Genotoxicity studies on green tea catechin

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Abstract

The beneficial effects of tea catechins are well documented. We evaluated the genotoxic potential of a green tea catechin preparation using established genotoxicity assays, including a bacterial reverse mutation assay (Ames test), a chromosomal aberration assay in cultured Chinese hamster lung cells (CHL/IU), a mouse lymphoma L5178Y/tk assay, and a bone marrow micronucleus (MN) assay in ICR CD mice and SD rats. No significant increases in the number of revertant colonies were observed in the Ames test, but positive responses were observed in two *in vitro* assays: the chromosomal aberration assay and mouse lymphoma L5178/tk assay. However, the *in vivo* study demonstrated no significant increase in micronucleated polychromatic erythrocytes (MNPCE) in the bone marrow of both ICR CD mice and SD rats administered a high dose of the green tea catechin preparation up to 2000 mg/kg. Combined with favorable epidemiological information suggesting a chemopreventive effect of tea catechins on carcinogenesis, we conclude that green tea catechin presents no significant genotoxic concern under the anticipated conditions of use. These results are consistent with other genotoxicity studies of tea catechins, which show minimal, if any, genotoxic potential.
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1. Introduction

Tea (*Camellia sinensis*) is one of most popular beverages consumed worldwide and has a long history of consumption dating from ancient times. Among the various types of tea, green tea contains a relatively high level of polyphenols, which consist of flavanol monomers (flavan-3-ols), also referred to as catechins. Tea catechins have garnered considerable attention as a result of beneficial effects on health, such as their observed antioxidant activity (Yoshino et al., 1994) and anti-viral activity (Nakayama et al., 1993), as well as their ability to serve as an anti-plaque-forming agent (Hattori et al., 1990), anti-carcinogenic agent (Bemis et al., 2006; Katiyar and Mukhtar, 1996; Lee et al., 2006; Yuan et al., 2007), anti-cardiovascular disease agent (Wolfam, 2007), hypotensive agent (Henry and Stephens-Larson, 1984), hypocholesterogenic agent (Ikeda et al., 1992), and blood sugar reductive agent (Matsumoto et al., 1993).

Recently, the ingestion of tea catechins or tea beverage enriched with catechins was shown to be effective in reducing body fat (Nagao et al., 2005). Although the mechanism

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Abbreviations: PE, plating efficiency; RS, relative survival; RSG, relative survival growth; RTG, relative total growth; MF, mutation frequency; L-MF, MF of large colonies; S-MF, MF of small colonies; T-MF, MF of total colonies; MN, micronucleus; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte; MNPCE, micronucleated PCE; MMS, methyl methanesulphonate; ENNG, *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine; 2NF, 2-Nitrofluorene; AF-2, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; 2AA, 2-aminoanthracene; 9AA, 9-aminoacridine hydrochloride hydrate; NaN3, sodium azide; CMC-Na, carboxymethyl cellulose sodium aqueous solution; MMC, mitomycin C; B(*a*)P, benzo(*a*)pyrene.

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of action for this effect is not known, it is thought to be due to the stimulation of lipid catabolism in the liver (Murase et al., 2002) and/or the enhancement of energy consumption (Osaki et al., 2001). Based on these findings, a catechin-rich tea beverage that claims to have a preventive effect on body fat accumulation has been marketed in Japan as a "Food for Specified Health Use."

During the manufacture of catechin-rich beverages, green tea extract is prepared from green tea leaves and then formulated into beverages. The green tea catechins consist of isomers including catechin (C), catechin gallate (Cg), gallocatechin (GC), gallocatechin gallate (GCg), epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), and epigallocatechin gallate (EGCg) (Fig. 1). When subjected to high heat conditions, tea catechin monomers undergo epimerization (Seto et al., 1997). Hence, the composition of catechin isomers in beverages varies, primarily according to the heat-sterilization conditions employed during the manufacturing process. The heat sterilization conditions for different beverages are determined based on certain characteristics of the beverage, including pH and packaging; thus, the relative epimerization of catechins is greater in certain beverages.

In the present study, we evaluated the genotoxic potential of green tea catechin preparations for the catechin-rich beverages. Regarding the genotoxic aspects of tea catechins, many studies demonstrated that tea catechins could suppress the genotoxic activity of various carcinogens with both *in vitro* and *in vivo* systems (Nikaidou et al., 2005; Sinha et al., 2005; Muto et al., 1999; Kuroda, 1996; Hayatsu et al., 1992; Cheng et al., 1991; Wang et al., 1989; Jain et al., 1989). As to the genotoxic profile of tea catechins when tested alone, Chang et al. (2003) have shown that there is minimal genotoxic concern with a decaffeinated green tea catechin mixture (Polyphenon E) that contains about 50% epigallocatechin gallate and 30% other catechins. Isbrucker et al. (2006) also have found no genotoxic concern with a epigallocatechin gallate (EGCG) preparation, Teavigo™.

Green tea catechin preparations, GTC type-1 and GTC type-2 evaluated in the present study reflect the catechin profile in the catechin-rich beverages (Healthya® green tea beverage and Healthya® water sports drink) introduced into the marketplace. GTC type-1 is a heat-treated preparation of green tea catechins made to simulate the catechin characteristics following the heat sterilization process. In contrast, the GTC type-2 preparation is not subjected to heat treatment.

To evaluate the potential genotoxic effect of these preparations, consistent with the International Conference of Harmonization Guidelines, we conducted a bacterial reverse mutation assay (Ames test), a chromosomal aberration assay in cultured Chinese hamster lung cells (CHL/IU), a mouse lymphoma cell thymidine kinase (Tk) gene mutation assay in L5178Y cells, and a bone marrow micronucleus (MN) assay in ICR CD mice and SD rats (ICH Guidelines, 1997).

2. Materials and methods

2.1. Study design

The genotoxicity tests were performed in compliance with the Good Laboratory Practice Regulations (except for the rat MN study) and in accordance with standard guidelines for genotoxicity tests including the OECD guidelines.

