285 chemicals revealed that 39 of the 90 past-positive chemicals were estimated to be negative under the new test guidelines (Fujita *et al.*, 2016b). In particular, 14

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chemicals were considered particularly likely to become negative when applying the new indices because of the prioritization index < 1 (Fujita *et al.*, 2016b). The prioritization index was calculated by dividing the $eRICC_{D5}$ by 40. $eRICC_{D5}$ is the estimated RICC with a 5% chromosomal aberration rate (according to the positive criteria reported by Ishidate and Odashima (1977)), and an eRICC < 40 indicates strong cytotoxicity beyond the recommended cyototoxicity in the OECD test guidelines (OECD, 2014b). Thus, a prioritization index < 1 ($eRICC_{D5}$/40) indicates that chromosomal aberration might only be observed under a condition of strong cytotoxicity; therefore, chemicals with a prioritization index < 1 might become negatives when tested under the new OECD test guidelines. However, since these suggested false-positive chemicals were not experimentally re-evaluated, the usefulness of our strategy was not validated.

Accordingly, the aims of the present study were 1) to confirm the validity of the retrospective evaluation methods for identifying false-positive chemicals based on *in vitro* chromosomal aberration and micronucleus tests by re-evaluation of the suggested 14 past-positive chemicals that were predicted to be negative under the new test guidelines; and 2) to investigate the effect on the reduction of the false-positive results by adopting the new cytotoxicity indices experimentally. Improvement of *in vitro* mammalian cell genotoxicity tests has been promoted by the Cosmetics Europe research group (Pfuhler *et al.*, 2014). In the European Union, the cosmetic industry is no longer permitted to conduct the *in vivo* micronucleus test owing to a ban on animal testing, and the notes of guidance 9th edition have recommends the *in vitro* micronucleus test as the standard genotoxicity test (SCCS NfG, 2016). Furthermore, *in vitro* micronucleus test can evaluate both of aneugenicity and clastogenicity, while chromosomal aberration test is not designed to detect aneugenicity. Therefore, given the crucial impact and relevance of the *in vitro* micronucleus test to accurately judge the suggested false positives as negatives for the cosmetic industry, we employed the *in vitro* micronucleus test for the re-evaluation of past-positive chemicals in the present study.

MATERIALS AND METHODS

Test chemicals

Fourteen available chemicals (Table 1) were selected from the probably and possibly negative chemicals from the screen in the previous study (Fujita *et al.*, 2016b). The selected chemicals showed negative results of the Ames test and were considered to be of high priority for re-evaluation (prioritization index < 1). The probably negative chemicals were those for which a certain level of cytotoxicity was observed at the lowest observed genotoxicity effect level (LOGEL) and the no observed genotoxicity effect level (NOGEL). The possibly negative chemicals were those that showed strong cytotoxicity at the LOGEL, whereas adequate cytotoxicity was not indicated at the NOGEL. Thus, the probably negative chem-

Table 1. Chemicals selected for testing.

Chemical category (Fujita *et al.*, 2016b)	Test chemicals[1]	Abbreviation	Cas No.	solvent[2]	S-9[3]	treatment time (hr)[3]	Prioritization index (Fujita *et al.*, 2016b)
Probably negative chemicals	2,2,6,6-Tetramethyl-4-hydroxypiperidine	TMHP	2403-88-5	Saline	-	6-18	0.00
	Acenaphthene	ANT	83-32-9	0.5% CMC	+	6-18	0.00
	2-(4-Morpholinyldithio)benzothiazole	MDTBT	95-32-9	DMSO	-	6-18	0.00
	4,4'-Sulfonyldiphenol	SDP	80-09-1	DMSO	-	24-0	0.10
	4-Methylbenzoic acid	MBA	99-94-5	1% CMC	+	6-18	0.34
	2,3,6-Trimethylphenol	TMP	2416-94-6	Acetone	+	6-18	0.39
	o-Dichlorobenzene	DCB	95-50-1	DMSO	+	6-18	0.47
	1,3-Bis(2-methylphenyl)guanidine	BMPG	97-39-2	DMSO	+	6-18	0.63
	2,4-Di-tert-butylphenol	DBP	96-76-4	DMSO	+	6-18	0.79
	o-Acetoacetotoluidine	AT	93-68-5	1% CMC	-	24-0	0.86
Possibly negative chemicals	Thymol	T	89-83-8	DMSO	+	6-18	0.00
	3-Methylzenzoic acid	MZA	99-04-7	DMSO	+	6-18	0.07
	2-tert-Butylphenol	BP	88-18-6	DMSO	+	6-18	0.48
	4-(1-Methylethenyl)phenol	MEP	4286-23-1	DMSO	-	6-18	0.67

