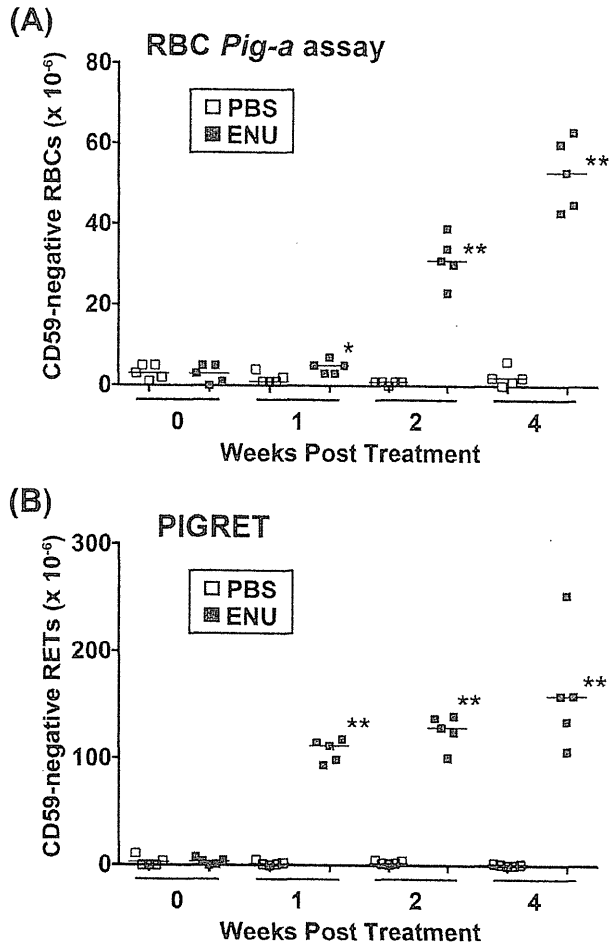

Fig. 1. Comparative analyses of the RBC *Pig-a* (A) and PIGRET (B) assays. At 1, 2, and 4 weeks after treatment with 40 mg/kg ENU or PBS solvent, peripheral blood was withdrawn from the tail vein and analyzed by flow cytometry for the presence of surface CD59 on RBCs or RETs. $^{*}p<0.05$, $^{**}p<0.01$.

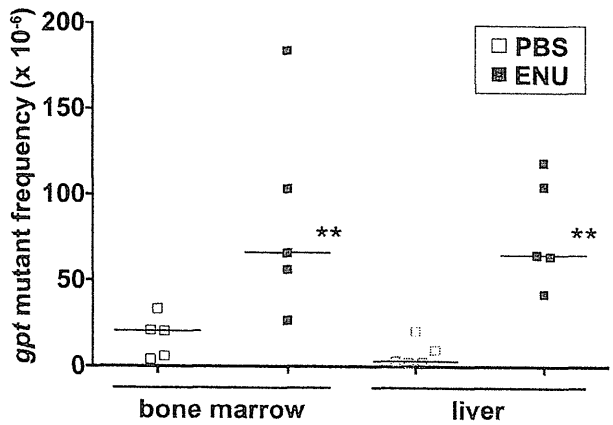

Fig. 2. *gpt* Mutation frequencies in the bone marrow and liver samples. Four weeks after treatment, all rats were sacrificed and their bone marrow and liver samples were collected and analyzed by the *gpt* assay. $^{**}p<0.01$.

mutation assays in rats. Although TGR mutation assays, such as the *gpt* gene mutation assay performed here, are well established methods and permit the *in vivo* evaluation of genotoxicity in more than one organ concurrently (1,4,21), they are costly and need TGR animals. While the *Pig-a* gene mutation assays, including the PIGRET assay, analyze only one type of cells (i.e., blood cells), these assays have the advantage of not using transgenic animals (5,6) and strong potential to be integrated into repeat-dose toxicology studies because accumulated effects can be evaluated (8–10). Additionally, compared with the RBC *Pig-a* assay, the PIGRET assay can detect increases in *Pig-a* MF sooner after exposure (20).

The results of our RBC *Pig-a* and PIGRET assays indicated that the latter more consistently detected ENU-induced increases in *Pig-a* MF at early sampling times than the former (Fig. 1). These results obtained using a single oral administration of ENU were consistent with those previously reported (15,19,20). The ENU-induced *gpt* MFs on the bone marrow and liver samples were well detected as MFs of the RBC *Pig-a* and PIGRET assays (Fig. 2), suggesting that both assays were equally able to detect ENU genotoxicity.

The OECD guideline for TGR assays recommends a tissue sampling time of 3 days after 28 consecutive daily treatments (4), making it difficult to integrate TGR assays into standard repeat-dose toxicology studies. Because the *Pig-a* gene is an endogenous gene, the *Pig-a* assay can be combined with a TGR assay as was done in this present and a previous study (14), and it also can potentially be integrated into repeat-dose toxicology studies that do not use TGRs (8,9,11,12,16,17,22–24). Additionally, the PIGRET assay has strong potential to detect genotoxicity in an early stage of the study, e.g., at 1 week after exposure. Currently, however, we need additional studies that compare mutational responses in the *Pig-a* gene and TGR transgenes to help validate the *Pig-a* assays.

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Short communication

Evaluation of *in vivo* mutagenicity of hydroquinone in Muta™ mice

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ABSTRACT

Hydroquinone (HQ) is used in skin bleaching agents, hair dyes, and finger nail treatments. Many skin-lightening cosmetics that contain HQ are currently marketed in Japan. Concerns have been expressed regarding health risks to the general population because the carcinogenicity of HQ was previously suggested in animal studies. HQ induced hepatocellular adenomas and forestomach hyperplasias in mice and renal tubular cell adenomas in male rats. In the present study, the *lacZ* transgenic mutation assay was conducted according to OECD test guideline 488 to determine whether mutagenic mechanisms were involved in HQ-induced carcinogenesis. Male Muta™ mice were repeatedly administered HQ orally at dosages of 0, 25, 50, 100, or 200 mg/kg bw/day for 28 days. Body weight gain was decreased in all treatment groups. No significant differences were observed in mutant frequencies in the liver, stomach, lung, or kidney between HQ-treated mice and the concurrent negative controls, whereas the significant induction of mutations was noted in the positive control, *N*-ethyl-*N*-nitrosourea. These results suggest that a mutagenic mechanism is not responsible for HQ-induced carcinogenesis.

1. Introduction

Hydroquinone (HQ) is used as an antioxidant in the rubber industry and as a developing agent in photography [1]. It is also used in skin bleaching agents, hair dyes, and finger nail treatments [1]. Many kinds of HQ skin-lightening cosmetics are currently available in Japan, up to 10% of which contain >2% HQ. HQ is not listed as a prohibited or limited ingredient for cosmetic use in Japan [2]. However, the cosmetic use of HQ for skin-lightening has been banned in the UK and EU due to the potential carcinogenic risk of HQ [3]. Approximately 200 different types of skin-lightening products contained 0.4–5.0% HQ in the US in 2006, whereas only prescription skin-lightening products can now contain >2–4% HQ and 2% or less is allowed for cosmetic use [1]. The prolonged use of HQ products (1–2%) has been associated with exogenous ochronosis, which was first reported by Findlay et al. [4], and a worldwide total of 789 cases of exogenous ochronosis had been reported by 2007 [5]. In addition to this topical local effect, concerns have been raised regarding the carcinogenic potential of HQ.

Two previous studies examined the carcinogenicity of HQ in rats and mice by oral administration [6,7]. HQ induced hepatocellular adenomas in female mice in one study [6] and in male mice in the other study [7]. Although epithelial hyperplasia of the forestomach was observed in both sexes in these two studies, tumors did not develop. Furthermore, HQ induced renal tubule adenomas in male rats in both of these studies. Increased rates of leukemia were observed in female rats, but the kidneys remained unaffected. A subsequent histopathological evaluation suggested that the interaction between the development of renal tumors and HQ enhanced chronic progressive nephropathy [8], and the relevance of renal carcinogenic effects in male rats to humans was reported to be questionable based on strain- and sex-specific metabolic pathways [9,10].

The initiating and/or promoting activity of HQ was examined in assays for thyroid, bladder, stomach, liver, lung, esophagus, and kidney carcinogenesis in rats [11–17]. The initiating activity of HQ was not observed in any of these studies, and promoting activity was absent in most assays; an increase in the multiplicity of esophageal tumors was reported in one study [12] while that of renal cell tumors was described in another [17]. No initiating effect was observed on skin tumors in a study using mice [18], and no

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promoting effect was found on pancreatic carcinogenesis in a study with hamsters [19].

Humans are exposed to HQ by oral, dermal, and inhalation routes. The primary route of exposure to HQ during its manufacture is considered to be the inhalation route. The highest average estimated inhalation dose of HQ during manufacturing is 0.0363 mg/kg bw/day for loader packagers. HQ occurs naturally in plants, and humans can consume it *via* foods or cigarette smoking [20]. HQ was previously shown to be dermally absorbed in humans with a bioavailability of 45.3 ± 11.2% for a 24-h application [21]; therefore, it can be absorbed through cosmetic use. Limited information is currently available on the carcinogenic potential of HQ in humans. A mortality study of 879 workers involved in the manufacture and use of HQ in the US reported no significant increases in death due to kidney cancer, liver cancer, or leukemia [22]. Another cohort study in Denmark found that a total of 24 cancer cases among 837 lithographers, and five cases of malignant melanoma were identified with a relative risk of 3.4. Two of five lithographers developed malignant melanoma following exposure to HQ [23].

HQ showed positive results in chromosomal aberration tests and micronuclei tests both *in vivo* (intraperitoneal or subcutaneous injection) and *in vitro* [24–28]. Ciranni et al. reported that the positive result was observed for micronuclei tests after intraperitoneal administration but not after oral administration [25], indicating routs of administration can affect genotoxic responses of HQ. Oxidative stress associated with HQ was shown to induce cytotoxicity [29] and has also been implicated in DNA damage [30]. A comet assay revealed DNA damage in human embryo lung fibroblasts treated with HQ [31]. Two out of three *in vitro* reverse mutation studies with *Salmonella typhimurium* strains were negative with and without metabolic activation [32,33], while one study showed a positive result in S. *typhimurium* TA104 (−S9) and negative results in another 4 strains of *S. typhimurium* (+/−S9). Mutagenic carcinogens are generally considered to have irreversible effects. If HQ carcinogenesis is related to mutagenic events, the no-threshold concept should be applied for risk assessment. However, no information is currently available for the *in vivo* mutagenicity of HQ. A transgenic mouse mutation assay in the target organs of carcinogenicity is useful for determining whether carcinogenesis is related to mutagenic events. In the present study, we evaluated the *in vivo* mutagenicity of HQ using a transgenic mouse mutation assay.

2. Materials and methods

This study was performed at the BioSafety Research Center (BSRC; Shizuoka, Japan) in accordance with "the Act on Welfare and Management of Animals", "the standards relating to the care and management of laboratory animals and relief of pain" and "Guideline for Animal Experimentation, BSRC". Animals were treated in accordance with "Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms", and "Safety Management Regulations for Recombinant DNA Experiment, BSRC". The study was conducted according to OECD TG 488 (28 July 2011: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays).

2.1. Chemicals

HQ (CAS: 123-31-9, Lot no. WEJ0292, purity: 99.3%) was purchased from Wako Pure Chemical Industries, Ltd. A positive control substance, *N*-ethyl-*N*-nitrosourea, was purchased from Toronto Research Chemicals Inc.

2.2. Animals and treatment

Nine-week old male Muta™ mice were purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan), and 33 animals found to be in good health were selected for use after an 8-day acclimation period. These animals were reared on a basal diet, CRF-1 (Oriental yeast) and water *ad libitum*. Animals were maintained at a room temperature of 20–26 °C, relative humidity of 35–70%, 12 h light/dark cycle, and 12 air changes per hour. Groups of five or six mice were administered HQ by gavage once a day for four weeks at a volume of 10 ml/kg, and at levels of 25, 50, 100, and 200 mg/kg. The highest dose level was set based on the results of the NTP fourteen-day gavage study using B6C3F1 mice in which HQ-related deaths (2/5) were observed within 3 days in males receiving 250 mg/kg bw/day [6]. Separate groups (5 animals/group) of the vehicle control (distilled water) were maintained in the same manner. The positive control was treated with *N*-ethyl-*N*-nitrosourea (i.p.) at 100 mg/kg bw/day once a day for 2 days. Animals were observed once a day every day. Body weight was recorded on days 1, 8, 15, and 22 of the administration period, and 1 and 3 days after the last treatment for HQ-treated animals, and one day before the treatment and 10 days after the last treatment for the positive control animals.

2.3. Tissue and DNA isolation

The liver, stomach, kidney, lung, and thyroid were collected 3 days after the last treatment, and a gross pathological examination was conducted. Positive control animals were sacrificed 10 days after the last treatment, and their organs were collected in the same manner. Tissue samples were quickly frozen in liquid nitrogen and then stored at −80 °C until analysis. Genomic DNA was extracted from the liver and stomach at 0, 50, 100, and 200 mg/kg bw/day, and the lung and kidney at 0, and 200 mg/kg bw/day as follows. Frozen tissue was placed into a Dounce homogenizer and homogenized with a pestle. The homogenized tissue fragments were poured into an ice-cooled centrifuge tube containing sucrose solution. After centrifugation by a centrifuge (LC-122, TOMY) at 3000 r/min (1710 G) for 10 min, the organic layer was incubated with RNase and proteinase K at 50 °C for 3 h. A mixture of phenol and chloroform (1:1) was added, and the water layer was separated after centrifugation at 2500 r/min (1190 G) for 10 min; this operation was repeated two times. Chloroform and isoamyl alcohol (24:1) and the water layer were mixed, and similarly centrifuged. The water layer was added in another centrifuging tube, and ethanol was added to precipitate the DNA. DNA was washed by soaking in 70% ethanol for 10 min. The DNA collected following the evaporation of ethanol was dissolved in TE buffer (NIPPON GENE) at room temperature overnight. The DNA solution was stored in a refrigerator.