2.2. Preparation of tea catechins

The green tea catechins used in the present study, GTC type-1 and GTC type-2, were prepared consistent with the manufacturing process for the green tea catechin preparations added to catechin-rich beverages. Tea catechins were first extracted from green tea leaves with hot water, and the extract was reduced to a powder under aerated conditions. The powder was then mixed with ethanol solution (60–95%) to dissolve the catechin monomers present in green tea extract. Insoluble macromolecules were removed with a micropore filter and the tea catechin solution was diluted with ion-exchanged water to 10% total catechins (weight/volume). The tea catechin solution for GTC type-1 was further heat-treated at 121 °C for 40 min and freeze-dried. For the GTC type-2 preparation, the tea catechins were directly freeze-dried after filtering, without dilution and heat-sterilization.

The chemical compositions of both GTC type-1 and GTC type-2 are shown in Table 1. The ratio of total catechins among polyphenols was reduced by the heat-treatment. In addition, a reduced ratio of epi-catechins (EGC, EC, EGCg, and ECg) among total catechin monomers also was evident in GTC type-1. This reduced ratio observed in the GTC type-1 preparation consistently reflects the relative proportion of individual
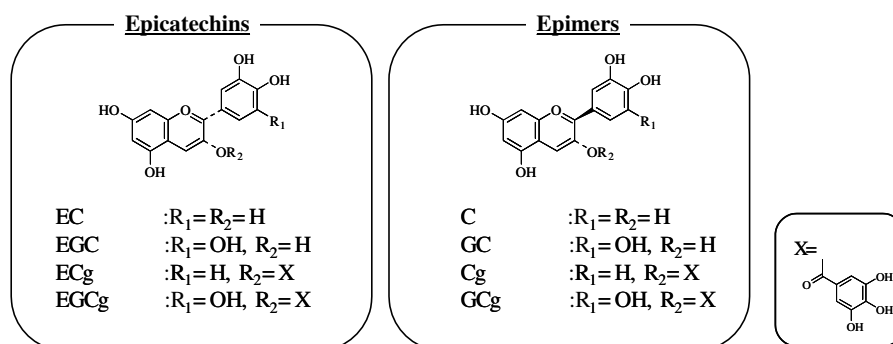

Fig. 1. Structure of catechin monomers.

Table 1
Chemical analysis of green tea catechin GTC type-1 and GTC type-2

Composition	Green tea catechin	
	Type-1	Type-2
Total catechin[a] (%)	33.1	63.7
Gallocatechin (GC)	6.8	4.8
Epigallocatechin (EGC)	4.9	18.0
Catechin (C)	1.8	1.9
Epicatechin (EC)	1.8	4.9
Epigallocatechin gallate (EGCg)	6.9	25.1
Gallocatechin gallate (GCg)	6.9	1.3
Epicatechin gallate (ECg)	2.4	7.1
Catechin gallate (Cg)	1.6	0.6
Epimerization ratio[b]	0.48	0.86
Caffeine (%)	3.7	1.2
Polyphenol (%)	48.5	66.0

[a] Sum of eight catechins (GC, EGC, C, EC, EGCg, GCg, ECg, Cg).
[b] (sum of EGC, EC, EGCg, and ECg)/total catechins.

catechin monomers found in catechin-rich beverages that undergo heat sterilization. As shown in Table 1, epimerization from epi-catechins to catechins preferentially occurred during the heat processing.

2.3. Preparation of S9 mixture

The S9 mix used in the present *in vitro* genotoxicity studies for metabolic activation condition was prepared as follows. For the GTC type-2 assay in the Ames test, the S9 fraction (Kikkoman Corporation, Japan) was prepared from phenobarbital and 5,6-benzoflavone-induced Sprague-Dawley rats. The S9 mixture was prepared by mixing with 9 volumes of Cofactor-I (Oriental Yeast Co. Ltd., Japan) dissolved in water (the NADPH regenerating system) with one volume of the S9 fraction. For the GTC type-1 assay in the Ames test, the S9 fraction (prepared in-house at Inveresk, Scotland) was prepared from Aroclor 1254-induced Fischer 344 rats. The S9 mixture was then prepared by mixing nine volumes of Cofactor (prepared in-house at Inveresk, Scotland) dissolved in water (the NADPH regenerating system) to one volume of the S9 fraction. For chromosomal aberration assay and mouse lymphoma L5178Y/tk assay, the S9 mixture was prepared similarly to that described in the Ames test for GTC type-2.

2.4. Bacterial reverse mutation assay (Ames test)

Assays for GTC type-1 and GTC type-2 were independently conducted in different institutions. Accordingly, some experimental conditions between the assays on the two preparations varied slightly from each other.

The following chemicals were used as positive controls, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2), 2-aminoanthracene (2AA), and sodium azide (NaN$_3$) (Wako Pure Chemical Industries, Ltd., Japan) 9-aminoacridine hydrochloride (9AA) (Aldrich Chemical Company, Inc., USA, GTC type-2 study), 2AA and 9AA (Sigma Chemical Company Ltd., USA), methylmethane sulphonate (MMS) (Fluka USA) and *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (ENNG) and 2-nitrofluorene (2NF) (Aldrich Chemical Company USA, GTC type-1 study).

The strains of *Salmonella typhimurium* and *Escherichia coli* were obtained from the National Institute of Health Sciences, Tokyo, Japan (GTC type-2 study) or *S. typhimurium* was obtained from Professor B.N. Ames, Department of Biochemistry, University of California, Berkeley, CA, USA, and *E. coli* was obtained from the national collection of industrial bacteria, Aberdeen, Scotland (GTC type-1 study).

Histidine-dependent auxotrophic mutants of *S. typhimurium* (strains TA98, TA100, TA1535, and TA1537), and a tryptophan-dependent auxotrophic mutant of *E. coli*, strain WP2uvrA, were exposed to the test

substance dissolved in dimethylsulphoxide (GTC type-1) and physiological saline (GTC type-2). Treatments with only physiological saline or dimethylsulphoxide were used as a negative control. Both experiments employed a pre-incubation method (Gatehouse et al., 1994), and were performed in the presence and absence of metabolic activation (S9 mix). Specifically, 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) (absence of metabolic activation) or 0.5 mL of S9 mix (presence of metabolic activation), and 0.1 mL of bacterial inoculate was added to a test tube. The test substance (0.1 mL) was then added and incubated for 20 min at 37 °C. After incubation, 2.0 mL of top agar was added to the mixture and poured onto a minimal glucose agar plate. The plates were incubated for 48 h at 37 °C. Following the incubation, the number of revertant colonies was evaluated.