[1] These chemicals were estimated to be false-positive chemicals in our previous study (Fujita *et al.*, 2016b).
[2] The solvents were reported in the previous study (Japan Existing Chemical Data Base (JECDB)). DMSO: dimethyl sulfoxide; CMC: carboxymethylcellulose sodium
[3] Test condition of the *in vitro* micronucleus test in the present study for those showing positive results in the previous *in vitro* chromosome aberration test (Japan Existing Chemical Data Base (JECDB)).

icals could be considered to be more likely to become negative when adopting the new indices than the possibly negative chemicals.

Acenaphthene, 2-(4-morpholinyldithio)benzothiazole, 4,4'-sulfonyldiphenol, 4-methylbenzoic acid, o-dichlorobenzene, 2,3,6-trimethylphenol, 1,3-bis(2-methylphenyl)guanidine, o-acetoacetotoluidine, and thymol were all purchased from Tokyo Chemical Industry (Tokyo, Japan); 2,4-di-tert-butylphenol, 3-methylzenzoic acid, and 2-tert-butylphenol were purchased from Sigma-Aldrich (St. Louis, MO, USA); 4-(1-methylethenyl)phenol was purchased from Nacalai Tesque (Kyoto, Japan); and 2,2,6,6-tetramethyl-4-hydroxypiperidine was purchased from Kanto Chemical (Tokyo, Japan).

As a positive control, mitomycin C (MMC; Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) and benzo(*a*)pyrene (B(*a*)P; Tokyo Chemical Industry, Tokyo, Japan) were used for conditioning without and with metabolic activation, respectively.

Cell culture

CHL/IU cells were obtained from the JCRB cell bank (JCRB0030) and were maintained in 10-cm dishes (Iwaki, Tokyo, Japan) containing minimum essential medium (MEM; Thermo Fisher Scientific, Kanagawa, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Kanagawa, Japan). Cultures were incubated at 37°C.

In vitro micronucleus test

Dosage selection and test conditions

Based on the past test reports of the Japan Existing Chemical Database (JECDB) (Japan Existing Chemical Data Base (JECDB)), the maximum concentration was set to that able to induce around 50-60% cytotoxicity (40-50% cell viability as the RICC), and three different concentrations were analyzed for each chemical. In the case of strong cytotoxicity in narrow exposure ranges and the 50-60% cytotoxicity was not obtained, three different concentrations, including an analyzable dose below 50-60% cytotoxicity, were evaluated. The cytotoxicity data (eRICC) taken from Fujita *et al.* (2016b) were used as the basis for the selection of test concentrations. At the end of treatment (i.e., 24 hr after the start of treatment), cytotoxicity was measured using the RICC, and the highest dose inducing 50-60% cytotoxicity (55 ± 5%) was selected. The CHL/IU cells were seeded at 0.8×10^4 cells/mL in a 6-well plate (Becton, Dickinson & Co., Oxnard, CA, USA) and cultured for around 48 hr. After replacing with fresh medium, according to the previous test condition for each chemical (Morita *et al.*, 2012), short-term treatment (6 hr treatment and 18 hr recovery) with or without metabolic activation, or extended treatment (24 hr treatment) without metabolic activation was conducted. The rat liver fraction S9 (Kikkoman Corporation, Chiba, Japan) was used for metabolic activation. For the short-term treatment, at 6 hr after treatment, the test chemicals were removed and washed twice with Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific, Kanagawa, Japan). Then, fresh medium was added and the culture was continued for another 18 hr. Stock solutions of the test chemicals were added directly to the cultures or diluted (10% in saline [Otsuka Pharmaceutical, Tokyo, Japan], 10% in carboxymethylcellulose sodium [Wako Pure Chemical Industries, Osaka, Japan], 1% in dimethyl sulfoxide [MP Biomedicals, CA, USA], or 1% in acetone [Wako Pure Chemical Industries, Osaka, Japan]) in the medium as final concentrations and treated after the medium was replaced. As a positive control, MMC and B(*a*)P were added at 0.1 μg/mL and 15 μg/mL, respectively.