The DNA of the thyroid was not able to be extracted and, therefore, was excluded from the evaluation.

2.4. In vitro packaging

DNA packaging was performed according to the Instruction Manual of Transpack (Stratagene). The DNA solution (200–600 μg/mL) was gently mixed with the Transpack packaging extract and incubated at 30 °C for 1.5 h twice, and SM buffer (NaCl, $MgSO_4 \cdot 7H_2O$, Tris–HCl [pH: 7.5], and gelatin) was then added.

2.5. Mutant frequency determination

The phage solution absorbed *Escherichia coli* at room temperature for 20–30 min. An appropriately diluted *E. coli* solution was mixed with LB top agar for the titer plates. The remaining phage-*E. coli* solution was mixed with LB top agar containing P-gal (phenyl-β-D-galactoside, Sigma–Aldrich) for the selection plates. These plates were then incubated overnight at 37 °C. Packaging was repeated to reach a total number of 300,000 plaques. The mutant frequency (MF) was calculated by the following formula: MF = total number of plaques on selection plates/total number of plaques on titer plates.

2.6. Statistical analysis

To assess the homogeneity of data, MFs in the treatment and negative control groups were analyzed with Bartlett's test. When homogeneity was recognized, data were analyzed using the Dunnett test. The Steel test was used for non-homogenous data. MFs between the negative and positive controls were compared by the Student's *t*-test or Aspin–Welch's *t*-test. Five percent levels of probability were used as the criterion for significance.

3. Results

No deaths were recorded in any of the treatment groups; therefore, animals at 25 mg/kg bw/day were excluded for the evaluation of mutagenicity. Body weight gain was decreased in all treatment groups (Fig. 1). No clinical signs of toxicity were observed. No abnormal effects were observed in the gross pathological examination. MFs induced by HQ in the liver, stomach, lung, and kidney are shown in Tables 1–4. MFs in the *lacZ* genes of the liver, stomach, lung, and kidney were not significantly higher than those in the respective negative controls. The positive control, *N*-ethyl-*N*-nitrosourea, induced mutations at a frequency that was 2-fold higher in the liver, 11-fold higher in the stomach, 5-fold higher in the lung, and 3-fold higher in the kidney than in their respective negative control organs.

Table 1
Mutation frequencies in the livers of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water	0	1001	666,000	47	70.6	
(Negative control)		1002	348,300	24	68.9	
		1003	722,700	48	66.4	75 ± 11.5
		1004	652,500	62	95	
		1005	673,200	50	74.3	
Hydroquinone	50	1201	734,400	36	49	
		1202	598,500	15	25.1	
		1203	938,700	46	49	42.4 ± 13.3
		1204	722,700	23	31.8	
		1205	719,100	41	57	
	100	1301	1,159,200	45	38.8	
		1302	754,200	29	38.5	
		1303	1,125,000	50	44.4	44.1 ± 8.5
		1304	816,300	48	58.8	
		1305	919,800	37	40.2	
	200	1401	1,036,800	44	42.4	
		1402	1,673,100	232	138.7	
		1403	760,500	42	55.2	69 ± 40.1
		1404	784,800	51	65	
		1405	527,400	23	43.6	
N-ethyl-N-nitrosourea	100	1501	596,700	81	135.7	
(Positive control[b])		1502	611,100	107	175.1	
		1503	640,800	112	174.8	158 ± 27.5[a]
		1504	803,700	147	182.9	
		1505	650,700	79	121.4	

[a] Significantly different from the negative control ($P < 0.05$) by the Student's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

4. Discussion

In the current *in vivo* mutagenicity study, no deaths were recorded in mice treated with HQ up to the highest dose. The highest dose was set as the maximum tolerated dose based on the results of the NTP fourteen-day gavage study using B6C3F1 mice, in which HQ-related deaths (2/5) were observed in males within three days at 250 mg/kg bw/day [6]. In the NTP study, tremors, convulsions, and decreases in body weight (8%) were also observed at 250 mg/kg bw/day. Toxicity in the current study was slightly weaker than expected; however, body weight gain decreased in all treatment groups, indicating that HQ was absorbed and distributed to a sufficient degree to manifest toxicity.

The MF of 138.7 ($\times 10^{-6}$) in one animal (ID number: 1402) at 200 mg/kg bw/day for the liver was higher than the historical negative control data [Mean ± S.D. = 47.6 ± 17.2 ($\times 10^{-6}$); an acceptable range of 0.00–99.2 ($\times 10^{-6}$)] in the facility. However, this change was considered to be spontaneous because the livers of other

Table 2
Mutation frequencies in the stomachs of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water	0	1001	609,300	25	41	
(Negative control)		1002	420,300	21	50	
		1003	831,600	26	31.3	39.6 ± 7.5
		1004	846,900	36	42.5	
		1005	419,400	14	33.4	
Hydroquinone	50	1201	831,600	33	39.7	
		1202	736,200	41	55.7	
		1203	993,600	41	41.3	54.7 ± 14
		1204	588,600	41	69.7	
		1205	761,400	51	67	
	100	1301	741,600	27	36.4	
		1302	651,600	25	38.4	
		1303	914,400	45	49.2	46.7 ± 9
		1304	805,500	46	57.1	
		1305	763,200	40	52.4	
	200	1401	855,900	43	50.2	
		1402	721,800	40	55.4	
		1403	943,200	73	77.4	55.9 ± 12.3
		1404	1,445,400	70	48.4	
		1405	434,700	21	48.3	
N-ethyl-N-nitrosourea	100	1501	621,900	321	516.2	
(Positive control[b])		1502	327,600	150	457.9	
		1503	745,200	369	495.2	473 ± 31.3[a]
		1504	882,900	399	451.9	
		1505	582,300	258	443.1	

[a] Significantly different from the negative control ($P < 0.05$) by Aspin–Welch's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

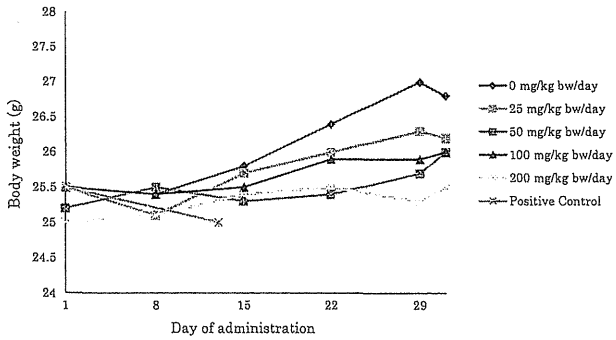

Fig. 1. Body weights of transgenic mice treated with hydroquinone for 28 days. Positive control: *N*-ethyl-*N*-nitrosourea was dosed once a day for 2 days (i.p) and tissues were collected 10 days after the last treatment.

animals at 200 mg/kg bw/day were not similarly affected. This kind of increase in MF could have occurred from a single mutation and clonal expansion [34].

HQ is one of the principal metabolites of benzene. The formation of DNA adducts in the bone marrow was previously reported in male mice exposed to benzene [35], and the same DNA adducts were detected in HL-60 cells or bone marrow treated with HQ *in vitro* [36,37]. However, no DNA adducts were observed in the bone marrow, Zymbal gland, liver, or spleen of female rats given HQ with phenol by gavage [38]. NTP carcinogenicity studies in mice and rats showed different carcinogenic properties between HQ and benzene; benzene showed clearer carcinogenicity in various organs [6,39]. Benzene is known to be leukemogenic in animals and humans [39,40], but no clear evidence has yet been reported to show that HQ induces leukemia in laboratory animals. In the NTP study, female rats showed increased rates of leukemia [6], but these were not significantly higher than those in the historical controls. Leukemogenic effects were not detected in humans who were occupationally exposed to HQ [22,23]. Therefore, HQ itself does not appear to be responsible for the carcinogenicity of benzene.

The main purpose of this study was investigating mutagenicity of HQ responsible to the carcinogenic effects caused by the oral administration. Our current study demonstrated that a mutagenic mechanism was not responsible for the carcinogenesis of HQ, suggesting that HQ can be a threshold carcinogen. Because orally administrated HQ is well absorbed [41], findings of the current study will be applicable for risk assessment for systemic effects of HQ despite of routes of administration. The lowest LOAEL (lowest observed adverse effect level) of a repeated dose was previously reported to be 17.9 mg/kg bw/day (25 mg/kg bw, 5 days/week for 103 weeks) for general toxicity due to lowered body weight and carcinogenicity due to renal tubule adenomas in rats given HQ by gavage [6]. This value can be used

Table 3
Mutation frequencies in the lungs of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water (Negative control)	0	1001	824,400	50	60.7	
		1002	501,300	31	61.8	
		1003	936,000	43	45.9	56.3 ± 10.9
		1004	709,200	49	69.1	
		1005	682,200	30	44.0	
Hydroquinone	200	1401	1,115,100	68	61.0	
		1402	631,800	49	77.6	
		1403	715,500	47	65.7	61.4 ± 26.1
		1404	684,900	58	84.7	
		1405	334,800	6	17.9	
N-ethyl-N-nitrosourea (Positive control[b])	100	1501	681,300	141	207.0	
		1502	458,100	151	329.6	
		1503	848,700	178	209.7	260.2 ± 67.8[a]
		1504	613,800	208	338.9	
		1505	959,400	207	215.8	

[a] Significantly different from the negative control ($p < 0.05$) by Aspin–Welch's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

Table 4
Mutation frequencies in the kidneys of transgenic mice treated with hydroquinone for 28 days.

Substance	Dose (mg/kg bw/day)	Animal ID number	No. of plaques	No. of mutants	MF ($\times 10^{-6}$)	Mean ± SD
Distilled water (Negative control)	0	1001	572,400	24	41.9	
		1002	512,100	36	70.3	
		1003	753,300	52	69.0	53.4 ± 14.9
		1004	558,000	24	43.0	
		1005	633,600	27	42.6	
Hydroquinone	200	1401	551,700	25	45.3	
		1402	681,300	31	45.5	
		1403	475,200	33	69.4	47.0 ± 13.8
		1404	666,000	29	43.5	
		1405	540,000	17	31.5	
N-ethyl-N-nitrosourea (Positive control[b])	100	1501	431,100	87	201.8	
		1502	305,100	39	127.8	
		1503	543,600	92	169.2	147.8 ± 37.7[a]
		1504	590,400	79	133.8	
		1505	479,700	51	106.3	

[a] Significantly different from the negative control ($p < 0.05$) by the Student's *t*-test
[b] Positive control: dosed once a day for 2 days (i.p) and expression period of 10 days.

as a starting point for risk assessments of the cosmetic use of HQ in humans. However, uncertainty may remain for local effects, because routs of administration may affect genotoxic outcomes.

In conclusion, HQ is considered to be a threshold carcinogen because mutagenic activity was not observed in the liver, stomach, lung, or kidney of HQ-treated mice.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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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

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ABSTRACT

For the *in vitro* chromosomal aberration (CA) test, the proposed top-concentration limit will be reduced to '10 mM or 2 mg/mL' (whichever is lower) in the draft revised OECD (r-OECD) test guideline (TG) 473, down from '10 mM or 5 mg/mL' in the current OECD TG, which was adopted in 1997 (1997-OECD). It was previously reduced to 1 mM or 0.5 mg/mL in the International Conference of Harmonization (ICH) S2 (R1) guideline for pharmaceuticals. Reduction of the top-concentration limit is expected to reduce the number of false or misleading positives. However, this reduction may affect the sensitivity or specificity to predict rodent carcinogenicity. Thus, the effect of a reduction in the top-concentration limit on sensitivity and specificity was investigated by use of a dataset on 435 chemicals obtained from the 'Carcinogenicity and Genotoxicity eXperience' (CGX) database (267 CA-positives and 168 CA-negatives; 317 carcinogens and 118 non-carcinogens) where three TGs (*i.e.*, 1997-OECD, r-OECD and ICH) were applied. The application of the r-OECD TG did not affect the sensitivity (63.1%) or specificity (59.3%) against carcinogenicity, compared with the 1997-OECD TG (sensitivity 63.1%, specificity 59.3%). However, the application of the ICH TG had certain effects, *i.e.*, a decrease in sensitivity (45.4%) and an increase in specificity (72.9%). A change in the number of CA-positives by the application of each TG was also investigated by use of 124 CA-positives from the Japanese Existing Chemical (JEC) database. The application of r-OECD TG showed a small reduction in CA-positives, but the ICH TG reduced this number by approximately half. More than half of the CA-positives had a molecular weight <200. These results suggest that the r-OECD TG will not affect the sensitivity or specificity for the detection of rodent carcinogens, indicating the usefulness of the guideline. However, nearly no improvement with respect to a reduction in the number of false positives should be expected.