The study consisted of two independent tests, a dose-range finding test and a primary test. The primary test was performed twice to confirm reproducibility. A response was classified as positive if the test substance caused a dose-dependent increase of at least two fold in the mean number of revertants per plate compared to the negative control value, in conjunction with confirmation that the three independent tests were reproducible.

2.5. Chromosomal aberration assay in cultured cells

Chemicals for positive controls, mitomycin C (MMC) and benzo(*a*)-pyrene (B(*a*)P), were purchased from Wako Pure Chemical Industries, Ltd., Japan.

Chinese hamster lung cells (CHL/IU) were plated at 1×10^4 cells/mL and incubated for 72 h. The culture medium was then replaced with fresh medium that contained the test substance (either GTC type-1 or GTC type-2). The test substance was dissolved in physiological saline. For short-term treatment, cells were exposed to each test substance for 6 h with either additional culture medium (without metabolic activation) or S9 mix at a final concentration of 5% (with metabolic activation). After a 6-h exposure, the cells were washed and then incubated in fresh medium for an additional 18 h. For continuous treatment, the cells were treated with each dose for 24 or 48 h. Colcemid was added (0.1 μg/mL final concentration) 2 h prior to harvesting. The cells were then harvested, swollen in 0.075 M KCl solution, fixed in methanol:acetic acid, placed onto clean glass slides, and Giemsa (3.0%) stained. One hundred metaphases per slide (200 metaphases per dose) were analyzed. Results were evaluated according to the following criteria for structural or numerical chromosomal aberration frequencies (Matsuoka et al., 1991): Negative < 5%; Uncertain 5–10%; Positive ⩾ 10%.

2.6. Mouse lymphoma L5178Y/tk assay

Methylmethanesulfonate (MMS) and cyclophosphamide monohydrate (CP) were purchased from Aldrich Chemical Company, Inc. and Sigma-Aldrich Co., USA.

L5178Y (tk +/− −3.7.2 c) mouse lymphoma cells (American Type Culture Collection (ATCC)) were maintained at a logarithmic growth phase and suspended to 10^6 cells/mL. The test substance (either GTC type-1 or GTC type-2) was dissolved in physiological saline. Ten mL of cell suspension were mixed with 7 mL of culture medium, 1 mL of 150 mmol/L KCl (without metabolic activation) or S9 mix (with metabolic activation), and 2 mL of the test substance. The cell suspension was incubated at 37 °C for either 3 h for short-term treatment, or 24 h for continuous treatment. After treatment, the cells were washed and re-suspended to a concentration of 2×10^5 cells/mL. An aliquot of each cell suspension was diluted to 8 cells/mL and transferred to 96-well plates at 0.2 mL/well (averaging 1.6 cells/well). The plates were incubated for 10 or 11 days and the number of wells containing colonies was counted macroscopically for evaluation of cytotoxicity (plating efficiency (PE0) and relative survival (RS0) at Day 0).

The remaining cell suspension was cultured for two days to allow for expression of the mutant phenotype. After this incubation period, the cell suspension in each culture was diluted to 10^4 cells/mL. An aliquot of each

cell suspension was transferred to 96-well plates for incubation to evaluate cell viability (plating efficiency (PE2) and relative survival (RS2) at Day 2) as described above for evaluation of cytotoxicity at Day 0. Trifluorothymidine (TFT) was added to the remaining cell suspension at 3 µg/mL for the selection of mutant colonies. The cell suspension was transferred to 96-well plates at 0.2 mL/well (2000 cells/well) and incubated for 11–12 days. After the incubation period, the number of wells containing colonies was counted macroscopically for calculation of mutant frequency (MF).

Statistical analysis was performed according to the method of Omori et al. (2002) to determine the significance of MF and dose dependency in the test substance group. The conclusion of a positive result is made upon observation of a significant increase in MF that is dose-dependent in the test substance groups when compared with the negative control group.

2.7. Bone marrow MN assay in mice

The positive control, mitomycin C (MMC), was purchased from Wako Pure Chemical Industries, Ltd., Japan.

The test substance (500, 1000, and 2000 mg/kg b.w.), was suspended in physiological saline and each dose was administered two times to nine-week-old male ICR (Crj: CD-1) mice by oral gavage. The interval between the two administrations was 24 h. There were six animals per treatment group. The negative control group received only physiological saline. Mice in the positive control group were given a single i.p. injection of mitomycin C dissolved in physiological saline at a dose of 2 mg/kg b.w. Bone marrow smears were prepared from each treatment group 24 h after last administration. Each smear was stained with Giemsa and examined using optical microscopy.

Polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) were identified according to the method of Hayashi et al. (1983). The number of micronucleated PCEs (MNPCEs) per 2000 PCEs was then counted. The PCE/NCE ratio was also determined by examination of 500 erythrocytes from each animal. A response was classified as positive if the test substance caused a significant dose-dependent increase in the total number of MNPCEs. The Student's *t*-test was applied at a significance level of 5% in order to assess the significance of the differences in PCE/NCE ratio between the test substance-treated groups and vehicle control group. For analysis of the incidence of MNPCEs, the tables of Kastenbaum and Bowman were applied at a significance level of 5% (Kastenbaum and Bowman, 1970). Then, further analysis, the Cochran–Armitage trend test, was applied to evaluate dose dependency if the test substance-treated groups showed a significant difference when compared with the concurrent vehicle control group.

2.8. Bone marrow MN assay in rats

The experiment essentially followed the procedure of the mouse MN assay described above. Two different concentrations of the test substance, 1000 and 2000 mg/kg b.w., were suspended in physiological saline and administered to seven-week-old male SD rat by oral gavage in two separate doses administered within 24 h. There were five animals per treatment group. Sample preparation and evaluation of the results were conducted by the same methods as the mouse MN assay, described above.

3. Results

3.1. Ames test

No dose-dependent increase in revertant colonies or bacterial toxicity were observed in the dose-range finding test at concentrations of GTC type-1 or GTC type-2 up to 5000 µg/plate in the presence or absence of metabolic activation. The primary test was performed in a similar manner at concentrations ranging from 156 to 5000 µg/plate (type-1) or from 78.1 to 5000 µg/plate (type-2), at a common ratio of 2. While the primary test was conducted twice to confirm reproducibility, the data of these two experiments were merged and are summarized in Tables 2 and 3, respectively.