Cell fixation and slide preparation

Cells were recovered in 10 mL MEM including 10% FBS and centrifuged at 1000 rpm for 5 min after 0.25% trypsin/ethylenediaminetetraacetic acid (Thermo Fisher Scientific, Kanagawa, Japan) treatment. Then, cell numbers were counted on an automated cell counter (TC20™, Bio-Rad, CA, USA). After hypotonic treatment with 0.075% KCl, the cells were temporarily fixed with Carnoy solution (methanol:acetic acid = 3:1) and finally fixed with 99% methanol solution (methanol:acetic acid = 99:1). The cell suspension liquid (20-40 μL) was dropped onto the glass slide and air-dried. Finally, the cells were stained by acridine orange (Thermo Fisher Scientific, Kanagawa, Japan).

Scoring of micronucleated cells

At each tested concentration, 2000 cells were counted according to the OECD test guidelines (OECD, 2014b). Micronucleated cells were identified when they showed the same staining characteristics as the main nuclei and a clear nuclear membrane, with a size approximately less than half that of the main nuclei.

Data analysis for micronucleated cells

According to the OECD test guidelines (OECD, 2014b), the chemicals were judged to be clearly positive when three criteria were fulfilled: 1) the proportion of micronucleated cells was increased compared to that of the solvent control based on Fisher's exact test; 2) the dose-dependent increase was confirmed by the Cochran-

Armitage test ($p \leq 0.05$ accepted as significant); 3) the numbers of micronucleated cells were greater than those in the historical data. When none of the criteria was satisfied, the result was judged to be clearly negative, and if any one criterion was not satisfied, the result was judged as negative. In case only two of the three criteria were fulfilled, a reproducibility test was conducted. When reproducibility was confirmed, these chemicals were judged to be positive. The experiments were considered to have been conducted successfully when a statistically significant increase of micronucleated cells was observed for the positive control chemicals compared with the solvent control.

RESULTS

We re-evaluated 14 chemicals that previously showed positive results in the *in vitro* chromosomal aberration test based on the old OECD test guidelines (OECD, 1997), which were considered to be likely to become negative when applying the new cytotoxicity indices (Fujita *et al.*, 2016b) (Fig. 1). All of the positive control chemicals showed statistically significant increases in the number of micronucleated cells induced, thereby validating the test results. Eleven of the 14 chemicals evaluated (78.6%) showed negative results (clearly negative or negative) in the *in vitro* micronucleus test using the RICC. The percentage of becoming negatives among the chemicals classified as probably negative chemicals was higher (90%, 9/10) than that of the chemicals classified as possibly negative chemicals (50%, 2/4). Consequently, at least 28.2% (11 in 39 chemicals) of the suspected false-positive chemicals previously judged as positive were re-evaluated as clearly negative or negative. Moreover, all of the chemicals with high prioritization (prioritization index < 0.45) were likely to become negatives regardless of whether they are classified as possibly or probably negative.

Among the 10 probably negative chemicals, 9 were judged as clearly negative or negative, and one was judged as positive. Eight chemicals (acenaphthene, 2-(4-morpholinyldithio)benzothiazole, 4,4'-sulfonyldiphenol, 4-methylbenzoic acid, 2,3,6-trimethylphenol, 1,3-bis(2-methylphenyl)guanidine, 2,4-di-tert-butylphenol, and o-acetoacetotoluidine) became clearly negative. Although one chemical (2,2,6,6-tetramethyl-4-hydroxypiperidine) showed a significant increase of micronucleated cells (1.2%) at 0.707 mg/mL, the response was within the range of the background data for solvent controls and dose dependency was not confirmed; therefore, it was judged as negative. By contrast, in the evaluation