1. Introduction

Unless limited by cytotoxicity or solubility, the top concentration suggested for use in the *in vitro* chromosomal aberration (CA) test has been 10 mM or 5 mg/mL (whichever is lower) in the Organization for Economic Co-operation and Development (OECD) test-guideline (TG) number 473 [1] for industrial chemicals and in the International Conference of Harmonization (ICH) S2A guideline [2] for pharmaceuticals, after recommendation from the first International Workshop on Genotoxicity Test Procedures (IWGTP) held in Melbourne in 1993 [3]. The 10-mM limit was defined as a limit that was low enough to avoid artificial increases in chromosomal damage due to excessive osmolality and was sufficiently high to ensure the detection of *in vivo* clastogens [4]. However, there has been much discussion on reducing of this top concentration-limit, in particular to diminish the number of false or misleading positive results obtained from mammalian cell genotoxicity tests in recent years [5–10]. Such results are the consequence of biologically non-relevant experimental conditions at very high concentrations used *in vitro*, *e.g.*, low pH, high osmolality, or precipitation of test material in the culture medium. Excessive cellular metabolism, activation or defense, and extremely high concentrations that would not be reached *in vivo* also induce false/misleading positives. Although several recommendations on the new top-concentration limits have been proposed, the recent ICH S2(R1) guideline for pharmaceuticals specified 1 mM or 0.5 mg/mL, whichever is lower, as the

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concentration limit [11]. The rationale for a maximum concentration of 1 mM is as follows: (1) a test battery that includes the Ames test and an *in vivo* genotoxicity assay optimizes the detection of genotoxic carcinogens without relying on any individual assay alone. There is a very low likelihood that the compounds of concern (DNA-damaging carcinogens) – when they are not detected in the Ames test or an *in vivo* genotoxicity assay – can be detected in an *in vitro* mammalian assay above 1 mM; (2) a limit of 1 mM maintains the element of hazard identification, because it is higher than clinical exposures to known pharmaceuticals, including those concentrated in tissues, and is also above the levels generally achieved in preclinical studies *in vivo*. Even beyond the 1-mM limit, the *in vivo* tests ultimately determine the relevance for human safety. However, for pharmaceuticals with an unusually low molecular weight (*e.g.*, less than 200), higher test concentrations should be considered [11]. On the other hand, the draft revised OECD TG 473 proposes a limit of 10 mM or 2 mg/mL, whichever is lower [12]. The rationale for this top-concentration limit is based on the analysis of the data set generated by Parry et al. [6], suggesting that 10 mM is required to detect biologically relevant effects from lower molecular weight non-cytotoxic substances. A simulation study by Brookmire et al. [10] suggested that a test sensitivity at 10 mM is most similar to 2 mg/mL. These findings suggest that the combination of 10 mM or 2 mg/mL, whichever is lower, represents the best balance between the mM and mg/mL concentrations. For complex mixtures, the recommended top concentration remains 5 mg/mL. New top-concentration limits recommended by these TGs are expected to reduce the number of false or misleading positives. However, a reduction of the top-concentration limit may affect the sensitivity or specificity for rodent carcinogenicity, although this reduction should result in an improvement in the specificity of tests without a loss in sensitivity. Here, sensitivity is the ratio of positive *in vitro* CA test results to rodent carcinogens, while specificity is the ratio of negative *in vitro* CA test results to rodent non-carcinogens. In addition, a quantitative structure–activity relationship and software tools have been used recently for to predict genotoxicity [13]. Chromosome damage is also one of the predictive endpoints in in-silico models, *e.g.*, Deductive Estimation of Risk from Existing Knowledge (DEREK) [9,14] or TIssue MEtabolism Simulator (TIMES) [9,15]. Alerts for chromosome damage are based primarily on data from the *in vitro* CA test. Therefore, in-silico evaluation may be affected by changes (from positive to negative) in the CA data. Thus, the effects of reductions of the top-concentration limit on sensitivity and specificity were investigated by use of a set of chemical data, *i.e.*, the Carcinogenicity and Genotoxicity eXperience (CGX) database. To assess the effects in terms of reduction of potential false positives, another chemical data set, *i.e.*, the Japan Existing Chemical (JEC) database, which refers to the Chemical Substances Control Law (CSCL), was used to determine the usefulness of the reduction. These analyses, based on real data obtained from many different chemicals, will be useful for understanding the potential impact of changes in the top concentration used in the *in vitro* CA test.

2. Materials and methods

2.1. Databases used

2.1.1. CGX database

The CGX database provides genotoxicity information on 756 rodent carcinogens and 183 non-carcinogens [16]. The chemicals included in the database comprise all types of chemical, such as industrial chemicals, agrochemicals, pesticides, pharmaceuticals, natural products, and others. For some of these chemicals *in vitro* CA test data are available. The 756 carcinogens included 231 CA-positives, 107 CA-negatives and 14 CA-equivocal. In addition, the 183 non-carcinogens included 61 CA-positives, 61 CA-negatives and 14 CA-equivocal. Data for the *in vitro* CA test were obtained from compilations, such as that from Ishidate et al. [17], and from reports of NTP studies published by Galloway et al. [18], Loveday et al. [19,20], Anderson et al. [21] and other published literature in the CGX database [16]. Thus, various protocols were applied, with different cell types (CHO, CHL, human lymphocytes, etc.), sampling times, top-concentration limit, and cytotoxicity, or different applications of the test guideline or the Good Laboratory Practice (GLP) regulations. The lowest effective concentrations (LECs) were confirmed in all 292 CA-positives (231 carcinogens and 61 non-carcinogens) using the NTP database [22] or original studies [17–21,23–46]. The LEC was defined as the lowest concentration with a statistically significant induction of CA or with a 10% or more CA induction if no statistical analysis was performed, regardless of the test conditions, *e.g.*, different duration of treatment and the presence or absence of S9 mix. The rationale for selecting a 10% cut-off for a positive response is as follows: Ishidate classified test results as positive (≥10% cells with CAs), equivocal (≥5–10% cells with CAs) or negative (less than 5% cells with CAs) in the CA test using Chinese hamster lung (CHL) cells in a similar study protocol [24], and many test results by this author were cited in the CGX database [17]. The 10% cut-off rule is not fully applicable to other cell types with various background data on CA induction in different test protocols. However, it is reasonable to use this cut-off value in order to avoid any overestimation of the CA induction in this analysis. The molecular weight (MW) of each chemical substance was also recorded. When the LEC or MW of the chemical substance could not be identified due to the absence of any description in the paper, *e.g.*, in the case of chemical mixtures or polymers, the substance was excluded from the analysis. Thus, a total of 267 CA-positive chemicals (210 carcinogens and 57 non-carcinogens) were selected for analysis (Table 1). In addition, 168 CA-negatives (107 carcinogens and 61 non-carcinogens) from the CGX database [16] were included. The test concentrations were usually expressed as the weight per volume (*e.g.*, mg/mL). Therefore, LECs identified as mg/mL were converted to equivalent mol concentration (*e.g.*, mM) based on the MW of each chemical.

2.1.2. JEC database

The JEC database, which is based on CSCL regulations, provides toxicity information, *e.g.*, results of a 28-day repeat oral dose study, an Ames test or an *in vitro* CA test, on 277 Japanese existing chemicals (as of January 2012; test data generated from 1991 to 2007) [47]. All chemicals in the database are industrial chemicals with a high production volume in Japan. The *in vitro* CA test was performed with CHL cells according to the OECD TG 473 (first version 1983; revised version 1997 [1]) or the Japanese test guideline for new chemicals [24,48] under GLP conditions. LECs (mg/mL or mM) were defined as those in the CGX database. Of the 272 chemicals with *in vitro* CA data, 124 CA-positives and 148 CA-negatives were found according to their original call (evaluation). Importantly, the old Japanese test guideline employed a long exposure time (48-h of continuous treatment) and the assessment of numerical aberrations for polyploidy was the same as that recently found using TGs. The top-concentration limit was 5 mg/mL (or the equivalent of 10 mM) when no cytotoxicity was observed. The LECs in CA-positives or their MWs were confirmed by use of the original reports [47,49]. All chemicals were identified according to their LECs and MWs; thus, there were no exclusive chemicals identified from the analysis, and 124 CA-positives were used for the analysis (Table 2).

2.2. Application of the test guidelines

The following TGs issued by the OECD and ICH were applied in the analysis: (1) current OECD TG 473 adopted in 1997 (1997-OECD) [1], (2) draft revised OECD TG 473 (r-OECD) [12] for industrial chemicals and (3) ICH S2(R1) TG (ICH) [11] for pharmaceuticals. These TGs specify different top-concentration limits when not limited by solubility or cytotoxicity, namely, 10 mM or 5 mg/mL, whichever is lower, in the 1997-OECD; 10 mM or 2 mg/mL, whichever is lower, in the r-OECD; and 1 mM or 0.5 mg/mL, whichever is lower, in the ICH TG.

2.3. Sensitivity and specificity analyses

To analyze the sensitivity and specificity of the *in vitro* CA-test data against rodent carcinogenicity, a dataset on 435 chemicals (267 CA-positives and 168 CA-negatives; 317 carcinogens and 118 non-carcinogens) from the CGX database was used. Each LEC (in terms of mg/mL and mM) was applied to the three TGs, and the results were re-evaluated (positive or negative) based on the application of the concentration limit for each TG. The sensitivity and specificity against carcinogenicity were also calculated.

2.4. Analysis of the alterations in the number of CA-positives

Analysis of the altered numbers of CA-positives made use of 124 CA-positives from the JEC database. Each LEC (in terms of mg/mL and mM) was applied to the three TGs, and the results (positive or negative) were re-evaluated based on the application of the concentration limit of each TG.

2.5. Evaluation of the relevance of the in vitro CA results

The evaluation of the relevance of the *in vitro* CA results for the chemicals that showed 'different' results between the r-OECD (positive call) and ICH (negative call) TGs for chemicals from the JEC database, was based on a weight-of-evidence

Table 1
Re-evaluation of chromosomal aberration test results on the 267 CA-positive chemicals (210 carcinogens and 57 non-carcinogens).

CGX ID	C/NC	Chemical name	CAS no.	MW	CA (original call)	Equiv. to 10 mM (mg/mL)	LEC (mg/mL)	LEC (mM)	Ref.	1997-OECD[a]	r-OECD[b]	ICH[c]
										CA	CA	CA
1	C	*Acetaldehyde*	*75-07-0*	*44.1*	+	*0.44*	*0.0044*	*0.1*	*17*	+	+	+
2	C	*Acetaminophen*	*103-90-2*	*151.2*	+	*1.51*	*0.2*	*1.3*	*17*	+	+	[redacted]
3	C	*N*-Acetoxy-2-acetylaminofluorene	6098-44-8	281.3	+	2.81	0.0003	0.001	17	+	+	+
4	C	2-Acetylaminofluorene	53-96-3	223.3	+	2.23	0.5	2.2	17	+	+	[redacted]
5	C	*Acrylamide*	*79-06-1*	*71.1*	+	*0.71*	*2*	*28.14*	*22*	[redacted]		
6	C	*Acrylonitrile*	*107-13-1*	*53.1*	+	*0.53*	*0.0053*	*0.1*	*23*	+	+	+
7	C	Actinomycin D	50-76-0	1255.4	+	12.55	0.0018	0.0014	17	+	+	+
8	C	Aflatoxin B1	1162-65-8	312.3	+	3.12	0.0005	0.0016	17	+	+	+
9	C	Aldrin	309-00-2	364.9	+	3.65	0.019	0.052	17	+	+	+
10	C	*Allyl glycidyl ether*	*106-92-3*	*114.1*	+	*1.14*	*0.06*	*0.53*	*22*	+	+	+
11	C	*Allyl isothiocyanate*	*57-06-7*	*99.2*	+	*0.99*	*5.00E-07*	*0.000005*	*17*	+	+	+
12	C	*Allyl isovalerate*	*2835-39-4*	*142.2*	+	*1.42*	*0.3*	*2.11*	*22*	+	+	[redacted]
13	C	*4-Aminobiphenyl*	*92-67-1*	*169.2*	+	*1.69*	*0.05*	*0.30*	*22*	+	+	+
14	C	3-Amino-1,4-dimethyl-5H-pyrido{4,3-b]indoleacetate (Trp-P-1 acetate)	68808-54-8	271.3	+	2.71	0.00125	0.0046	17	+	+	+
15	C	3-Amino-1-methyl-5H-pyrido{4,3-b]indoleacetate (Trp-P-2 acetate)	72254-58-1	257.3	+	2.57	0.05	0.019	17	+	+	+
16	C	*2-Amino-4-nitrophenol*	*99-57-0*	*154.1*	+	*1.54*	*0.015*	*0.1*	*17*	+	+	+
17	C	*2-Amino-5-nitrophenol*	*121-88-0*	*154.1*	+	*1.54*	*0.00375*	*0.024*	*17*	+	+	+
18	C	*4-Amino-2-nitrophenol*	*119-34-6*	*154.1*	+	*1.54*	*0.16*	*1.04*	*22*	+	+	[redacted]
19	C	*2-Amino-5-nitrothiazole*	*121-66-4*	*145.1*	+	*1.45*	*0.1*	*0.69*	*22*	+	+	+
20	C	Atrazine	1912-24-9	215.7	+	2.16	0.0184	0.085	24	+	+	+
21	C	Auramine O	2465-27-2	303.8	+	3.04	0.0064	0.02	25	+	+	+
22	C	5-Azacytidine	320-67-2	244.2	+	2.44	0.002	0.008	17	+	+	+
23	C	Azathioprine	446-86-6	277.3	+	2.77	0.023	0.083	17	+	+	+
24	C	*Benzaldehyde*	*100-52-7*	*106.1*	+	*1.06*	*5.00E-06*	*0.00005*	*17*	+	+	+
25	C	*Benzene*	*71-43-2*	*78.1*	+	*0.78*	*0.009*	*0.11*	*17*	+	+	+
26	C	*Benzidine*	*92-87-5*	*184.2*	+	*1.84*	*0.0025*	*0.014*	*17*	+	+	+
27	C	Benzidine 2HCl	531-85-1	257.2	+	2.57	0.003	0.12	19	+	+	+
28	C	Benzo[*a*]pyrene	50-32-8	252.3	+	2.52	0.005	0.02	17	+	+	+
29	C	*Benzyl chloride*	*100-44-7*	*126.6*	+	*1.27*	*0.015*	*0.12*	*17*	+	+	+
30	C	2-Biphenylamine HCl	2185-92-4	205.7	+	2.06	0.2	0.97	22	+	+	+
31	C	2,2-Bis(bromomethyl)-1,3-propanediol, technical grade	3296-90-0	261.9	+	2.62	0.8	3.05	18	+	+	[redacted]
32	C	*Bis(2-chloro-1-methylethyl)ether, technical grade*	*108-60-1*	*171.1*	+	*1.71*	*0.124*	*0.72*	*22*	+	+	+
33	C	Bis(2,3-dibromopropyl)phosphate, magnesium salt	36711-31-6	201.4	+	2.01	2	9	17	+	+	[redacted]
34	C	*Bromate, potassium*	*7758-01-2*	*167.0*	+	*1.67*	*0.0625*	*0.37*	*17*	+	+	+
35	C	*Bromodichloromethane*	*75-27-4*	*163.8*	+	*1.64*	*0.24*	*1.5*	*17*	+	+	[redacted]
36	C	*Butylated hydroxyanisole*	*25013-16-5*	*180.3*	+	*1.80*	*0.125*	*0.69*	*26*	+	+	+
37	C	*N-n-Butyl-N-nitrosourea*	*869-01-2*	*145.2*	+	*1.45*	*0.1*	*0.7*	*17*	+	+	+
38	C	*Cadomium cholride*	*10108-64-2*	*183.3*	+	*1.83*	*0.0055*	*0.03*	*17*	+	+	+
39	C	Cadomium sulphate	10124-36-4	208.5	+	2.09	0.02	0.1	17	+	+	+
40	C	*Calcium chromate*	*13765-19-0*	*156.0*	+	*1.56*	*0.00015*	*0.001*	*17*	+	+	+
41	C	Carbaryl	63-25-2	201.2	+	2.01	0.015	0.075	17	+	+	+
42	C	*Carboxymethylnitrosourea*	*60391-92-6*	*147.1*	+	*1.47*	*0.0625*	*0.42*	*17*	+	+	+
43	C	*Caffeic acid*	*331-39-5*	*180.2*	+	*1.80*	*0.26*	*1.4*	*17*	+	+	[redacted]
44	C	Captafol	2425-06-1	349.1	+	3.49	0.0035	0.01	17	+	+	+
45	C	Captan	133-06-2	300.6	+	3.00	0.007	0.023	17	+	+	+
46	C	*Chloral hydrate*	*302-17-0*	*165.4*	+	*1.65*	*0.6*	*3.63*	*27*	+	+	[redacted]
47	C	Chloramben	133-90-4	206.0	+	2.06	1.51	7.33	22	+	+	[redacted]
48	C	Chlorambucil	305-03-3	304.2	+	3.04	0.00025	0.0008	17	+	+	+
49	C	Chlorendic acid	115-28-6	388.8	+	3.89	1.95	5.015	28	+	+	[redacted]