No bacterial toxicity was observed at any dose tested. Neither GTC type-1 nor GTC type-2 caused more than a two fold increase in the mean number of revertants per plate compared to the negative control. The positive controls for each strain resulted in the expected increase in the number of revertant colonies. These data provide no evidence of gene mutagenic potential under the conditions used in this test for either GTC type-1 or GTC type-2.

Table 2
Results of the primary tests in the bacterial reverse mutation assay (Ames test) of GTC type-1

Treatment	Revertants per plate[a]									
	TA98		TA100		TA1535		TA1537		WP2uvrA	
	S9+	S9−	S9+	S9−	S9+	S9−	S9+	S9−	S9+	S9−
Vehicle control (DMSO)	40	24	97	93	16	10	12	6	8	13
156 µg/plate	39	21	92	83	10	10	10	8	8	9
313 µg/plate	37	21	92	88	13	12	10	8	10	8
625 µg/plate	40	21	94	83	12	10	12	9	7	11
1250 µg/plate	39	23	90	86	10	11	13	8	10	10
2500 µg/plate	39	29	89	88	6	13	11	7	7	6
5000 µg/plate	47	25	103	92	11	9	13	7	0[b]	2[b]
2NF 1 µg/plate		1122								
MMS 200 µg/plate				553						
NaN$_3$ 1 µg/plate						405				
9 AA 80 µg/plate								3101		
ENNG 2 µg/plate										435
2AA 0.5 µg/plate	512		648							
2AA 2 µg/plate					383		212			
2AA 20 µg/plate									698	

[a] Mean value of six plates in total (three plates/experiment).
[b] Density of the bacterial background lawn was clearly lower than that of the negative control group.

Table 3
Results of the primary tests in the bacterial reverse mutation assay (Ames test) of GTC type-2

Treatment	Revertants per plate[b]									
	TA98		TA100		TA1535		TA1537		WP2uvrA	
	S9+	S9−	S9+	S9−	S9+	S9−	S9+	S9−	S9+	S9−
Vehicle control (physiological saline)	25	21	113	113	12	10	10	9	24	23
78.1 µg/plate						11				
156 µg/plate	24	19	119	110	11	14	9	8	24	26
313 µg/plate	26	23	120	112	11	15	10	7	29	26
625 µg/plate	26	20	114	114	11	10	10	7	25	24
1250 µg/plate	25	22	117	114	11	10	9	8	23	24
2500 µg/plate	26[a]	18	119[a]	113	10[a]	9	6[a]	6	22[a]	22
5000 µg/plate	24[a]	16	115[a]	79[c]	9[a]	0[d]	4[a,c]	2[c]	15[a]	19
AF-2 0.1 µg/plate		556								
AF-2 0.01 µg/plate				435						
AF-2 0.02 µg/plate										385
2AA 0.5 µg/plate	859									
2AA 1 µg/plate			1210							
2AA 2 µg/plate					311		364			
2AA 10 µg/plate									127	
9 AA 80 µg/plate								612		
NaN$_3$ 0.5 µg/plate						306				

[a] Test substance precipitation.
[b] Mean value of six plates in total (three plates/experiment).
[c] Density of the bacterial background lawn was clearly lower than that of the negative control group.
[d] Bacterial background lawn was not observed due to strong growth inhibition by the test substance, and minute colonies were not observed.

3.2. Chromosomal aberration assay

The results of the chromosomal aberration assay with CHL cells for GTC type-1 and GTC type-2 are shown in Tables 4 and 5, respectively. Based on the results of the concentration-range finding study, GTC type-1 was tested at concentrations ranging from 56.3 to 900 µg/mL, and GTC type-2 was tested at concentrations ranging from 35.1 to 700 µg/mL.

Dose-related increases in cytotoxicity were noted at every concentration tested. No increases in the number of cells with numerical aberrations were found in any treatment group.

For GTC type-1, the frequency of the cells with structural aberrations was increased >10% only in the absence of metabolic activation (S9 mix) in both short-term and continuous treatment (24–0 h). For GTC type-2, increases at a frequency of >10% in the cells with structural aberrations were found following short-term treatment in both the presence or absence of metabolic activation (S9 mix).

Both the positive controls, containing either mitomycin (MMC) for treatment without metabolic activation or benzo[a]pyrene (B[a]P) for treatment with metabolic activation, resulted in an increase in the frequency of cells with >10% chromosomal aberration, supporting the validity of the study. Based on pre-designated criteria, the observed responses were judged to be positive.

3.3. Mouse lymphoma L5178Y/tk assay

Based on the results of the concentration-range finding study, GTC type-1 was assayed at concentrations ranging

from 52.7 to 3600 µg/mL, and GTC type-2 was assayed at concentrations ranging from 21.2 to 4000 µg/mL. No further testing was conducted for concentrations that resulted in no viable cells remaining after the treatment with the test substance (GTC type-2: 4000 µg/mL in short-term treatment with metabolic activation). Those concentrations where relative survival (RS0) or relative total growth (RTG) was less than 10% were excluded from the evaluation (GTC type-1: 2700 µg/mL in short-term treatment with metabolic activation, 3600 µg/mL in short-term treatment without metabolic activation, and 400 µg/mL in continuous treatment; GTC type-2: 2000 µg/mL in short-term treatment with metabolic activation, and 223 and 401 µg/mL in continuous treatment). The results of the mouse lymphoma L5178Y/tk assay for GTC type-1 and GTC type-2 are shown in Tables 6 and 7, respectively.

For GTC type-1, a concentration-related increase in MF was noted in the short-term treatment, both with and without metabolic activation. In the 24-h continuous treatment, a marked decrease in cell growth was observed at the highest concentration, 400 µg/mL. While this dose was excluded from evaluation, a significant increase in MF was noted, but no dose dependency was confirmed.

For GTC type-2, a concentration-related increase in MF was noted in the short-term treatment, both with and without metabolic activation, as well as in the 24-h continuous treatment.