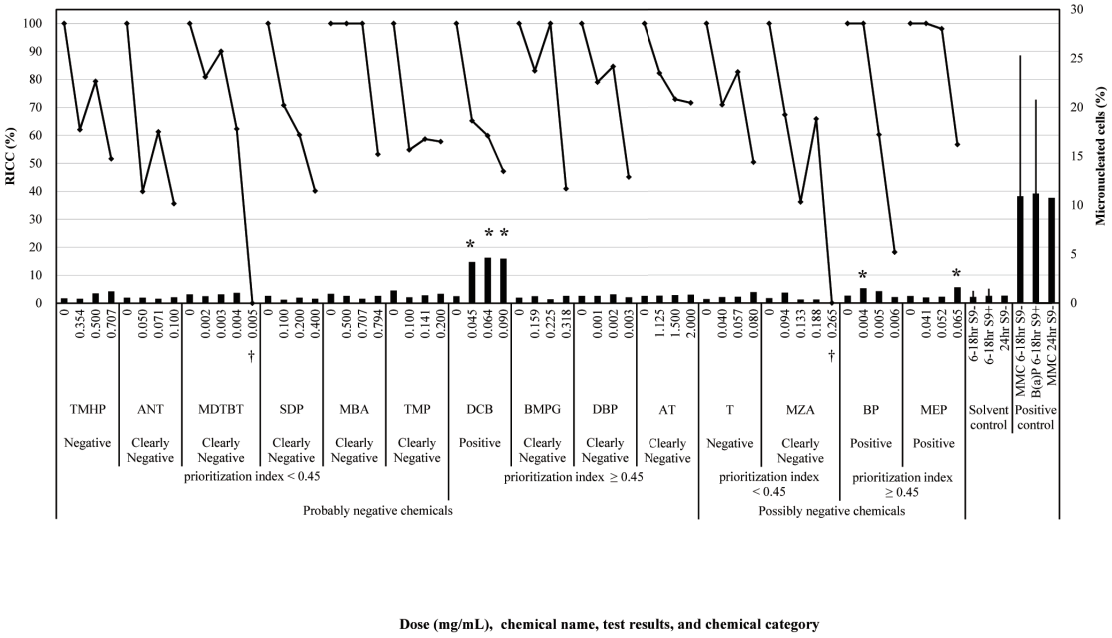

Fig. 1. *In vitro* micronucleus test results of 14 chemicals. Data are the means ± 3 SD. * Fisher exact test $p < 0.05$ for the comparison of the solvent and positive control. †Micronucleus cells could not be counted because of strong cytotoxicity. The abbreviations of test chemicals are listed in Table 1.

of o-dichlorobenzene, significant increases in the numbers of micronucleated cells were found at 0.045 mg/mL, 0.064 mg/mL, and 0.090 mg/mL (4.2%, 4.7%, and 4.6%, respectively) and the responses were beyond the range of the background data. Moreover, since reproducibility was confirmed in the confirmation test (data not shown), o-dichlorobenzene was judged to be positive according to the present criteria.

Among the four possibly negative chemicals, two were judged as clearly negative or negative, and the other two were judged as positive. One chemical, 3-methylzenzoic acid, became clearly negative. Thymol showed a significant increase in the number of micronucleated cells (1.1%) at 0.080 mg/mL; however, since the response was within the range of the background data and dose dependency was not confirmed, it was judged as negative. Both 2-tert-butylphenol and 4-(1-methylethenyl)phenol showed significant increases in the numbers of micronucleated cells at 0.004 mg/mL and 0.065 mg/mL (1.5% and 1.6%, respectively), and the responses were beyond the range of the background data, whereas the dose-dependent increase was not confirmed. Consequently, since reproducibility was confirmed (data not shown), 2-tert-butylphenol and 4-(1-methylethenyl)phenol were judged as positives according to the present criteria.

DISCUSSION

The aims of the present study were to evaluate the reliability of our strategy for the experimental re-evaluation of chemicals showing potential false-positive results, and to investigate the effect of applying new cytotoxicity indices for reducing the false positive rate. Two major conclusions were derived from this study. First, we showed that false positive chemicals could be effectively screened among past-positive chemicals using our approach, and second, approximately 30% (11 in 39 chemicals) of the suspected false-positive chemicals previously judged as positive were re-evaluated as negative in the *in vitro* micronucleus test using the new cytotoxicity indices.