50	C	*Chlorobenzene*	*108-90-7*	*112.6*	+	*1.13*	*0.15*	*1.33*	*19*	+	+		
51	C	Chlorodibromomethane	124-48-1	208.3	+	2.08	0.72	3.457	28	+	+		
52	C	*3-Chloro-2-methylpropene, technical grade*	*563-47-3*	*90.6*	+	*0.91*	*0.12*	*1.33*	*22*	+	+		
53	C	*3-(Chloromethyl)pyridine HCl*	*6959-48-4*	*164.0*	+	*1.64*	*0.05*	*0.30*	*22*	+	+	+	
54	C	*1-Chloro-4-nitrobenzene*	*100-00-5*	*157.6*	+	*1.58*	*0.6*	*3.81*	*22*	+	+		
55	C	*3-(p-Chlorophenyl)-1,1-dimethylurea*	*150-68-5*	*198.7*	+	*1.99*	*1.3*	*6.54*	*22*	+	+		
56	C	*4-Chloro-m-phenylenediamine*	*5131-60-2*	*142.6*	+	*1.43*	*0.525*	*3.68*	*20*	+	+		
57	C	*4-Chloro-o-phenylenediamine*	*95-83-0*	*142.6*	+	*1.43*	*0.0101*	*0.07*	*20*	+	+	+	
58	C	Chlorothalonil	1897-45-6	265.9	+	2.66	0.0005	0.002	22	+	+	+	
59	C	Chrysazin	81-55-0	330.2	+	3.30	0.005	0.02	22	+	+	+	
60	C	C.I. Acid orange 3	6373-74-6	452.4	+	4.52	0.0891	0.20	22	+	+	+	
61	C	C.I. Disperse blue 1	2475-45-8	268.3	+	2.68	0.0075	0.03	22	+	+	+	
62	C	C.I. Disperse orange 2 (1-amino-2-methyl-anthrquinone)	82-28-0	237.3	+	2.37	0.3	1.26	22	+	+		
63	C	Ciprofibrate	52214-84-3	289.2	+	2.89	0.0289	0.1	29	+	+	+	
64	C	Clofibrate	637-07-0	242.7	+	2.43	0.25	1	17	+	+	+	
65	C	*Coumarin*	*91-64-5*	*146.2*	+	*1.46*	*1.6*	*10.95*	*18*				
66	C	*m-Cresidine*	*102-50-1*	*137.2*	+	*1.37*	*0.5*	*3.64*	*22*	+	+		
67	C	*Cupferron*	*135-20-6*	*155.2*	+	*1.55*	*1.163*	*7.50*	*22*	+	+		
68	C	Cytembena	21739-91-3	307.1	+	3.07	0.0249	0.08	22	+	+	+	
69	C	Danthron	117-10-2	240.2	+	2.40	0.017	0.07	22	+	+	+	
70	C	*p,p'*-DDE	72-55-9	318.0	+	3.18	0.0088	0.028	17	+	+	+	
71	C	DDT	50-29-3	354.5	+	3.55	0.0081	0.023	17	+	+	+	
72	C	2,4-Diaminoanisole sulphate	39156-41-7	236.2	+	2.36	0.06	0.025	17	+	+	+	
73	C	*2,4-Diaminotoluene*	*95-80-7*	*122.2*	+	*1.22*	*0.0985*	*0.81*	*20*	+	+	+	
74	C	1,2-Dibromo-3-chloropropane	96-12-8	236.3	+	2.36	0.047	0.2	17	+	+	+	
75	C	*1,2-Dibromoethane*	*106-93-4*	*187.9*	+	*1.88*	*0.38*	*2*	*17*	+	+		
76	C	Dibromomannitol	488-41-5	308.0	+	3.08	0.15	0.49	22	+	+	+	
77	C	1,3-Dibutyl-1-nitrosourea	56654-52-5	201.3	+	2.01	0.0625	0.31	17	+	+	+	
78	C	*Dichloroacetic acid*	*79-43-6*	*128.9*	+	*1.29*	*1.25*	*9.69*	*27*	+	+		
79	C	*1,2-Dichloroethane*	*107-06-2*	*99.0*	+	*0.99*	*0.5*	*5.05*	*22*	+	+		
80	C	*Dichloromethane*	*75-09-2*	*84.9*	+	*0.85*	*0.0005*	*0.06*	*17*	+	+	+	
81	C	*2,6-Dichloro-p-phenylenediamine*	*609-20-1*	*177.0*	+	*1.77*	*0.25*	*1.41*	*22*	+	+		
82	C	*1,2-Dichloropropane*	*78-87-5*	*113.0*	+	*1.13*	*0.66*	*5.84*	*22*	+	+		
83	C	Dichlorvos	62-73-7	221.0	+	2.21	0.01	0.045	17	+	+	+	
84	C	Dieldrin	60-57-1	380.9	+	3.81	0.001	0.003	17	+	+	+	
85	C	Diethylstilbestrol	56-53-1	268.4	+	2.68	0.0001	0.00037	17	+	+	+	
86	C	Diglycidyl resorcinol ether, technical grade	101-90-6	222.2	+	2.22	0.0005	0.002	22	+	+	+	
87	C	*Dimethoxane*	*828-00-2*	*174.2*	+	*1.74*	*0.0126*	*0.07*	*22*	+	+	+	
88	C	3,3'-Dimethoxybenzidine-4,4'-diisocyante	91-93-0	296.3	+	2.96	0.093	0.31	22	+	+	+	
89	C	*N,N*-Dimethyl-4-aminoazobenzene	60-11-7	225.3	+	2.25	0.025	0.11	17	+	+	+	
90	C	*N,N-Dimethylaniline*	*121-69-7*	*121.2*	+	*1.21*	*0.083*	*0.69*	*19*	+	+	+	
91	C	7,12-Dimethylbenz[*a*]anthracene	57-97-6	256.4	+	2.56	0.001	0.0039	17	+	+	+	
92	C	3,3'-Dimethylbenzidine	119-93-7	212.3	+	2.12	0.46	2.17	22	+	+		
93	C	3,3'-Dimethylbenzidine 2HCl	612-82-8	285.2	+	2.85	0.005	0.02	22	+	+	+	
94	C	*Dimethylcarbamoyl chloride*	*79-44-7*	*107.5*	+	*1.08*	*0.02*	*0.185*	*17*	+	+	+	
95	C	*Dimethyl hydrogen phosphite*	*868-85-9*	*110.0*	+	*1.10*	*1.6*	*14.54*	*22*				
96	C	*Epichlorhydrin*	*106-89-8*	*92.5*	+	*0.93*	*0.005*	*0.054*	*17*	+	+	+	
97	C	*1,2-Epoxybutane*	*106-88-7*	*92.5*	+	*0.93*	*0.05*	*0.54*	*22*	+	+	+	
98	C	*Ethionamide*	*536-33-4*	*166.2*	+	*1.66*	*0.4*	*2.4*	*17*	+	+		
99	C	*Ethyl acrylate*	*140-88-5*	*100.1*	+	*1.00*	*0.011*	*0.11*	*17*	+	+	+	
100	C	*Ethylene oxide*	*75-21-8*	*44.1*	+	*0.44*	*0.22*	*5*	*17*	+	+		

Table 1 (*Continued*)

CGX ID	C/NC	Chemical name	CAS no.	MW	CA (original call)	Equiv. to 10 mM (mg/mL)	LEC (mg/mL)	LEC (mM)	Ref.	1997-OECD[a]	r-OECD[b]	ICH[c]
										CA	CA	CA
101	C	*Ethyl methanesulphonate*	*62-50-0*	*124.2*	+	*1.24*	*3.00E-06*	*0.000024*	*17*	+	+	+
102	C	*N-Ethyl-N'-nitro-N-nitrosoguanidine*	*63885-23-4*	*161.1*	+	*1.61*	*0.0025*	*0.016*	*17*	+	+	+
103	C	*1-Ethyl-1-nitrosourea*	*759-73-9*	*117.1*	+	*1.17*	*0.0117*	*0.1*	*17*	+	+	+
104	C	*5-Fluorouracil*	*51-21-8*	*130.1*	+	*1.30*	*0.001*	*0.008*	*17*	+	+	+
105	C	*Formaldehyde*	*50-00-0*	*30.0*	+	*0.30*	*0.006*	*0.2*	*17*	+	+	+
106	C	Fumonisin B1	116355-83-0	721.8	+	7.22	0.001	0.0014	30	+	+	+
107	C	*Furan*	*110-00-9*	*68.1*	+	*0.68*	*0.16*	*2.35*	*22*	+	+	[redacted]
108	C	*Furfural*	*98-01-1*	*96.1*	+	*0.96*	*0.2*	*2.08*	*22*	+	+	[redacted]
109	C	Furosemide	54-31-9	330.7	+	3.31	2	6	17	+	+	[redacted]
110	C	Furylfuramide (AF-2)	3688-53-7	248.2	+	2.48	0.005	0.02	17	+	+	+
111	C	*Glycidol*	*556-52-5*	*74.1*	+	*0.74*	*0.03*	*0.4*	*17*	+	+	+
112	C	Griseofulvin	126-07-8	352.8	+	3.53	0.04	0.11	17	+	+	+
113	C	Haloperidol	52-86-8	375.9	+	3.76	0.01	0.026	31	+	+	+
114	C	HC Blue 1 (impure and purified)	2784-94-3	255.3	+	2.55	0.96	3.76	20	+	+	[redacted]
115	C	Heptachlor	76-44-8	373.3	+	3.73	0.025	0.07	22	+	+	+
116	C	*Hexanamide*	*628-02-4*	*115.2*	+	*1.15*	*4*	*34.73*	*22*	[redacted]		
117	C	*Hydrazine sulphate*	*10034-93-2*	*130.1*	+	*1.30*	*0.158*	*1.2*	*17*	+	+	[redacted]
118	C	*Hydrazobenzene*	*122-66-7*	*184.2*	+	*1.84*	*0.0014*	*0.01*	*22*	+	+	+
119	C	*Hydrogen peroxide*	*7722-84-1*	*34.0*	+	*0.34*	*0.00034*	*0.01*	*17*	+	+	+
120	C	N-Hydroxy-2-acetylaminofluorene	53-95-2	239.3	+	2.39	0.001	0.0042	17	+	+	+
121	C	*Isobutyl nitrite*	*542-56-3*	*103.1*	+	*1.03*	*0.051*	*0.49*	*22*	+	+	+
122	C	*Isoniazid*	*54-85-3*	*137.1*	+	*1.37*	*0.44*	*3.2*	*17*	+	+	[redacted]
123	C	*Isophorone*	*78-59-1*	*138.2*	+	*1.38*	*1.25*	*9.044*	*28*	+	+	[redacted]
124	C	Lasiocarpine	303-34-4	411.5	+	4.12	0.206	0.5	17	+	+	+
125	C	Lead acetate	301-04-2	325.3	+	3.25	0.0033	0.01	17	+	+	+
126	C	Manganese ethylenebisthiocarbamate	12427-38-2	265.3	+	2.65	0.015	0.057	17	+	+	+
127	C	Melphalan	148-82-3	305.2	+	3.05	0.0001	0.0033	17	+	+	+
128	C	*2-Mercaptobenzothiazole*	*149-30-4*	*167.2*	+	*1.67*	*0.374*	*2.23*	*22*	+	+	[redacted]
129	C	Methapyrilene hydrochloride	135-23-9	297.8	+	2.98	0.747	2.51	22	+	+	[redacted]
130	C	*Methimazole*	*60-56-0*	*114.2*	+	*1.14*	*0.37*	*3.2*	*17*	+	+	[redacted]
131	C	*4-Methoxyphenol*	*150-76-5*	*124.1*	+	*1.24*	*0.031*	*0.25*	*32*	+	+	+
132	C	8-Methoxypsoralen	298-81-7	216.2	+	2.16	0.1	0.46	22	+	+	+
133	C	*Methylazoxymethanol acetate*	*592-62-1*	*132.1*	+	*1.32*	*0.00013*	*0.001*	*17*	+	+	+
134	C	*alpha-Methylbenzyl alcohol*	*98-85-1*	*122.2*	+	*1.22*	*1*	*8.19*	*22*	+	+	[redacted]
135	C	3-Methylcholanthrene	56-49-5	268.3	+	2.68	0.002	0.0075	17	+	+	+
136	C	3'-Methyl-4-dimethylaminoazobenzene	55-80-1	239.3	+	2.39	0.05	0.21	17	+	+	+
137	C	4,4'-Methylenedianiline 2HCl	13552-44-8	271.2	+	2.71	0.8	2.95	22	+	+	[redacted]
138	C	*Methyl methanesulphonate*	*66-27-3*	*110.1*	+	*1.10*	*3.00E-06*	*0.000027*	*17*	+	+	+
139	C	2-Methyl-1-nitroanthraquinone	129-15-7	267.2	+	2.67	0.005	0.02	22	+	+	+
140	C	*N-Methyl-N'-nitro-N-nitrosoguanidine*	*70-25-7*	*147.1*	+	*1.47*	*3.00E-06*	*0.00002*	*17*	+	+	+
141	C	*Methylnitrosocyanamide*	*33868-17-6*	*85.1*	+	*0.85*	*0.00085*	*0.01*	*17*	+	+	+
142	C	*N-Methylolacrylamide*	*924-42-5*	*101.1*	+	*1.01*	*0.25*	*2.47*	*22*	+	+	[redacted]
143	C	Methylphenidate HCl	298-59-9	267.0	+	2.67	1	3.71	18	+	+	[redacted]
144	C	*Metronidazole*	*443-48-1*	*171.2*	+	*1.71*	*0.0001*	*0.0006*	*33*	+	+	+
145	C	Mitomycin C	50-07-7	334.3	+	3.34	0.00017	0.00005	17	+	+	+
146	C	Monocrotaline	315-22-0	325.4	+	3.25	0.065	0.2	17	+	+	+
147	C	Nafenopin	3771-19-5	310.4	+	3.10	0.0093	0.03	29	+	+	+
148	C	*Naphthalene*	*91-20-3*	*128.2*	+	1.28	*0.03*	*0.23*	*22*	+	+	+
149	C	*1,5-Naphthalenediamine*	*2243-62-1*	*158.2*	+	1.58	*0.001*	*0.01*	*22*	+	+	+
150	C	*2-Naphthylamine*	*91-59-8*	*143.2*	+	1.43	*0.00333*	*0.023*	*17*	+	+	+
151	C	*Nitrite sodium*	*7632-00-0*	*69.0*	+	0.69	*4*	*58.0*	*17*	[redacted]		
152	C	*o-Nitroanisole*	*91-23-6*	*153.1*	+	1.53	*1.06*	*6.92*	*18*	+	+	[redacted]