Positive controls (MMS and CP) showed clear positive responses for all tests. Background mutant frequencies of negative controls, as well as plating efficiency at Day 0 and Day 2, were within the acceptable range. These data

Table 4
Results of the chromosomal aberration assay in CHL cells induced by GTC type-1

Treatment	S9 Mix	Structural aberrations						Gaps	Numerical aberrations			Cell growth (%)
		ctb	cte	csb	cse	Others	Total (%)		pol	end	Total (%)	
Short-term treatment (6–18 h)												
Negative control physiological saline	–	1	0	0	0	0	0.5	0.5	1	0	0.5	100
118.5 µg/mL	–	0	1	0	0	0	0.5	0	0	0	0	106.8
177.8 µg/mL	–	2	1	0	0	0	1.5	0	1	0	0.5	106.8
266.7 µg/mL	–	4	19	0	0	0	10.5	0	2	2	2.0	84.9
400.0 µg/mL	–	16	26	0	0	0	15.0	0	2	2	2.0	64.4
600.0 µg/mL	–	6	14	1	0	2	17.0	0	1	2	1.5	37.0
Positive control MMC 0.15 µg/mL	–	12	42	0	0	0	22.0	0	0	0	0.0	60.3
Negative control physiological saline	+	0	0	0	0	0	0.0	1	0	0	0	100
56.3 µg/mL	+	0	0	0	0	0	0	0	1	0	0.5	102.7
112.5 µg/mL	+	0	1	0	0	0	0.5	1	1	0	0.5	81.1
225.0 µg/mL	+	1	1	0	1	0	1.5	0	1	0	0.5	72.1
450.0 µg/mL	+	4	5	0	0	0	4.0	0	0	0	0	71.2
900.0 µg/mL	+	1	2	0	0	0	1.5	0	0	0	0	39.6
Positive control B(*a*)P 20 µg/mL	+	8	38	0	0	0	20.5	0	0	0	0	59.5
Continuous treatment (24–0 h)												
Negative control physiological saline	–	0	0	0	0	0	0	0	1	0	0.5	100
177.8 µg/mL	–	4	6	0	0	0	5.0	0	2	0	1.0	95.5
266.7 µg/mL	–	11	24	0	0	0	16.5	0	0	0	0	81.8
400.0 µg/mL	–	18	26	0	0	2	37.0	0	0	0	0.5	63.6
600.0/900.0 µg/mL	–	Tox										57.6/24.2
Positive control MMC 0.05 µg/mL	–	12	38	0	0	0	22.0	0	0	0	0.0	80.3
Continuous treatment (48–0 h)												
Negative control physiological saline	–	0	0	0	0	0	0	1	1	0	0.5	100
79.0 µg/mL	–	0	0	0	0	0	0	0	2	0	1.0	81.1
118.5 µg/mL	–	1	2	0	0	0	1.5	0	4	0	2.0	73.4
177.8 µg/mL	–	3	3	0	0	0	2.0	1	1	0	0.5	50.3
266.7/400.0 µg/mL	–	Tox										31.5/22.4
Positive control MMC 0.05 µg/mL	–	13	51	0	0	0	26.5	0	0	0	0	40.6

Structural aberrations: ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange.
Numerical aberrations: pol, polyploidy; end, endo-reduplication.
Test substance precipitation in the culture medium was not observed up to 900 µg/mL at the start or end of test substance treatment.

3.4. Bone marrow MN assay in mice

The results of the bone marrow MN assay in mice for GTC type-1 and GTC type-2 are presented in Tables 8 and 9, respectively. No deaths occurred as a result of the administration of either test substances. Compared with vehicle control, no significant increase in the incidence of MNPCEs for either the GTC type-1- or GTC type-2- treated groups was observed. With regard to the PCE/NCE ratio, there was no significant difference compared to controls for either the test substance-treated groups. These data indicate no significant growth suppression in erythrocytes, and may raise the question of whether the test substance was systemically available in the animals treated.

In a separate experiment, GTC type-1 and GTC type-2 were administered orally to male ICR (Crj: CD-1) mice at a dose of 2000 mg/kg, and the plasma concentration of catechins was measured at 0.5 h after administration. As a result, the average concentrations of total free catechins were 173 ng/mL (GTC type-1) and 572 ng/mL (GTC type-2), respectively (data not shown). Accordingly, it was determined that the test substances were adequately exposed to the target tissue (bone marrow) in the experiment. The positive control compound, cyclophosphamide, induced a substantial increase in the incidence of MNPCEs ($p < 0.01$). Under the conditions used in this assay, neither GTC type-1 nor GTC type-2 showed any evidence of genotoxic potential.

3.5. Bone marrow MN assay in rats

A single oral administration of green tea catechin equivalent to GTC type-1 at levels of 2000 mg/kg per weight to SD rats (under non-fasting conditions, same as the MN assay) gives a significantly higher plasma concentration of total free catechins (2321 ng/mL, average of 3 animals) at 0.5 h after administration relative to that achieved in the mice used in the previous assay, described above. Accordingly, in order to assess the potential mutagenic effect of the test substances under the most stringent

Table 5
Results of the chromosomal aberration assay in CHL cells induced by GTC type-2

