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Short communication

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

Mariko Matsumoto[a,d], Shoji Masumori[b], Mutsuko Hirata-Koizumi[a], Atsushi Ono[a,*], Masamitsu Honma[c], Kazuhito Yokoyama[d], Akihiko Hirose[a]

[a] Division of Risk Assessment, National Institute of Health Sciences, Tokyo, Japan
[b] Public Interest Incorporated Foundation Biosafety Research Center, Shizuoka, Japan
[c] Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo, Japan
[d] Department of Epidemiology and Environmental Health, Juntendo University Faculty of Medicine, Tokyo, Japan

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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

* Corresponding author at: Division of Risk Assessment, Biological Safety Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel.: +81 33700 9878; fax: +81 33700 1408.

E-mail address: atsushi@nihs.go.jp (A. Ono).

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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 (Negative control)	0	1001	666,000	47	70.6	
		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 (Positive control[b])	100	1501	596,700	81	135.7	
		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 (Negative control)	0	1001	609,300	25	41	
		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 (Positive control[b])	100	1501	621,900	321	516.2	
		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.

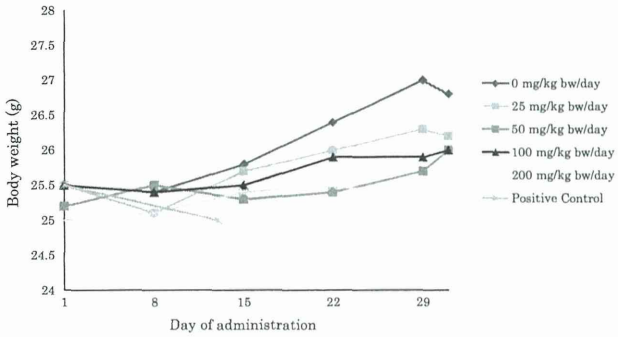

Fig. 1. Body weights of transgenic mice treated with hydroquinone for 28 days. Positive control: *N*-ethyl-*N*-nitrosourea was dosed once a day for 2 days (i.p) and tissues were collected 10 days after the last treatment.

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

Takeshi Morita[a,*], Atsuko Miyajima[b], Akiko Hatano[a], Masamitsu Honma[c]

[a] Division of Safety Information on Drug, Food and Chemicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[b] Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[c] Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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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

* Corresponding author.
E-mail address: morita-tk@nihs.go.jp (T. Morita).

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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	+	+	- (+)
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	+	+	-
5	C	Acrylamide	79-06-1	71.1	+	0.71	2	28.14	22	-	-	-
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	+	+	- (+)
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	+	+	- (+)
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	+	+	-
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	+	+	-
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	+	+	- (+)
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	+	+	- (+)
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	+	+	- (+)
47	C	Chloramben	133-90-4	206.0	+	2.06	1.51	7.33	22	+	+	-
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	+	+	-

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	+	+	- (+)