153	C	*Nitrobenzene*	*98-95-3*	*123.1*	+	1.23	*6.15*	*50*	*34*			
154	C	*6-Nitrobenzimidazole*	*94-52-0*	*163.1*	+	1.63	*0.5*	*3.06*	*22*	+	+	
155	C	*p-Nitrobenzoic acid*	*62-23-7*	*167.1*	+	1.67	*0.875*	*5.24*	*22*	+	+	
156	C	*5-Nitro-2-furaldehyde semicarbazone*	*59-87-0*	*198.1*	+	1.98	*0.023*	*0.12*	*22*	+	+	+
157	C	1-[(5-nitrofurfurylidene)amino]hydantoin	67-20-9	238.2	+	2.38	0.747	3.14	22	+	+	
158	C	*Nitrogen mustard*	*51-75-2*	*156.1*	+	*1.56*	*0.00002*	*0.0001*	*17*	+	+	+
159	C	*2-Nitro-p-phenylenediamine*	*5307-14-2*	*153.1*	+	*1.53*	*0.3*	*1.96*	*22*	+	+	
160	C	1-Nitropyrene	5522-43-0	247.2	+	2.47	0.1	0.404	35	+	+	+
161	C	*4-Nitroquinoline-N-oxide*	*56-57-5*	*190.2*	+	*1.90*	*0.00002*	*0.00011*	*17*	+	+	+
162	C	*p-Nitrosodiphenylamine*	*156-10-5*	*198.2*	+	*1.98*	*0.00025*	*0.0013*	*22*	+	+	+
163	C	*N-Nitrosodiethylamine (diethylnitrosamine)*	*55-18-5*	*102.1*	+	*1.02*	*3*	*29*	*17*			
164	C	*N-Nitrosodimethylamine (dimethylnitrosamine)*	*62-75-9*	*74.1*	+	*0.74*	*0.5*	*6.7*	*17*	+	+	
165	C	*N-Nitroso-N-methylurea*	*684-93-5*	*103.1*	+	*1.03*	*0.01*	*0.1*	*17*	+	+	+
166	C	*5-Nitro-o-toluidine*	*99-55-8*	*152.2*	+	*1.52*	*0.5*	*3.29*	*22*	+	+	
167	C	4,4'-Oxydianiline	101-80-4	200.2	+	2.00	0.1	0.50	22	+	+	+
168	C	*N*-Oxydiethylene thiocarbamyl-*N*-oxydiethylene sulphenamide	13752-51-7	248.4	+	2.48	0.005	0.02	17	+	+	+
169	C	Pentachloroethane	76-01-7	202.3	+	2.02	0.008	0.395	28	+	+	+
170	C	Pentachloronitrobenzene	82-68-8	295.3	+	2.95	0.0024	0.01	22	+	+	+
171	C	Petasitenine	60102-37-6	381.4	+	3.81	1.91	5	17	+	+	
172	C	*Phenacetin*	*62-44-2*	*179.2*	+	*1.79*	*0.4*	*2.2*	*17*	+	+	
173	C	Phenazopyridine HCl	136-40-3	249.7	+	2.50	0.105	0.42	22	+	+	+
174	C	Phenobarbital	50-06-6	232.2	+	2.32	0.1	0.43	17	+	+	+
175	C	Phenolphthalein	28-37-6	318.3	+	3.18	0.05	0.16	22	+	+	+
176	C	Phenoxybenzamine HCl	63-92-3	340.3	+	3.40	0.03	0.09	22	+	+	+
177	C	Phenylbutazone	50-33-9	308.4	+	3.08	1.6	5.19	18	+	+	
178	C	*o-Phenylphenol*	*90-43-7*	*170.2*	+	*1.70*	*0.1*	*0.59*	*25*	+	+	+
179	C	*Propane sultone*	*1120-71-4*	*122.1*	+	*1.22*	*0.012*	*0.1*	*17*	+	+	+
180	C	*beta-Propiolactone*	*57-57-8*	*72.1*	+	*0.72*	*0.03*	*0.42*	*17*	+	+	+
181	C	*1,2-Propylene oxide*	*75-56-9*	*58.1*	+	*0.58*	*0.5*	*8.61*	*22*	+	+	
182	C	*N-Propyl-N'-nitro-N-nitrosoguanidine*	*13010-07-6*	*175.2*	+	*1.75*	*0.01*	*0.057*	*17*	+	+	+
183	C	Pyrimethamine	58-14-0	248.7	+	2.49	0.05	0.201	36	+	+	+
184	C	Quercetin	117-39-5	302.2	+	3.02	0.006	0.02	17	+	+	+
185	C	*p-Quinone dioxime*	*105-11-3*	*138.1*	+	*1.38*	*0.01*	*0.07*	*22*	+	+	+
186	C	Retinol acetate	127-47-9	328.5	+	3.29	0.0656	0.2	23	+	+	+
187	C	Saccharin, sodium	128-44-9	205.2	+	2.05	8	39	17			
188	C	*Safrole*	*94-59-7*	*162.2*	+	*1.62*	*0.0833*	*0.5*	*17*	+	+	+
189	C	*Selenium sulphide*	*7446-34-6*	*111.0*	+	*1.11*	*0.0005*	*0.0045*	*20*	+	+	+
190	C	Sodium dichromate	10588-01-9	262.0	+	2.62	0.0001	0.0019	17	+	+	+
191	C	*Styrene*	*100-42-5*	*104.2*	+	*1.04*	*0.25*	*2.4*	*17*	+	+	
192	C	*Styrene oxide*	*96-09-3*	*120.2*	+	*1.20*	*0.00375*	*0.031*	*17*	+	+	+
193	C	*1,1,1,2-Tetrachloroethane*	*630-20-6*	*167.8*	+	*1.68*	*0.1*	*0.596*	*28*	+	+	+
194	C	12-O-tetradecanoylphorbol 13-acetate	16561-29-8	616.8	+	6.17	6.20E-06	0.00001	17	+	+	+
195	C	*Tertanitromethane*	*509-14-8*	*196.0*	+	*1.96*	*0.02*	*0.10*	*22*	+	+	+
196	C	4,4'-Thiodianiline	139-65-1	216.3	+	2.16	0.1	0.46	22	+	+	+
197	C	*Thio-tepa*	*52-24-4*	*189.2*	+	*1.89*	*0.00094*	*0.0049*	*17*	+	+	+
198	C	*o-Toluidine*	*95-53-4*	*107.2*	+	*1.07*	*0.012*	*0.13*	*17*	+	+	+
199	C	Trenimon	68-76-8	231.3	+	2.31	1.00E-08	4.3E-08	17	+	+	+
200	C	Triamterene	396-01-0	253.3	+	2.53	0.00375	0.015	17	+	+	+
201	C	Tribromomethane	75-25-2	252.7	+	2.53	0.116	0.46	17	+	+	+
202	C	*1,1,2-Trichloroethane*	*79-00-5*	*133.4*	+	*1.33*	*0.377*	*2.83*	*22*	+	+	
203	C	*N*-(Trichloromethylthio)phthalimide	133-07-3	296.6	+	3.00	0.005	0.017	37	+	+	+
204	C	*1,2,3-Trichloropropane*	*96-18-4*	*147.4*	+	*1.47*	*0.0595*	*0.40*	*22*	+	+	+

Table 1 (*Continued*)

CGX ID	C/NC	Chemical name	CAS no.	MW	CA (original call)	Equiv. to 10 mM (mg/mL)	LEC (mg/mL)	LEC (mM)	Ref.	1997-OECD[a]	r-OECD[b]	ICH[c]
										CA	CA	CA
205	*C*	*2,4,5-Trimethylaniline*	*137-17-7*	*135.2*	+	*1.35*	*0.415*	*3.07*	*20*	+	+	
206	*C*	*Trimethylphosphate*	*512-56-1*	*140.1*	+	*1.40*	*3*	*21.42*	*22*			
207	C	Tris(2,3-dibromopropyl)phosphate	126-72-7	697.9	+	6.98	0.125	0.18	17	+	+	+
208	*C*	*Urethane*	*51-79-6*	*89.1*	+	*0.89*	*8*	*90*	*17*			
209	C	Zearalenone	17924-92-4	318.4	+	3.18	0.015	0.05	22	+	+	+
210	C	Zinc dimethyldithioearbamate (Ziram)	137-30-4	305.8	+	3.06	0.000025	0.00008	22	+	+	+
211	NC	Acetohexamide	968-81-0	324.4	+	3.24	2	6	17	+	+	
212	*NC*	*o-Anthranilic acid*	*118-92-3*	*137.1*	+	*1.37*	*4*	*29.2*	*22*			
213	*NC*	*Benzoate, sodium*	*532-32-1*	*144.1*	+	*1.44*	*0.29*	*2*	*17*	+	+	
214	NC	Benzoin	119-53-9	212.2	+	2.12	0.02	0.1	17	+	+	+
215	*NC*	*1H-Benzotriazole*	*95-14-7*	*119.1*	+	*1.19*	*1.257*	*10.55*	*22*			
216	*NC*	*Benzyl alcohol*	*100-51-6*	*108.1*	+	*1.08*	*4*	*36.99*	*22*			
217	*NC*	*Caffeine*	*58-08-2*	*194.2*	+	*1.94*	*0.08*	*0.4*	*17*	+	+	+
218	NC	Carbromal	77-65-6	237.1	+	2.37	1	4.22	22	+	+	
219	NC	4-(Chloroacetyl)-acetanilide	140-49-8	211.6	+	2.12	0.0025	0.01	22	+	+	+
220	*NC*	*p-Chloroaniline*	*106-47-8*	*127.6*	+	*1.28*	*0.5*	*3.92*	*22*	+	+	
221	*NC*	*o-Chlorobenzalmalonitrile*	*2698-41-1*	*188.6*	+	*1.89*	*0.006*	*0.03*	*22*	+	+	+
222	*NC*	*2-(Chloromethyl)pyridine HCl*	*6959-47-3*	*164.0*	+	*1.64*	*0.0302*	*0.18*	*22*	+	+	+
223	NC	Chlorpheniramine maleate	113-92-8	390.9	+	3.91	0.5	1.28	22	+	+	
224	NC	Chlorpropamide	94-20-2	276.7	+	2.77	1	3.6	17	+	+	
225	NC	C.I. acid orange 10	1936-15-8	452.4	+	4.52	1.25	2.76	22	+	+	
226	NC	Diallyl phthalate	131-17-9	246.3	+	2.46	0.2	0.81	22	+	+	+
227	NC	2,5-Diaminotoluen sulphate	6369-59-1	220.3	+	2.20	0.04	0.18	22	+	+	+
228	*NC*	*2,6-Diaminotoluene 2HCl*	*15481-70-6*	*195.1*	+	*1.95*	*1*	*5.13*	*22*	+	+	
229	NC	Diazinon	333-41-5	304.4	+	3.04	0.1	0.32	17	+	+	+
230	*NC*	*2,4-Dichlorophenol*	*120-83-2*	*163.0*	+	*1.63*	*0.0978*	*0.6*	*38*	+	+	+
231	NC	Dimethoate	60-51-5	229.2	+	2.29	0.5	2.2	17	+	+	
232	*NC*	*Dimethoxane, commercial grade*	*828-00-2*	*174.2*	+	*1.74*	*0.0126*	*0.07*	*22*	+	+	+
233	*NC*	*2,4-Dimethoxyaniline HCl*	*54150-69-5*	*189.6*	+	*1.90*	*0.5*	*2.64*	*22*	+	+	
234	NC	Diphenhydramine HCl	147-24-0	291.8	+	2.92	0.1	0.34	22	+	+	+
235	NC	Diphenyl-*p*-phenylenediamine	74-31-7	260.3	+	2.60	0.001	0.0038	39	+	+	+
236	NC	Ethyl tellurac	20941-65-5	720.7	+	7.21	0.000032	0.00004	22	+	+	+
237	*NC*	*Eugenol*	*97-53-0*	*164.2*	+	*1.64*	*0.125*	*0.76*	*18*	+	+	+
238	NC	FD & C red no. 3 (MW as anhydrous)	16423-68-0	879.9	+	8.80	0.6	0.68	17	+	+	
239	NC	FD & C yellow no. 5 [AKA tartrazine]	1934-21-0	534.4	+	5.34	2	3.7	17	+	+	
240	NC	Fenthion	55-38-9	278.3	+	2.78	0.0015	0.005	40	+	+	+
241	NC	Fenvalerate	51630-58-1	419.9	+	4.20	0.01	0.024	41	+	+	+
242	*NC*	*Fluoride sodium*	*7681-49-4*	*42.0*	+	*0.42*	*0.02*	*0.48*	*17*	+	+	+
243	NC	Hexachlorocyclopentadiene	77-47-4	272.8	+	2.73	0.0075	0.03	22	+	+	+
244	*NC*	*8-Hydroxyquinoline*	*148-24-3*	*145.2*	+	*1.45*	*0.0058*	*0.04*	*42*	+	+	+
245	NC	4,4'-Isopropylidenediphenol	80-05-7	228.3	+	2.28	0.0912	0.4	43	+	+	+
246	NC	Lead dimethyldithiocarbamate	19010-66-3	447.6	+	4.48	0.000025	0.000056	18	+	+	+
247	NC	Lithocholic acid	434-13-9	376.6	+	3.77	0.56	1.5	17	+	+	
248	NC	Malathion	121-75-5	330.4	+	3.30	<0.303	<0.92	18	+	+	+
249	*NC*	*Manganese(II) sulfate monohydrate*	*10034-96-5*	*169.0*	+	*1.69*	*0.18*	*1.065*	*18*	+	+	
250	NC	Methotrexate	59-05-2	454.4	+	4.54	0.001	0.0022	17	+	+	+
251	*NC*	*Methyl methacrylate*	*80-62-6*	*100.1*	+	*1.00*	*1.6*	*15.98*	*44*			
252	NC	N-(1-Naphthyl)ethylenediamine 2HCl	1465-25-4	259.2	+	2.59	0.2	0.77	45	+	+	+
253	*NC*	*p-Nitroaniline*	*100-01-6*	*138.1*	+	*1.38*	*1.6*	*11.58*	*18*			
254	*NC*	*4-Nitroanthranilic acid*	*619-17-0*	*182.1*	+	*1.82*	*2.2*	*12.08*	*22*			
255	*NC*	*1-Nitronaphthalene*	*86-57-7*	*173.2*	+	*1.73*	*0.016*	*0.09*	*45*	+	+	+