Treatment	S9 Mix	Structural aberrations						Gaps	Numerical aberrations			Cell growth (%)
		ctb	cte	csb	cse	Others	Total (%)		pol	end	Total (%)	
Short-term treatment (6–18 h)												
Negative control physiological saline	–	0	0	0	0	0	0.0	0	1	0	0.5	100
229 µg/mL	–	6	15	0	0	0	9.0	0	4	1	2.5	86.8
287 µg/mL	–	4	19	0	0	1	11.0	0	6	0	3.0	76.5
358 µg/mL	–	10	16	1	0	1	11.5	0	3	0	1.5	66.2
448 µg/mL	–	7	9	0	0	3	13.0	1	3	0	2.3	42.6
560 µg/mL	–	Tox										35.3
Positive control MMC 0.15 µg/mL	–	14	38	1	0	0	22.0	1	1	0	0.5	80.9
Negative control physiological saline	+	0	0	0	0	0	0	0	2	0	1.0	100
287 µg/mL	+	8	8	0	0	1	7.5	0	2	0	1.0	71.7
358 µg/mL	+	4	16	0	0	2	10.0	0	2	0	1.0	60.6
448 µg/mL	+	4	17	0	0	2	10.5	0	2	0	1.0	46.5
560 µg/mL	+	4	9	0	0	2	6.5	0	1	1	1.0	32.3
700 µg/mL	+	1	3	0	0	3	3.5	0	2	0	1.0	22.2
Positive control B(*a*)P 20 µg/mL	+	6	39	0	0	0	19.5	0	0	0	0	66.7
Continuous treatment (24–0 h)												
Negative control physiological saline	–	0	0	0	0	0	0	0	1	0	0.5	100
79.0 µg/mL	–	1	2	0	0	0	1.5	0	2	0	1.0	83.6
119 µg/mL	–	2	7	0	0	0	4.5	0	2	0	1.0	73.1
178 µg/mL	–	6	10	0	1	1	8.0	0	2	0	1.0	49.3
267/400 µg/mL	–	Tox										35.8/34.3
Positive control MMC 0.05 µg/mL	–	21	26	0	0	0	19.5	0	0	0	0.0	62.7
Continuous treatment (48–0 h)												
Negative control physiological saline	–	1	0	0	0	0	0.5	0	2	0	1.0	100
35.1 µg/mL	–	1	0	0	0	0	0.5	0	3	0	1.5	92.6
52.7 µg/mL	–	1	0	0	0	0	0.5	0	3	0	1.5	95.7
79.0 µg/mL	–	1	1	0	0	0	1.0	0	2	0	1.0	80.9
119 µg/mL	–	2	7	0	0	0	4.0	0	1	0	0.5	70.2
178 µg/mL	–	Tox										37.2
Positive control MMC 0.05 µg/mL	–	17	37	0	0	0	22.5	0	1	0	0.5	62.8

Structural aberrations: ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange.
Numerical aberrations: pol, polyploidy; end, endo-reduplication.
Test substance precipitation in the culture medium was not observed at 5000 µg/mL at the start or end of test substance treatment.

in vivo conditions; a subsequent MN assay was conducted in SD rats. The results of the study for both GTC type-1 and GTC type-2 are presented in Table 10.

No deaths occurred following administration of the test substances. There was no significant increase in the incidence of MNPCEs in either of the test substance-treated groups when compared to vehicle control. The positive control compound, mitomycin C, induced a remarkable increase in the incidence of MNPCEs ($p < 0.01$), indicating that the study was valid. Therefore, under the conditions used in this assay, neither GTC type-1 nor GTC type-2 showed any evidence of genotoxic potential.

4. Discussion

The test substances, GTC type-1 and GTC type-2, have the same tea catechin composition of catechin-enriched beverages (Healthya® green tea beverage and Healthya® water sports drink) in the marketplace. The results of the studies described above demonstrate that there is no substantial difference in the genotoxic potential between GTC type-1 and GTC type-2. Regardless of whether the

preparation of tea catechins was heat treated, neither test substance showed mutagenic potential in the bacterial reverse mutation assay, but positive responses were observed in the *in vitro* mammalian mutation assays – i.e., the chromosomal aberration assay and the mouse lymphoma L5178Y/tk assay. However, oral administration of the test substances in both mice and rats, at the dosages up to 2000 mg/kg, showed no evidence of genotoxicity (i.e., no increase of MNPCE) in the *in vivo* test.

These results are consistent with the study results of Chang et al. (2003), which assessed the genotoxic potential of a decaffeinated green tea catechin mixture, Polyphenon E, as well as those reported by Isbrucker et al. (2006), using an epigallocatechin gallate (EGCG) preparation, Teavigo™. A negative response in the bacterial reverse mutation assay and a positive response in the *in vitro* mouse lymphoma L5178Y/tk assay were observed in both of these studies. Similarly, the *in vivo* MN assay with both Polyphenon E and Teavigo™ showed no evidence of genotoxicity.

Chang et al. (2003) further assessed the mutagenic potential of Polyphenon E using transgenic Big Blue mice administered Polyphenon E at levels of up to 2000

Table 6
Results of the mouse lymphoma cell thymidine kinase (Tk) gene mutation assay in L5178Y cells induced by GTC type-1

Treatment	S9Mix	PE0	RS0[a]	RSG[b]	PE2	RS2[c]	RTG[d]	L-MF[e]	S-MF[e]	T-MF[e]
Short-term treatment (3 h)										
Negative control physiological saline	–	103	100	100	101	100	100	54.7	34.6	91.6
257 µg/mL	–	82	79.3	88.8	130	128.4	114.0	67.7	31.1	105.4
412 µg/mL	–	55	53.2	56.0	217	213.9	119.9	47.9	25.4	76.2
659 µg/mL	–	63	61.3	61.5	130	128.4	79.0	75.0	51.4	129.8
1055 µg/mL	–	36	34.9	26.2	155	153.4	40.2	108.7	90.4	223.2
1688 µg/mL	–	19	18.3	9.8	185	182.4	17.9	63.2	105.6	187.7
2700 µg/mL	–	5	5.3	0.9	nt	nt	nt	nt	nt	nt
Positive control MMS 10 µg/mL	–	120	116.9	80.6	89	88.2	71.1	184.9	95.1	306.8
Negative control physiological saline	+	108	100	100	116	100	100	40.0	25.4	67.9
343 µg/mL	+	95	87.8	90.7	130	112.0	101.6	60.6	24.8	87.4
549 µg/mL	+	98	90.6	80.9	95	81.9	66.3	64.1	34.0	92.7
879 µg/mL	+	56	52.0	61.9	101	87.3	54.0	34.6	45.8	80.9
1406 µg/mL	+	47	43.8	39.5	173	149.4	58.9	75.1	58.1	147.9
2250 µg/mL	+	36	33.2	29.0	87	74.7	21.7	119.8	77.1	203.3
3600 µg/mL	+	3	2.5	0.0	nt	nt	nt	nt	nt	nt
Positive control CP 3 µg/mL	+	50	46.4	65.4	79	68.3	44.7	217.5	222.1	513.9
Continuous treatment (24 h)										
Negative control physiological saline	–	110	100	100	127	100	100	44.3	24.2	69.1
52.7 µg/mL	–	79	72.0	98.1	105	82.1	80.5	81.2	38.9	124.4
79.0 µg/mL	–	148	134.4	103.3	120	94.4	97.6	63.0	22.2	89.0
119 µg/mL	–	130	118.1	102.1	112	87.9	89.8	38.8	23.9	62.3
178 µg/mL	–	84	76.4	97.7	130	102.0	99.6	44.6	24.8	70.1
267 µg/mL	–	141	128.4	104.8	120	94.4	98.9	55.5	24.5	83.6
400 µg/mL	–	15	13.3	10.4	98	76.9	8.0	119.1	59.1	187.2
Positive control MMS 10 µg/mL	–	65	58.9	78.4	79	62.2	48.8	212.9	122.9	362.9