Although the concept for the strategy and the calculation procedure for determining the prioritization index were previously reported (Fujita *et al.*, 2016b), experimental verification was not conducted. Thus, we conducted the *in vitro* micronucleus test for the past-positive chemicals and could confirm the usefulness of our strategy based on the prioritization index. That is, chemicals with a low prioritization index value (i.e., a high priority to be evaluated) tended to become negative, especially for those classified as probably negative chemicals and possibly negative chemicals with a prioritization index < 0.45. Moreover, the probably negative chemicals were more likely to become negative than the possibly negative chemicals. Because the chemicals with a NOGEL with a certain level of cytotoxicity ($RICC_{NOGEL} < 50$) were identified as probably negative chemicals, while not possibly negative chemicals. Therefore, the estimation of the $eRICC_{D5}$ index for probably negative chemicals was more accurate than that for possibly negative chemicals, which would affect the likelihood of becoming negative.

With respect to the reduction effect of false positives achieved by changing the cytotoxicity measurement, Fowler *et al*. (2012) reported that three of six (50%) well-known false-positive chemicals changed to negative results using the new cytotoxicity indices. However, there has been no large-scale evaluation to confirm this effect. Thus, the present study demonstrates that at least 28.2% (11 chemicals) of the 39 suspected false-positive chemicals were judged as negative by changing the cytotoxicity index. This ratio corresponds to approximately 10% of the past-positive chemicals, since 90 chemicals were analyzed in the previous study (Fujita *et al.*, 2016b). Therefore, changing the cytotoxicity measurement according to the updated OECD test guideline may help to effectively reduce the amount of false positives.

The adequacy of the results obtained in the present study was further confirmed since the 11 chemicals that were re-evaluated as negative were shown to have no reactivity to DNA, based on the negative result of the Ames test. Moreover, 2,2,6,6-tetramethyl-4-hydroxypiperidine (Tomita and Tokuwame, 2012) and 1,3-bis(2-methylphenyl)guanidine (Japan Existing Chemical Data Base (JECDB)) were negative in the *in vivo* micronucleus tests. Therefore, the negative results of 11 chemicals in the *in vitro* micronucleus test were considered to be reasonable. Nevertheless, three chemicals were still judged to be positive. The mechanism evaluation and additional *in vivo* tests have been conducted for these chemicals (U.S. Environmental Protection Agency, 2006; Japan Existing Chemical Data Base (JECDB); Matsumoto *et al.*, 2011). For example, since o-dichlorobenzene has positive result in *in vivo* micronucleus test (Mohtashamipur *et al.*, 1987), positive result in the present study might be reasonable. Meanwhile, alkylphenols, including 2-tert-butylphenol, are considered to undergo phase II conjugation and excretion before ionization *in vivo*, and the genotoxicity effect can be observed *in vitro* with S9 but not *in vivo* (Matsumoto *et al.*, 2011). Thus, *in vitro*-specific positive results would remain even if the cytotoxicity index is changed, and indicates a limitation of using the new cytotoxicity indices. The new OECD test guidelines update two other points (maximum dose and variation of

cell types) in addition to the cytotoxicity index changes in an effort to reduce false positive results. Although the maximum dose has little effect on the reduction of false positives (Morita *et al.*, 2014), changing the cell type is considered to be a promising method for re-evaluating *in vitro*-specific positive chemicals (Fowler *et al.*, 2014). Furthermore, additional *in vitro* and *in vivo* data would be useful for interpretation of positive results of *in vitro* tests in the future.

In conclusion, changes to the cytotoxicity index employed in the *in vitro* micronucleus assay could reduce the amount of false positives, and our strategy and method are considered to be useful to efficiently evaluate large amounts of past false-positive chemicals. Our method is expected to contribute to updating the past database effectively and will help to re-develop false-positive materials as negative chemicals in the future.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Bradley, M.O., Taylor, V.I., Armstrong, M.J. and Galloway, S.M. (1987): Relationships among cytotoxicity, lysosomal breakdown, chromosome aberrations, and DNA double-strand breaks. Mutat. Res., **189**, 69-79.

Fowler, P., Smith, R., Smith, K., Young, J., Jeffrey, L., Kirkland, D. Pfuhler, S. and Carmichael, P. (2012): Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. II. Importance of accurate toxicity measurement. Mutat. Res., **747**, 104-117.

Fowler, P., Smith, R., Smith, K., Young, J., Jeffrey, L., Carmichael, P., Kirkland, D. and Pfuhler, S. (2014): Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. III: sensitivity of human cell types to known genotoxic agents. Mutat. Res. Genet. Toxicol. Environ. Mutagen., **767**, 28-36.