256	NC	Penicillin VK	132-98-9	388.5	+	3.89	1.25	3.2	46	+	+	
257	NC	*Phenol*	*108-95-2*	*94.1*	+	*0.94*	2	*21.25*	22			
258	NC	*p-Phenylenediamine 2HCl*	*624-18-0*	*181.1*	+	*1.81*	*0.016*	*0.09*	45	+	+	+
259	NC	*1-Phenyl-2-thiourea*	*103-85-5*	*152.2*	+	*1.52*	3	*19.71*	22			
260	NC	*Phthalic anhydride*	*85-44-9*	*148.1*	+	*1.48*	*1.48*	*10*	38	+	+	
261	NC	*Resorcinol*	*108-46-3*	*110.1*	+	*1.10*	4	*36.33*	22			
262	NC	*Sodium chlorite*	*7758-19-2*	*90.4*	+	*0.90*	*0.02*	*0.22*	17	+	+	+
263	NC	Tetracycline HCl	64-75-5	480.9	+	4.81	0.01	0.02	17	+	+	+
264	NC	Tetraethylthiuram disulfide	97-77-8	296.5	+	2.97	5.00E-06	0.00002	22	+	+	+
265	NC	*Tetrakis(hydoxymethyl)phosphonium chloride*	*124-64-1*	*190.6*	+	*1.91*	*0.03*	*0.16*	22	+	+	+
266	NC	Tetrakis(hydoxymethyl)phosphonium sulphate	55566-30-8	251.2	+	2.51	0.005	0.02	44	+	+	+
267	NC	*Tin(II) chloride*	*7772-99-8*	*189.6*	+	*1.90*	*0.025*	*0.13*	22	+	+	+

C, Carcinogen; NC, Non-carcinogen; MW, Molecular weight; CA, Chromosomal aberration test; LEC, Lowest effective concentration; Equivalent to 10 mM means the equal concentration of weight per volume (mg/mL) to 10 mM.
+, Positive; –, Negative.
(+) shows positive after the appication of the r-OECD TGfor the chemicals MW less than 200 (n = 46).
Italics means chemicals MW less than 200 (n = 142).
Highiliht to the negative result by the re-evaluation.
[a] Current OECD test guideline adopted in 1997 (10 mM or 5 mg/mL whichever is lower).
[b] Draft revised OECD test guideline (10 mM or 2 mg/mL whichever is lower).
[c] ICH S2(R1) guideline (1 mM or 0.5 mg/mL whichever is lower).

approach, because there were no carcinogenicity data for nearly all the 124-CA positives from the JEC database. This approach consisted of the identification of effects from extreme culture conditions (*e.g.*, low pH, precipitation, cytotoxicity) and a review of the literature (*e.g.*, *in vivo* genotoxicity and carcinogenicity for the chemical, and for closely related chemicals). The level of concern for 'different' chemicals – to be used in human health-risk assessment – was defined and based on previously described analyses [9]. The general criteria were as follows: (1) negligible concern, negative result(s) in the *in vivo* genotoxicity or carcinogenicity test, clear evidence(s) of non-relevance (*e.g.*, extreme culture condition) for CA-induction and/or mode of action of the non-DNA target; (2) minimal concern, some evidence(s) of non-relevance of CA-induction or of an increasing level of negligible concern or negative result(s) in the *in vivo* genotoxicity tests with some limitations; (3) some concern, positive result(s) in the Ames test with negative result(s) or no data in the *in vivo* genotoxicity test, positive result(s) in the *in vivo* genotoxicity or carcinogenicity test in related chemicals or no supporting evidence(s) for reducing the level of concern; and (4) real concern, positive result(s) in the Ames or *in vivo* genotoxicity tests, or when mentioned in the list of IARC carcinogens in Group 2B or higher.

2.6. Distribution of the MWs of the chemicals

The distribution of the MWs of the 267 CA-positives from the CGX database and 124 CA-positives from the JEC database was investigated.

3. Results

3.1. Sensitivity and specificity analyses

Results from the re-evaluation of 267 CA-positive chemicals (210 carcinogens and 57 non-carcinogens) from the CGX database are shown in Table 1. The results of the sensitivity and specificity analyses on the 435 chemicals, including the 168 CA-negatives from the CGX database are shown in Table 3. In addition, 267 CA-positives in the original call of the CGX database included 19 positive chemicals (10 carcinogens, *i.e.*, CGX IDs 5, 65, 95, 116, 151, 153, 164, 187, 206, 208; and nine non-carcinogens, *i.e.*, CGX IDs 212, 215, 216, 251, 253, 254, 256, 259, 260) at more than 10 mM. The IARC Group-2A agents (probable carcinogens), acrylamide (CGX ID5), *N*-nitrosodiethylamine (CGX ID163) and urethane (CGX ID208) were also included in these 10 carcinogens. The number of CA-positive chemicals was reduced to 248, 248 or 176 from the 267 chemicals in the original call when the 1997-OECD, r-OECD or ICH TG was applied, respectively. Because these chemicals were considered negative, the number of CA-negative chemicals increased to 187, 187 or 259 from 168 in the original call by the application of the 1997-OECD, r-OECD or ICH TG, respectively. The sensitivity and specificity against carcinogenicity based on the re-evaluation for the 435 chemicals from the CGX database are shown in Table 3. The sensitivity was reduced to 63.1%, 63.1% or 45.4% from 66.2% based on the original call, and the specificity had increased to 59.3%, 59.3% or 72.9% from 51.7% based on the original call, by the application of the 1997-OECD, r-OECD or ICH TG, respectively. The application of the r-OECD TG did not affect the sensitivity and specificity of the application of the 1997-OECD TG. However, the application of the ICH TG reduced sensitivity and increased specificity by approximately 15%.

3.2. Analysis of the alteration of the number of CA-positives

The results of the re-evaluation of 124 CA-positives from the JEC database are shown in Table 2. Because the 124 CA-positives by the original call in the JEC database included six positive chemicals (*i.e.*, JEC IDs 2, 11, 87, 99, 106, 111) at more than 10 mM, 118 chemicals were considered positive under the 1997-OECD TG. Alterations in the number of positive chemicals are presented in Table 4. Application of r-OECD TG showed a small reduction in the number of CA-positives (113 out of 124 chemicals by 1997-OECD TG), but ICH TG reduced this number to approximately half (60 out of 124 chemicals). Moreover, the number of CA-positive chemicals decreased remarkably upon application of the ICH TG.

Table 2
Re-evaluation of chromosomal aberration test results on the 124 CA-positive chemicals from the JEC database, based on the different top-concentration limits in several test guidelines.