[a] RS0 (%) = (PE0 of test article/PE0 of negative control) × 100.
[b] RSG (%) = (product of cell growth[*] Day 1 and Day 2 on test article)/(product of cell growth Day 1 and Day 2 on negative control), [*] cell growth = cell concentration of designated day/cell concentration of initial day (Day 0).
[c] RS2 (%) = (PE2 of test article/PE2 of negative control) × 100.
[d] RTG (%) = RSG × RS2.
[e] MF = [−ln(number of wells without colonies/total wells)/mean number of cells per well]/PE2.

mg/kg, and observed no increase of mutagenic frequency in lung, liver, and spleen tissues. Based on these results, they concluded that Polyphenon E possesses minimal genotoxic activity. Polyphenon E is a concentrated catechin mixture wherein EGCg accounts for 50% of the total composition, with other catechins (details unknown) accounting for 30% of composition. In comparison, our green tea catechin contains a relatively lower amount of total catechins, and a different composition of each catechin monomer (Table 1). Nevertheless, there is no substantial difference in the genotoxicity profile between Polyphenon E, Teavigo™, and the green tea catechin preparations evaluated in the present study.

One potential cause of the positive response observed in *in vitro* mammalian mutation assays could be the pro-oxidant property of catechins. It has been shown that polyphenols, including catechins, can act not only as antioxidants, but also pro-oxidants under certain conditions (Sakihama et al., 2002). Several *in vitro* experiments have demonstrated that catechins can induce oxidative DNA damage, including formation of 8-oxodG (Oikawa et al., 2003; Srinivasan et al., 2002; Johnson and Loo, 2000). However, many *in vivo* experiments have shown that catechins prevent oxidative DNA damage induced by var-

ious oxidative stresses (Hasegawa et al., 1995; Inagake et al., 1995; Takabayashi et al., 1997; Lodovici et al., 2000). Moreover, in a phase II randomized controlled tea intervention trial, Hakim et al. (2003) demonstrated that urinary levels of 8-OHdG (8-oxodG) are significantly decreased among heavy smokers who consume 4 cups/day of decaffeinated green or black tea for four months. These findings suggest that, at least under physiological conditions, catechins primarily act as anti-oxidants in the presence of other oxidative stresses.

A report by Sugisawa and Umegaki (2002) supports this assertion by demonstrating that EGCg, a predominant component of catechin preparations, induced chromosomal damage in WIL2-NS cells at higher concentrations (100 µmol/L), but not at lesser concentration (<10 µmol/L), and could prevent chromosomal damage induced by reactive oxygen species at physiological concentration (<1 µmol/L) (2002). A similar observation was also reported in the *in vitro* study of Johnson and Loo (2000) discussed above. Considered in light of the negative results of the *in vivo* MN assay in the present study, these data further support the conclusion that tea catechins do not pose genotoxic concern under physiological conditions.

Table 7
Results of the mouse lymphoma cell thymidine kinase (Tk) gene mutation assay in L5178Y cells induced by GTC type-2

Treatment	S9Mix	PE0	RS0[a]	RSG[b]	PE2	RS2[c]	RTG[d]	L-MF[e]	S-MF[e]	T-MF[e]
Short-term treatment (3 h)										
Negative control physiological saline	−	118	100	100	91	100	100	49.5	31.0	80.2
68.8 µg/mL	−	116	98.2	104.3	89	98.5	102.8	58.3	23.8	81.5
124 µg/mL	−	116	98.2	88.6	120	132.7	117.6	29.1	22.2	50.6
223 µg/mL	−	87	73.3	83.6	98	108.1	90.3	32.9	38.6	74.2
401 µg/mL	−	77	65.2	71.9	130	143.3	103.1	53.7	35.7	92.4
722 µg/mL	−	47	40.1	34.0	164	180.5	61.4	96.5	92.2	215.0
1300 µg/mL	−	28	24.0	18.7	92	101.5	19.0	69.3	47.2	123.3
Positive control MMS 10 µg/mL	−	135	114.6	96.6	73	80.2	77.4	160.7	179.0	370.7
Negative control physiological saline	+	80	100	100	127	100	100	25.3	21.0	47.8
125 µg/mL	+	112	139.2	89.9	125	98.1	88.2	60.6	44.0	106.9
250 µg/mL	+	108	134.5	89.1	148	116.1	103.5	33.3	43.1	79.0
500 µg/mL	+	55	68.0	54.8	185	144.9	79.4	54.5	41.0	101.4
1000 µg/mL	+	49	60.6	52.5	141	110.9	58.3	53.6	71.2	135.2
2000 µg/mL	+	0	0.0	0.0	nt	nt	nt	nt	nt	nt
4000 µg/mL	+	nt	nt	nt	nt	nt	nt	nt	nt	nt
Positive control CP 3 µg/mL	+	40	49.1	64.9	69	53.9	35.0	295.3	348.3	766.6
Continuous treatment (24 h)										
Negative control physiological saline	−	133	100	100	108	100	100	35.0	26.0	61.7
21.2 µg/mL	−	148	111.6	114.5	92	85.1	97.4	17.2	29.0	44.2
38.2 µg/mL	−	116	87.5	100.8	84	77.7	78.3	51.7	18.9	72.4
68.8 µg/mL	−	130	98.0	113.6	101	93.6	106.3	68.9	23.7	96.3
124 µg/mL	−	112	84.4	100.8	120	111.2	112.1	78.4	31.5	116.7
223 µg/mL	−	13	9.8	5.8	135	125.1	7.3	300.9	124.6	468.4
401 µg/mL	−	1	0.5	0.0	nt	nt	nt	nt	nt	nt
Positive control MMS 10 µg/mL	−	52	39.0	68.7	65	60.0	41.2	227.1	381.8	694.3

[a] RS0 (%) = (PE0 of test article/PE0 of negative control) × 100.
[b] RSG (%) = (product of cell growth* Day 1 and Day 2 on test article)/(product of cell growth Day 1 and Day 2 on negative control), * cell growth = cell concentration of designated day/cell concentration of initial day (Day 0).
[c] RS2 (%) = (PE2 of test article/PE2 of negative control) × 100.
[d] RTG (%) = RSG × RS2.
[e] MF = [−ln (number of wells without colonies/total wells)/mean number of cells per well]/PE2.