Fujita, Y., Kasamatsu, T., Ikeda, N., Nishiyama, N. and Honda, H. (2016a): A retrospective evaluation method for *in vitro* mammalian genotoxicity tests using cytotoxicity index transformation formulae. Mutat. Res. Genet. Toxicol. Environ. Mutagen., **796**, 1-7.

Fujita, Y., Morita, T., Matsumura, S., Kawomoto, T., Ito, Y., Nishiyama, N. and Honda, H. (2016b): Comprehensive retrospective evaluation of existing *in vitro* chromosomal aberration test data by cytotoxicity index transformation. Mutat. Res. Genet. Toxicol. Environ. Mutagen., **802**, 38-49.

Galloway, S.M., Deasy D.A., Bean, C.L., Kraynak, A.R., Armstrong, M.J. and Bradley, M.O. (1987): Effects of high osmotic strength on chromosome aberrations, sister-chromatid exchanges and DNA strand breaks, and the relation to toxicity. Mutat. Res., **189**, 15-25.

Ishidate, M.Jr., Sofuni, T., Yoshikawa, K., Hayashi, M., Nohmi, T., Sawada, M. and Matsuoka, A. (1984): Primary mutagenicity screening of food additives currently used in Japan. Food Chem. Toxicol., **22**, 623-636.

Ishidate, M. and Odashima, S. (1977): Chromosome tests with 134 compounds on Chinese hamster cells *in vitro* — A screening for chemical carcinogens. Mutat. Res. Mol. Mech. Mutagen., **48**, 337-353.

Japan Existing Chemical Data Base (JECDB) as website at http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPageENG.jsp.

Matsumoto, M., Harada, T., Shibuya, T., Hamad, S., Honma, M. and Hirose, A. (2011): Chemical category approach of genotoxicity studies for branched alkylphenols. Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku., **129**, 68-75.

Mohtashamipur, E., Triebel, R., Straeter, H. and Norpoth, K. (1987) The bone marrow clastogenicity of eight halogenated benzenes in male NMRI mice. Mutagenesis, **2**, 111-113.

Morita, T., Honma, M. and Morikawa, K. (2012): Effect of reducing the top concentration used in the *in vitro* chromosomal aberration test in CHL cells on the evaluation of industrial chemical genotoxicity. Mutat. Res., **741**, 32-56.

Morita, T., Miyajima, A., Hatano, A. and Honma, M. (2014): Effects of lowering the proposed top-concentration limit in an *in vitro* chromosomal aberration test on assay sensitivity and on the reduction of the number of false positives. Mutat. Res. Genet. Toxicol. Environ. Mutagen., **769**, 34-49.

Morita, T., Hamada, S., Masumura, K., Wakata, A., Maniwa, J., Takasawa, H., Yasunaga, K., Hashizume, T. and Honma, M. (2016): Evaluation of the sensitivity and specificity of *in vivo* erythrocyte micronucleus and transgenic rodent gene mutation tests to detect rodent carcinogens. Mutat. Res. Genet. Toxicol. Environ. Mutagen., **802**, 1-29.

OECD (1997): OECD TG 473: *In vitro* mammalian chromosomal aberration test. OECD Guidelines for the Testing of Chemicals, Section 4.

OECD (2014a): OECD TG 473: *In vitro* mammalian chromosomal aberration test. OECD Guidelines for the Testing of Chemicals, Section 4, 1-20.

OECD (2014b): OECD TG487: *In vitro* mammalian cell micronucleus test. OECD Guidelines for the Testing of Chemicals, Section 4, 1-26.

Pfuhler, S., Fautz, R., Ouedraogo, G., Latil, A., Kenny, J., Moore, C., Diembeck, W., Hewitt, N.J., Reisinger, K. and Barroso, J. (2014): The Cosmetics Europe strategy for animal-free genotoxicity testing: Project status up-date. Toxicol. In Vitro., **28**, 18-23.

SCCS NfG (2016): The SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation. SCCS/1564/15.

Tomita, M.K. and Tokuwame, M.M. (2012): SIDS Initial Assessment Report for SIAM 14. OECD SIDS, 1-119.

U.S. Environmental Protection Agency (2006) Toxicological review of dichlorobenzenes as website at https://ofmpub.epa.gov/eims/eimscomm.getfile?p_download_id=457549.