JEC ID	Chemical name	CAS No.	MW	CA (original call)	Equiv. to 10 mM (mg/mL)	LEC (mg/mL)	LEC (mM)	Ref.	1997-OECD[a]	r-OECD[b]	ICH[c]
									CA	CA	CA
1	*Acenaphthene*	*83-32-9*	*154.2*	+	*1.54*	*0.2*	*1.3*	*47*	+	+	
2	*o-Acetoacetotoluidine*	*93-68-5*	*191.2*	+	*1.91*	*2.5*	*13.1*	*47*			
3	*3-Aminobenzenesulfonic acid*	*121-47-1*	*173.2*	+	*1.73*	*0.4*	*2.3*	*47*	+	+	
4	2-Amino-5-chloro-4-methylbenzenesulfonic acid	88-53-9	221.5	+	2.22	2.0	9.0	*47*	+	+	
5	*N-(Aminoethyl)ethanolamine*	*111-41-1*	*104.2*	+	*1.04*	*1.0*	*9.6*	*47*	+	+	
6	*2-Amino-5-methylbenzenesulfonic acid*	*88-44-8*	*187.2*	+	*1.87*	*1.0*	*5.1*	*47*	+	+	
7	2-Amino-1-naphthalenesulfonic acid	81-16-3	223.3	+	2.23	1.1	4.9	*47*	+	+	
8	*3-Aminophenol*	*591-27-5*	*109.1*	+	*1.09*	*0.03*	*0.3*	*47*	+	+	+
9	*4-Aminophenol*	*123-30-8*	*109.1*	+	*1.09*	*0.003*	*0.03*	*47*	+	+	+
10	*Azodicarbonamide*	*123-77-3*	*116.1*	+	*1.16*	*0.9*	*7.8*	*47*	+	+	
11	*Benzyltrimethylammonium chloride*	*56-93-9*	*185.7*	+	*1.86*	*1.9*	*10.2*	*47*			
12	*4,4'-Biphenyldiol*	*92-88-6*	*186.2*	+	*1.86*	*0.03*	*0.2*	*47*	+	+	+
13	*1,3-Bis(aminomethyl)cyclohexane (mixtures of cis-, trans-)*	*2579-20-6*	*142.3*	+	*1.42*	*0.4*	*2.8*	*47*	+	+	
14	*1,2-Bis(2-chloroethoxy)ethane*	*112-26-5*	*187.1*	+	*1.87*	*0.06*	*0.3*	*47*	+	+	+
15	Bis(1-methylethyl)naphthalene	38640-62-9	212.3	+	2.12	0.14	0.7	*47*	+	+	+
16	1,3-Bis(2-methylphenyl)guanidine	97-39-2	239.3	+	2.39	0.6	2.5	*47*	+	+	
17	*1-Bromo-3-chloropropane*	*109-70-6*	*157.4*	+	*1.57*	*0.3*	*1.6*	*47*	+	+	
18	N-*tert*-Butyl-2-benzothiazolesulfenamide	95-31-8	238.4	+	2.38	0.2	0.8	*47*	+	+	+
19	*tert-Butyl-methacrylate*	*585-07-9*	*142.2*	+	*1.42*	*0.4*	*2.8*	*47*	+	+	
20	*o-sec-Butylphenol*	*89-72-5*	*150.2*	+	*1.50*	*0.02*	*0.1*	*47*	+	+	+
21	*6-tert-Butyl-m-cresol*	*88-60-8*	*164.3*	+	*1.64*	*0.01*	*0.06*	*47*	+	+	+
22	*2-tert-Butylphenol*	*88-18-6*	*150.2*	+	*1.50*	*0.01*	*0.07*	*47*	+	+	+
23	*p-tert-Butylphenol*	*98-54-4*	*150.2*	+	*1.50*	*0.03*	*0.2*	*47*	+	+	+
24	Cadmium nitrate tetrahydrate	10022-68-1	308.5	+	3.09	0.01	0.02	*47*	+	+	+
25	*1-Chloro-2-(chloromethyl)benzene*	*611-19-8*	*161.0*	+	*1.61*	*0.1*	*0.6*	*47*	+	+	+
26	*4-Chloro-o-cresol*	*1570-64-5*	*142.6*	+	*1.43*	*0.1*	*0.7*	*47*	+	+	+
27	Chloropentabromocyclohexane	87-84-3	513.1	+	5.13	0.03	0.06	*47*	+	+	+
28	*2-Chlorophenol*	*95-57-8*	*128.6*	+	*1.29*	*0.3*	*2.3*	*47*	+	+	
29	*4-Chlorophenol*	*106-48-9*	*128.6*	+	*1.29*	*0.05*	*0.4*	*47*	+	+	+
30	Chromic acid disodium salt dihydrate	7789-12-0	297.8	+	2.98	0.001	0.003	*47*	+	+	+
31	C.I. Fluorescent brightner 271	41267-43-0	1347.1	+	13.47	5.0	3.7	*47*	+		
32	*2,4-Diamino-6-phenyl-s-triazine*	*91-76-9*	*187.2*	+	*1.87*	*0.08*	*0.4*	*47*	+	+	+
33	1,4-Dibromobenzene	106-37-6	235.9	+	2.36	0.6	2.5	*47*	+	+	
34	1,3-Dibromopropane	109-64-8	201.9	+	2.02	0.06	0.3	*47*	+	+	+
35	Dibutyl adipate	105-99-7	258.4	+	2.58	0.7	2.5	*47*	+	+	
36	2-(Di-n-butylamino)ethanol	*102-81-8*	*173.3*	+	*1.73*	*0.3*	*1.7*	*47*	+	+	
37	2,6-Di-tert-butyl-4-ethylphenol	4130-42-1	234.4	+	2.34	0.045	0.19	*47*	+	+	+
38	2,4-Di-*tert*-butylphenol	96-76-4	206.3	+	2.06	0.01	0.05	*47*	+	+	+
39	*o-Dichlorobenzene*	*95-50-1*	*147.0*	+	*1.47*	*0.2*	*1.4*	*47*	+	+	
40	*3,4-Dichloro-1-butene*	*760-23-6*	*125.0*	+	*1.25*	*0.01*	*0.08*	*47*	+	+	+
41	*1,2-Dichloro-3-nitrobenzene*	*3209-22-1*	*192.0*	+	*1.92*	*0.1*	*0.6*	*47*	+	+	+
42	*1,4-Dichloro-2-nitrobenzene*	*89-61-2*	*192.0*	+	*1.92*	*0.15*	*0.8*	*47*	+	+	+
43	*α,4-Dichlorotoluene*	*104-83-6*	*161.0*	+	*1.61*	*0.0125*	*0.08*	*47*	+	+	+
44	*1,2-Dicyanobenzene*	*91-15-6*	*128.1*	+	*1.28*	*0.3*	*2.3*	*47*	+	+	
45	*Dicyclohexylamine*	*101-83-7*	*181.3*	+	*1.81*	*0.6*	*3.3*	*47*	+	+	
46	*N,N*-Dicyclohexyl-2-benzothiazolesulfenamide	4979-32-2	346.6	+	3.47	0.2	0.6	*47*	+	+	+
47	*2-(Diethylamino)ethyl methacrylate*	*105-16-8*	*185.3*	+	*1.85*	*0.6*	*3.2*	*47*	+	+	- (+)
48	*O,O'-Diethyl dithiophosphate*	*298-06-6*	*186.2*	+	*1.86*	*0.12*	*0.6*	*47*	+	+	+
49	*Diethyl fumarate*	*623-91-6*	*172.2*	+	*1.72*	*0.01*	*0.06*	*47*	+	+	+
50	*2-(Dimethylamino)ethyl acrylate*	*2439-35-2*	*143.2*	+	*1.43*	*0.05*	*0.3*	*47*	+	+	+
51	*2-(Dimethylamino)ethyl methacrylate*	*2867-47-2*	*157.2*	+	*1.57*	*0.6*	*3.8*	*47*	+	+	
52	*2,3-Dimethylaniline (2,3-Xylidine)*	*87-59-2*	*121.2*	+	*1.21*	*0.6*	*5.0*	*47*	+	+	
53	*2,6-Dimethylaniline (2,6-Xylidine)*	*87-62-7*	*121.2*	+	*1.21*	*0.3*	*2.5*	*47*	+	+	

54	3,5-Dimethylaniline (3,5-Xylidine)	108-69-0	121.2	+	1.21	0.9	7.4	47	+	+	
55	N,N-Dimethylbenzylamine	103-83-3	135.2	+	1.35	0.4	3	47	+	+	
56	N-(1,3-Dimethylbutyl)-N'-phenyl-p-phenylenediamine	793-24-8	268.4	+	2.68	0.005	0.02	47	+	+	+
57	2,4-Dinitrophenol	51-28-5	184.1	+	1.84	1.2	6.5	47	+	+	
58	Diphenyl cresyl phosphate	26444-49-5	340.3	+	3.40	0.04	0.1	47	+	+	+
59	Disperse Red 206	26630-87-5	580.1	+	5.80	2.5	4.3	47	+		
60	Disperse Yellow 42	5124-25-4	369.4	+	3.69	0.08	0.2	47	+	+	+
61	2,3-Epoxypropyl methacrylate	106-91-2	142.2	+	1.42	0.02	0.1	47	+	+	+
62	Ethenyltrimethoxysilane	2768-02-7	148.2	+	1.48	0.8	5.4	47	+	+	
63	4-Ethoxybenzeneamine (p-Phenetidin)	156-43-4	137.2	+	1.37	0.05	0.4	47	+	+	+
64	N-Ethylaniline	103-69-5	121.2	+	1.21	1.1	9.1	47	+	+	
65	2-Ethylanthraquinone	84-51-5	236.3	+	2.36	0.16	0.6	47	+	+	+
66	2-Ethylbutyric acid	88-09-5	116.2	+	1.16	0.4	3.4	47	+	+	
67	3-Ethylphenol	620-17-7	122.2	+	1.22	0.05	0.4	47	+	+	+
68	4-Ethylphenol	123-07-9	122.2	+	1.22	0.04	0.3	47	+	+	+
69	Ferrous sulfate heptahydrate	7782-63-0	278.0	+	2.78	0.5	1.8	47	+	+	
70	Glycerol triacetate	102-76-1	218.2	+	2.18	2.2	10.0	47	+		
71	Hydrazine monohydrate	7803-57-8	50.1	+	0.50	0.06	1.2	47	+	+	
72	2-Hydroxybenzaldehyde	90-02-8	122.1	+	1.22	0.1	0.8	47	+	+	+
73	4-Hydroxy-benzenesulfonic acid, tin (2+) tetrahydride	70974-33-3	465.1	+	4.65	0.528	1.1	47	+	+	
74	4-Hydroxybenzoic acid	99-96-7	138.1	+	1.38	0.7	5.1	47	+	+	
75	2-Hydroxyethyl methacrylate	868-77-9	130.2	+	1.30	0.7	5.4	47	+	+	
76	3-Hydroxy-2-naphthalenecarboxylic acid	92-70-6	188.2	+	1.88	0.75	4.0	49	+	+	
77	2-Hydroxypropanenitrile	78-97-7	71.1	+	0.71	0.7	10.0	47	+	+	
78	2-Mercaptobenzimidazole	583-39-1	150.2	+	1.50	0.8	5.3	47	+	+	
79	Methacrylic acid, monoester with propane-1,2-diol	27813-02-1	144.2	+	1.44	0.7	4.9	47	+	+	
80	(Methacryloyloxyethyl)trimethylammonium chloride	5039-78-1	207.7	+	2.08	2.1	10.0	47	+		
81	Methacrylonitrile (Methyl Acrylonitrile)	126-98-7	67.1	+	0.67	0.07	1.0	47	+	+	+
82	3-Methoxybenzeneamine	536-90-3	123.2	+	1.23	0.8	6.5	47	+	+	
83	Methoxymethanol	4461-52-3	62.1	+	0.62	0.02	0.3	47	+	+	+
84	1-Methoxynaphthalene	2216-69-5	158.2	+	1.58	0.02	0.1	47	+	+	+
85	Methyl acetoacetate	105-45-3	116.1	+	1.16	1.2	10.0	47	+	+	
86	N-Methylaniline	100-61-8	107.2	+	1.07	0.6	5.6	47	+	+	
87	3-Methylbenzoic acid	99-04-7	136.2	+	1.36	1.5	11.0	47			
88	4-Methylbenzoic acid	99-94-5	136.2	+	1.36	1.2	8.8	47	+	+	
89	4,4'-Methylenebis(2-chloroaniline)	101-14-4	267.2	+	2.67	0.04	0.1	47	+	+	+
90	Methylenediphenol	1333-16-0	200.2	+	2.00	0.01	0.05	47	+	+	+
91	4,4'-Methylenediphenol	620-92-8	200.2	+	2.00	0.2	1.0	47	+	+	+
92	4-(1-Methylethenyl)phenol	4286-23-1	134.2	+	1.34	0.06	0.4	47	+	+	+
93	Methyl isothiocyanate	556-61-6	73.1	+	0.73	0.003	0.03	47	+	+	+
94	3-Methyl-4-nitrophenol	2581-34-2	153.2	+	1.53	0.04	0.3	47	+	+	+
95	3-Methylphenol (m-Cresol)	108-39-4	108.1	+	1.08	0.03	0.3	47	+	+	+
96	2-(4-Morpholinyldithio)benzothiazole	95-32-9	284.4	+	2.84	0.1	0.3	47	+	+	+
97	1-Naphthylacetic acid	86-87-3	186.2	+	1.86	1.7	9.1	47	+	+	
98	4-Nitro-o-anisidine	97-52-9	168.2	+	1.68	0.08	0.5	47	+	+	+
99	3-Nitrobenzenamine	99-09-2	138.1	+	1.38	1.6	11.6	47			
100	p-Nitrophenol sodium salt	824-78-2	161.1	+	1.61	0.6	3.7	47	+	+	
101	4,4'-Oxybis(benzenesulfonylhydrazide)	80-51-3	358.4	+	3.58	0.6	1.7	47	+	+	
102	2-Pentylanthraquinone	13936-21-5	278.4	+	2.78	0.06	0.2	47	+	+	+
103	N-Phenylmaleimide	941-69-5	173.2	+	1.73	0.01	0.02	47	+	+	+
104	N-Phenyl-N'-isopropyl-p-phenylenediamine	101-72-4	226.3	+	2.26	0.01	0.01	47	+	+	+
105	Phosphoric acid, dodecyl ester, sodium salt	50957-96-5	288.3	+	2.88	0.05	0.16	47	+	+	+
106	Phthalimide	85-41-6	147.1	+	1.47	2.5	17.0	47			
107	Sorbitan monooctadecanoate	1338-41-6	430.6	+	4.31	1.1	2.5	47	+	+	

Table 2 (*Continued*)

JEC ID	Chemical name	CAS No.	MW	CA (original call)	Equiv. to 10 mM (mg/mL)	LEC (mg/mL)	LEC (mM)	Ref.	1997-OECD[a]	r-OECD[b]	ICH[c]
									CA	CA	CA
108	4,4'-Sulfonyldiphenol	80-09-1	250.3	+	2.50	0.4	1.6	47	+	+	
109	*3a,4,7,7a-Tetrahydro-1H-indene*	*3048-65-5*	*120.2*	+	*1.20*	*0.004*	*0.8*	*47*	+	+	+
110	2,3,4,4'-Tetrahydroxybenzophenone	31127-54-5	246.2	+	2.46	0.0148	0.06	47	+	+	+
111	*2,2,6,6-Tetramethyl-4-hydroxypiperidine*	*2403-88-5*	*157.3*	+	*1.57*	*2.0*	*12.7*	*47*			
112	*Thiourea dioxide*	*4189-44-0*	*108.1*	+	*1.08*	*0.6*	*5.5*	*47*	+	+	
113	*Thymol*	*89-83-8*	*150.2*	+	*1.50*	*0.002*	*0.01*	*47*	+	+	+
114	*Tolylene diisocyanate (Toluene diisocyanate)*	*26471-62-5*	*174.2*	+	*1.74*	*0.3*	*1.8*	*47*	+	+	
115	2,4,6-Tribromophenol	118-79-6	330.8	+	3.31	0.05	0.2	47	+	+	+
116	*1,3,5-Trihydroxybenezene*	*108-73-6*	*126.1*	+	*1.26*	*0.1*	*1.0*	*47*	+	+	+
117	*2,4,6-Trimercapto-S-triazine*	*638-16-4*	*177.3*	+	*1.77*	*0.8*	*4.5*	*47*	+	+	
118	*Trimethoxyphosphine*	*121-45-9*	*124.1*	+	*1.24*	*1.2*	*10.0*	*47*	+	+	
119	*Trimethylamine*	*75-50-3*	*59.1*	+	*0.59*	*0.4*	*6.8*	*47*	+	+	
120	*2,3,6-Trimethylphenol*	*2416-94-6*	*136.2*	+	*1.36*	*0.05*	*0.4*	*47*	+	+	+
121	2,4,6-Trinitrophenol (Picric acid)	88-89-1	229.1	+	2.29	1.6	7.0	47	+	+	
122	Triphosphoric acid aluminium salt	13939-25-8	317.9	+	3.18	2.0	6.3	47	+	+	
123	1,3,5-Tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)isocyanuric acid	27676-62-6	784.1	+	7.84	2.5	3.2	47	+		
124	*2-Vinylpyridine*	*100-69-6*	*105.2*	+	*1.05*	*0.01*	*0.1*	*47*	+	+	+

MW, Molecular weight; CA, Chromosomal aberration test; LEC, Lowest effective concentration;
Equivalaent to 10 mM means the equal concentration of weight per volume (mg/mL) to 10 mM.
+, positive; –, negative.
(+) shows positive after the appication of the r-OECD TG for the chemicals MW less than MW 200 (n=41).
Italics means chemicals MW less than 200 (n=85).
Highilight to the negative result by the re-evaluation.