Table 8
Results of the bone marrow micronucleus assay for GTC type-1 in mice

Treatment[a]	Incidence of MNPCE (%)[d]	Incidence of PCE (%)[f]
Negative control (physiological saline), 2 times[a]	0.08 ± 0.03	43.37 ± 2.46
500 mg/kg, 2 times[b]	0.12 ± 0.06	40.63 ± 2.41
1000 mg/kg, 2 times[b]	0.10 ± 0.06	41.73 ± 4.27
2000 mg/kg, 2 times[b]	0.09 ± 0.04	41.77 ± 4.26
Positive control (MMC 2 mg/kg)[c]	3.67 ± 0.54[*e]	42.37 ± 4.26

[a] Each treatment consists of six animals per group.
[b] Administered twice by oral gavage separated 24 h.
[c] Administered once by intraperitoneal injection.
[d] 2000 polychromatic erythrocytes were scored.
[e] Significantly different from the negative control (*$p < 0.05$) by the Kastenbaum and Bowmans method.
[f] 1000 erythrocytes were scored.

Meanwhile, Elbling et al. (2005) demonstrated that EGCG could cause DNA damage in cultured cells and exhibited oxidant activity at pharmacologically attainable concentrations. However, whether polyphenols, including catechins, can act as pro-oxidants or antioxidants is likely dependent upon the relative redox-potentials of surrounding materials. Our unpublished data indicate that the *in vitro* culture condition promotes the pro-oxidant property of catechins to generate active oxygen species with ample supply of ambient or dissolved oxygen, whereas there are few sources of oxidants upon which catechins can act as an antioxidant. As mentioned by the authors, the relevance of the *in vivo* findings needs to be carefully addressed.

In fact, with respect to genotoxicity, catechins have attracted the attention of researchers because of their observed anti-genotoxic effect. The protective properties of catechins or tea extract in response to various mutagens has been demonstrated in bacteria (Nikaidou et al., 2005; Hayatsu et al., 1992; Wang et al., 1989; Jain et al., 1989), in mammalian cells (Sinha et al., 2005; Kuroda, 1996; Cheng et al., 1991; Wang et al., 1989), and experimental animals (Muto et al., 1999). Recently, Iwai et al. (2005) reported that consumption of a catechin-enriched beverage after supper for 5 weeks diminished a common 4977-bp deletion of mitochondrial DNA in leukocytes from nine human female volunteers. Based on this finding, the authors suggested that tea catechins might contribute to the maintenance of health status by reducing damage to mtDNA.

Table 9
Results of the bone marrow micronucleus assay for GTC type-2 in mice

Treatment[a]	Incidence of MNPCE (%)[d]	Incidence of PCE (%)[f]
Negative control (physiological saline), 2 times[b]	0.12 ± 0.05	44.60 ± 2.61
500 mg/kg, 2 times[b]	0.11 ± 0.07	47.97 ± 4.17
1000 mg/kg, 2 times[b]	0.11 ± 0.04	47.67 ± 1.44
2000 mg/kg, 2 times[b]	0.09 ± 0.06	42.23 ± 1.75
Positive control (MMC 2 mg/kg)[c]	2.73 ± 0.36[*e]	39.80 ± 1.89[**f, g]

[a] Each treatment consists of six animals per group.
[b] Administered twice by oral gavage at 24 h apart.
[c] Administered once by intraperitoneal injection.
[d] 2000 polychromatic erythrocytes were scored.
[e] Significantly different from the negative control ($^*p < 0.05$) by the Kastenbaum and Bowman's method.
[f] 1000 erythrocytes were scored.
[g] Significantly different from the negative control ($^{**}p < 0.01$) by Student's t-test.

Table 10
Results of the bone marrow micronucleus assay for GTC type-1 and GTC type-2 in rats

Treatment[a]	Incidence of MNPCE (%)[d]	Incidence of PCE (%)[f]
Negative control (physiological saline), 2 times[a]	0.08 ± 0.01	53.00 ± 5.74
type-1 1000 mg/kg, 2 times[b]	0.07 ± 0.01	54.80 ± 18.30
type-1 2000 mg/kg, 2 times[b]	0.09 ± 0.01	56.00 ± 26.15
type-2 1000 mg/kg, 2 times[b]	0.08 ± 0.01	56.00 ± 6.40
type-2 2000 mg/kg, 2 times[b]	0.09 ± 0.02	52.80 ± 14.33
Positive control (MMC 2 mg/kg)[c]	3.76 ± 0.43[*e]	39.80 ± 5.18[**g]

[a] Each treatment consists of 5 animals per group.
[b] Administered twice by oral gavage separated 24 h.
[c] Administered once by intraperitoneal injection.
[d] 2000 polychromatic erythrocytes were scored.
[e] Significantly different from the negative control ($^*p < 0.05$) by the Kastenbaum and Bowmans method.
[f] 200 erythrocytes were scored.
[g] Significantly different from the negative control (## $p < 0.01$) by Student's t-test.

Indeed, many studies on the beneficial characteristics of tea catechins, including a reduction in cancer risk, following administration have been reported, although careful evaluation is needed to confirm the clinical benefits alleged in these studies.

Based on the outcomes of the present study, as well as an abundance of evidence in the medical literature demonstrating a protective role for tea catechins in mutagenesis and carcinogenesis, we conclude that tea catechin preparations present no substantial genotoxic concern under the conditions for anticipated use in catechin-enriched beverages.

Conflict of interest statement

Several authors belong to Kao Corporation that markets the tea beverages enriched with catechins evaluated in this manuscript as indicated in their affiliation. No other authors have conflict of interest regarding this manuscript.

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