[a] Current OECD test guideline adopted in 1997 (10 mM or 5 mg/mL whichever is lower)

[b] Draft revised OECD test guideline (10 mM or 2 mg/mL whichever is lower)

[c] ICH S2(R1) guideline (1 mM or 0.5 mg/mL whichever is lower);

Table 3
Sensitivity and specificity for carcinogenicity upon application of each test guideline for the dataset on 435 chemicals from the CGX database.

Test guideline	Dataset	CA-negative	CA-positive	Total	Calculation
Original call[a]	Carcinogen	107	210	317	Sensitivity, 66.2% (210/317)
	Non-carcinogen	61	57	118	Specificity, 51.7% (61/118)
	Total	168	267	435	
1997-OECD[b]	Carcinogen	117	200	317	Sensitivity, 63.1% (200/317)
	Non-carcinogen	70	48	118	Specificity, 59.3% (70/118)
	Total	187	248	435	
r-OECD[c]	Carcinogen	117	200	317	Sensitivity, 63.1% (200/317)
	Non-carcinogen	70	48	118	Specificity, 59.3% (70/118)
	Total	187	248	435	
ICH[d]	Carcinogen	173	144	317	Sensitivity, 45.4% (144/317)
	Non-carcinogen	86	32	118	Specificity, 72.9% (86/118)
	Total	259	176	435	
ICH (modified)[e]	Carcinogen	133	184	317	Sensitivity, 58.0% (184/317)
	Non-carcinogen	80	38	118	Specificity, 67.8% (80/118)
	Total	213	222	435	

[a] Call in CGX database [16], including 19 CA-positives (10 carcinogens and 9 non-carcinogens) at >10 mM.
[b] Current OECD test guideline adopted in 1997 (10 mM or 5 mg/mL whichever is lower).
[c] Draft revised OECD test guideline (10 mM or 2 mg/mL whichever is lower).
[d] ICH S2(R1) guideline (1 mM or 0.5 mg/mL whichever is lower).
[e] Applied to the r-OECD TG for the chemicals MW less than 200.

3.3. Evaluation of the relevance of in vitro CA results

Fifty-three chemicals showed different results between r-OECD and ICH TGs (*i.e.*, positive call and negative call, respectively) (Table 2). Thus, these 53 different chemicals were detected as positive in the *in vitro* CA test with r-OECD TG but not with the ICH TG, indicating that the 53 chemicals would be missed if the ICH TG had been used. The relevance of the *in vitro* CA results was evaluated on the basis of the weight-of-evidence approach, and the level of concern on "different" chemicals was defined.

The 53 different chemicals included 34 chemicals that had their appropriate levels of concern evaluated in our previous study (four of 'some concern', seven of 'minimal concern', and 23 of 'negligible concern') [9]. All 34 chemicals were negative in the Ames test [9,47].

The remaining 19 chemicals were evaluated as a new level of concern. Fifteen out of the 19 chemicals were positive in the Ames test. To reveal the weight of the Ames-positives, the *in vivo* genotoxicity and carcinogenicity assays were reviewed for the 15 chemicals (Table 5). Seven of these, *i.e.*, *N*-(aminoethyl)ethanolamine (JEC ID5), azodicarbonamide (JEC ID10), 2-(dimethylamino)ethyl methacrylate (JEC ID51), 2,6-dimethylaniline (JEC ID53), 4,4′-oxybis(benzenesulfonylhydrazide) (JEC ID101), tolylene diisocyanate (JEC ID114) and 2,4,6-trinitrophenol (JEC ID121), were negative in the *in vivo* micronucleus (MN) test [47,49,50]. However, two (JECD IDs 53 and 114) were categorized in the IARC Group 2B (possible human carcinogen) [50]. Two other chemicals, hydrazine monohydrate (JEC ID71) and 3-methoxybenzeneamine (JEC ID82), were positive in the *in vivo* MN test [47,50]; the former chemical (JEC ID71) was categorized in IARC's Group 2B [50]. No *in vivo* genotoxicity and/or carcinogenicity data were available for the remaining six chemicals. On the basis of these data, four chemicals (JEC IDs 53, 71, 82 and 114) can be considered to be of real concern as a possible human carcinogen or an *in vivo* genotoxin. Genotoxic effects could not be ruled out for Ames-positive chemicals, despite the negative results obtained in an *in vivo* MN test. Thus, the remaining 11 chemicals (five *in vivo* MN-negatives and six without *in vivo* genotoxicity data) were considered to be of some concern.

For the last four chemicals, two (JEC IDs 76 and 117) were of negligible concern, one (JEC ID1) was of minimal concern, and one (JEC ID73) was of some concern on the basis of the following evaluations:

JEC ID 1. Acenaphthene (CAS No. 83-32-9): Acenaphthene induced CAs (16.4%, 195 cells analyzed) at the highest concentration of 0.20 mg/mL (1.3 mM) only with S9-mix; the relative cell growth, as measured by monolayer confluence, was 28.0%. A lower concentration of 0.10 mg/mL showed a CA frequency of 4.5%, with 30.0% relative cell growth [47]. In a bacterial reverse-mutation assay (*i.e.*, Ames test), acenaphthene was negative with or without S9. No *in vivo* genotoxicity data were available. The data did not explain that the CAs observed *in vitro* were irrelevant due to their high toxicity. Acenaphthene was classified in Group 3 by IARC due to inadequate evidence in experimental animals for its carcinogenicity [50]. There was insufficient evidence to classify this finding as a negligible level of concern; thus, we concluded that it fell in the category of a minimal level of concern.

JEC ID 73. 4-Hydroxy-benzenesulfonic acid, tin (2+) tetrahydride (CAS No. 70974-33-3): 4-Hydroxy-benzenesulfonic acid, tin (2+) tetrahydride induced CAs (4.5%, 12.5% or 24.0% at 0.528 mg/mL (1.1 mM), 0.755 mg/mL or 1.078 mg/mL, respectively) after 6-h treatment without S9; the relative cell growth, as measured by ATP contents, was 85%, 64% or 53% [47]. With S9, CAs (14.0%) were induced at 2.2 mg/mL after 6-h treatment; the relative cell growth was 43%. Precipitation was observed at the end of the treatment period with S9. The Ames test provided negative results, with or without the S9 mix [47]. No *in vivo* genotoxicity data were available. There was no supporting evidence for a reduced level of concern, and thus some concern remains.

Table 4
Alterations of the number of 124 CA-positives from the JEC database after the application of each test guideline.

Dataset	Original call[a]	1997-OECD[b]	r-OECD[c]	ICH[d]	ICH (modified)[e]
JEC 124 CA-positives	124	118	113	60	101

[a] Call in JEC database [47], including 6 CA-positives) at >10 mM.
[b] Current OECD test guideline adopted in 1997 (10 mM or 5 mg/mL whichever is lower).
[c] Draft revised OECD test guideline (10 mM or 2 mg/mL whichever is lower).
[d] ICH S2(R1) guideline (1 mM or 0.5 mg/mL whichever is lower).
[e] Applied to the r-OECD TG for the chemicals MW less than 200.

Table 5
Summary of *in vivo* genotoxicity and carcinogenicity (in terms of the IARC classification) data on the 15 different chemicals that were positive in the Ames test.

JEC ID	Chemical name	CAS No.	Ames	*in vivo* MN	Carcinogenicity[a]	Ref.
5	*N*-(Aminoethyl)ethanolamine	111-41-1	+	–		[47,49]
7	2-Amino-1-naphthalenesulfonic acid	81-16-3	+			[47]
10	Azodicarbonamide	123-77-3	+	–		[47,49]
13	1,3-Bis(aminomethyl)cyclohexane (mixtures of cis-, trans-)	2579-20-6	+			[47]
17	1-Bromo-3-chloropropane	109-70-6	+			[47]
51	2-(Dimethylamino)ethyl methacrylate	2867-47-2	+	–		[44,47]
52	2,3-Dimethylaniline (2,3-Xylidine)	87-59-2	+			[47]
53	2,6-Dimethylaniline (2,6-Xylidine)	87-62-7	+	–	2B	[47,49,50]
54	3,5-Dimethylaniline (3,5-Xylidine)	108-69-0	+			[47]
71	Hydrazine monohydrate	7803-57-8	+	+[b]	2B[b]	[47,50]
82	3-Methoxybenzeneamine	536-90-3	+	+		[47]
101	4,4'-Oxybis(benzenesulfonylhydrazide)	80-51-3	+	–		[47]
112	Thiourea dioxide	4189-44-0	+			[47]
114	Tolylene diisocyanate (Toluene diisocyanate)	26471-62-5	+	–	2B	[47,50]
121	2,4,6-Trinitrophenol (Picric acid)	88-89-1	+	–		[47,49]

+, positive; –, negative; MN, micronucleus.
[a] In terms of the IARC classification.
[b] As hydrazine (CAS No. 302-01-2).

JEC ID 76. 3-Hydroxy-2-naphthalenecarboxylic acid (CAS No. 92-70-6): 3-Hydroxy-2-naphthalenecarboxylic acid induced CAs in Chinese hamster V79 cells after a 6-h or 18-h treatment with the highest test concentration (0.75 mg/mL, *i.e.*, 4.0 mM) without S9. With S9, no CAs were observed [49]. No information about the frequency of CAs or cytotoxicity was available. The Ames test was negative with or without S9 [49,51]. In an *in vivo* CA test in bone-marrow cells of hamsters, no clastogenic activity and no toxicity were observed at the maximum recommended dose of 2000 mg/kg. However, the test had severe limitations (only 50 metaphases were examined per animal and there was no indication that the target tissue was reached by the chemical). Still, recent *in vivo* mouse bone-marrow MN tests were negative after oral administration of up to 500 mg/kg/day for 2 days. One animal died at 700 mg/kg/day in a dose-range finding study [47]. The weight-of-evidence suggests that the level of concern is negligible.

JEC ID 117. 2,4,6-Trimercapto-S-triazine (CAS No. 638-16-4): 2,4,6-Trimercapto-S-triazine induced CAs at the highest concentration of 0.8 mg/mL (4.5 mM) after 6-h treatment with or without S9 (19.0% or 5.5%, respectively); the relative cell growth, as measured by monolayer confluence, was 73% or 55%, respectively. The pH of the medium at 1.2 mg/mL or more was approximately 6.0 or less. The pH at 0.8 mg/mL was not measured. In a confirmatory test in pH-adjusted medium with S9, the chemical induced CAs (31.5%) at the highest concentration of 1.2 mg/mL, and precipitation was observed at the beginning of the treatment; the relative cell growth was 77%. No CAs were observed up to 0.31 mg/mL after 24-h treatment without the S9 mix [47]. The Ames test was negative with or without S9 [47]. An *in vivo* mouse bone-marrow MN test was negative after oral administration of up to 1000 mg/kg/day for 2 days. One animal died at 2000 mg/kg/day in a dose-range finding study [47]. The weight-of-evidence suggests that the level of concern is negligible.

The results of the evaluation of the level of concern are summarized in Table 6. Of the 53 different chemicals, four chemicals were of 'real concern', 16 were of 'some concern', eight were of 'minimal concern', and the remaining 25 chemicals were of 'negligible concern'. Importantly, the 'of some concern' category in some cases was due to the absence of relevant additional data and not based available data suggesting real concern [9]. In this analysis, 15 Ames-positive chemicals were included in the 53 different (*i.e.*, missed by the application of the ICH TG) chemicals (Table 5). All of the Ames-positives were classified as of 'some concern' or of 'real concern' (Table 6). If the Ames-positive chemicals were excluded from the analysis due to detection by the test-battery system, 38 chemicals would be missed. Among the 38 chemicals, five were of 'some concern'; eight were of 'minimal concern'; and the remaining 25 chemicals were of 'negligible concern' (Table 6).

3.4. Distribution of chemical MWs

The distribution of the MWs of the 267 CA-positives from the CGX database or 124 CA-positives from the JEC database is presented in Table 7. The MWs of the majority of chemicals (71.9% in CGX, 84.7% in JEC) were between 100 and 300. Approximately half (141/267) of the 267 CA-positives from the CGX database had a MW below 200. Similar distributions in MWs have been shown in carcinogens and non-carcinogens. Approximately 70% (85/124) of the 124 CA-positives from the JEC data set, based on CSCL for industrial chemicals, had a MW of less than 200. These distributions indicate that 10 mM can be considered equivalent to 2 mg/mL for industrial chemicals.

4. Discussion

The present reduction in the top-concentration limit in the *in vitro* CA test is expected to reduce the number of false or misleading positives, and hopefully, it will not greatly affect the assay's sensitivity or specificity for rodent carcinogenicity. We investigated the effects of this reduction by means of two chemical data sets from the CGX and JEC databases, by applying three test guidelines, *i.e.*, the 1997-OECD [1], r-OECD [12] and ICH [11] TGs. The chemical dataset from the CGX [16] or JEC [47] databases consisted of a variety of chemical categories, including natural products, pharmaceuticals and pesticides or industrial chemicals. The sensitivity and specificity analysis of the 435 chemicals from the CGX database revealed that application of the r-OECD TG (10 mM or 2 mg/mL) did not affect the sensitivity (63.1%) or specificity (59.3%) against carcinogenicity compared with those (sensitivity 63.1%, specificity 59.3%) seen with the 1997-OECD TG (10 mM or 5 mg/mL). However, the ICH TG (1 mM or 0.5 mg/mL) showed a different outcome, *i.e.*, approximately a 18% decrease in sensitivity (45.4%) and a 14% increase in specificity (72.9%) (Table 3). These results indicate that the r-OECD TG demonstrated the same ability to detect rodent carcinogens as the 1997-OECD TG for chemicals in the CGX database. However, the ICH TG showed a low sensitivity (less than 50%) and was not useful for its detection. Analysis of the changes in the number of 124 CA-positives from the JEC database revealed a small reduction in the number induced under the r-OECD TG, and a remarkable reduction (about half) under the ICH TG (Table 4). These data indicate that application of ICH TG did not lead to an effective detection of rodent carcinogens among non-pharmaceuticals (*e.g.*, general