Available online at www.sciencedirect.com
SCIENCE DIRECT
Food and Chemical Toxicology 44 (2006) 626–635
www.elsevier.com/locate/foodchemtox

Safety studies on epigallocatechin gallate (EGCG) preparations. Part 1: Genotoxicity

R.A. Isbrucker [a], J. Bausch [b], J.A. Edwards [b,*], E. Wolz [b]

[a] *Burdock Group, 888 17th St NW Suite 810, Washington, DC 20006, USA*
[b] *DSM Nutritional Products Ltd., Wurmisweg 576, CH-4303 Kaiseraugst, Switzerland*

Received 4 April 2005; accepted 12 July 2005

Abstract

Public interest in green tea has grown recently due to the potential health benefits from its consumption. Epigallocatechin gallate (EGCG), a principal polyphenolic component of green tea, is considered key to these healthful qualities. Although numerous studies have evaluated the anti-cancer effects of green tea and EGCG, few have examined the safety of EGCG consumption. The genotoxic potential of a concentrated EGCG preparation was tested in *Salmonella* and L5178Y $tk^{+/-}$ mouse lymphoma cell assays to further define the safety of Teavigo™, a high-concentration EGCG extract of *Camellia sinensis* leaves produced by the same novel method. No mutagenic activity was detected in the bacterial system; however, a clastogenic 'trend' from the formation of hydrogen peroxide was noted in the murine cells. The oral administration of 500, 1000, or 2000 mg EGCG/kg to mice did not induce micronuclei formation in bone marrow cells. Similarly, administering 400, 800, or 1200 mg EGCG/kg/day in their diet for 10 days did not induce bone marrow cell micronuclei and produced plasma EGCG concentrations comparable to those reported in human studies. The intravenous injection of 10, 25 and 50 mg EGCG/kg/day to rats resulted in much higher plasma concentrations and demonstrated an absence of genotoxic effects. From these studies, it is concluded that Teavigo™ (EGCG) is not genotoxic.

Keywords: Teavigo™; Epigallocatechin gallate; Genotoxicity; Bone marrow micronucleus; *Salmonella* mutagenicity

1. Introduction

Tea, made from the leaves of *Camellia sinensis*, has a long history of use, which extends for thousands of years in several Asian countries and now ranks as one of the most commonly consumed beverages in the world. Traditional medical practices have long espoused the healthful benefits of both black (fermented) and green (unfermented) tea and more recently they have been promoted for their cardioprotective and anti-carcinogenic properties (for reviews, see Higdon and Frei, 2003; Liao et al., 2001). In vitro studies have begun to identify the wide variety of biochemical and cellular mechanisms by which green tea components can potentially exert these favorable effects (Hou et al., 2004), such as the inhibition of mitogen-activated protein kinases, promoting cell cycle arrest, and blocking growth factor receptor signal transduction mechanisms. Epidemiological and clinical studies have demonstrated favorable or equivocal outcomes of green tea consumption. However, study design flaws or differences, and other confounding factors between these studies have made it difficult to derive a definitive conclusion on the healthful qualities of green tea. Nonetheless, green tea and green tea extract are gaining tremendous popularity in Western cultures and it is increasingly

Abbreviations: ADRS, adapted relative survival; AUC, area under the curve; C_{max}, maximum concentration; EGCG, epigallocatechin gallate; NCE, normochromatic erythrocytes; PCE, polychromatic erythrocytes; TFT, 5-trifluorothymidine.

* Corresponding author. Address. DSM Nutritional Products Ltd., P.O. Box 3255, Bldg 205/316a, CH-4002 Basel, Switzerland. Tel.: +41 (0)61 68 82281; fax: +41 (0)61 68 86819.
E-mail address: james-a.edwards@dsm.com (J.A. Edwards).

0278-6915/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2005.07.005

important that its safety for consumption be clearly established.

Teas, and green tea in particular, are known to be rich in polyphenolic substances, which have strong antioxidant properties. Epigallocatechin gallate (EGCG; Fig. 1), the more abundant of these compounds in green tea, is generally considered the principal antioxidant found in green tea extract. Numerous studies have illustrated the anti-genotoxic effect of EGCG in vitro (for example: Arimoto-Kobayashi et al., 2003; Hour et al., 1999; Roy et al., 2003). Similarly, studies in animals have found that EGCG inhibits the development of prostatic cancer in the murine TRAMP model (Adhami et al., 2003; Gupta et al., 2002) and reduces the chemically induced carcinogenesis of the intestines (Fujita et al., 1989; Hirose et al., 1993; Yamane et al., 1995, 1996), and lungs (Mimoto et al., 2000; Muto et al., 1999). However, others have reported a pro-oxidant and potentially genotoxic effect of EGCG at high concentrations (Bertram et al., 2003; Furukawa et al., 2003; Roy et al., 2003; Sugisawa and Umegaki, 2002). This dichotomy is not unique to EGCG, as pro-oxidant properties have been demonstrated with other compounds classically viewed as antioxidants, including vitamin C (Sakagami et al., 2001). At high concentrations, EGCG has been found to catalyze the formation of hydrogen peroxide (H_2O_2) under certain aqueous conditions (Long et al., 2000; Sugisawa and Umegaki, 2002), which can result in the promotion of genotoxic events. These detrimental effects are abrogated by the addition of catalase to the culture medium (Furukawa et al., 2003) further implicating the role of H_2O_2 formation.

In light of the increased public interest in purified EGCG consumption as a food additive, it is important that its safety at physiologically relevant concentrations and doses be clearly established. Although most genotoxicity studies involving EGCG or green tea extract examine their potential to counter the deleterious effects of known carcinogens and mutagens, very few reports have actually examined the safety of EGCG in the absence of other confounding agents or additives. DSM Nutritional Products Ltd. has recently developed a unique method for purifying EGCG from a hot water extract of *C. sinensis* leaves to produce Teavigo™, a crystalized product containing greater than 90% EGCG. The current study evaluates the safety of Teavigo™ in genotoxicity assays. Subsequent studies further this research and report on its safety in acute, dermal, subchronic and reproductive assays.

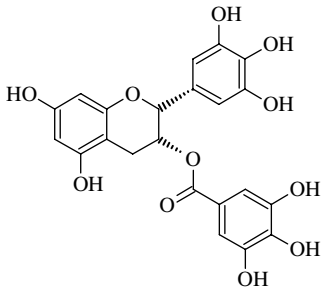

Fig. 1. Epigallocatechin gallate (EGCG, $C_{22}H_{18}O_{11}$). CAS No [989-51-5]; MW = 458.38.

2. Methods and materials

2.1. Isolation and purification of EGCG

EGCG was purified from a hot water extraction of green tea (*C. sinensis*) leaves. The initial hot water extract was further purified by separating the catechin fraction with ethylacetate. This was then subjected to chromatographic separation of EGCG from the catechin fraction in ethanol/water followed by crystallization or spray drying. This novel purification method is the basis for the production of Teavigo™, a preparation from *C. sinensis* leaves comprising greater than 90% EGCG. Although several different preparations of EGCG (of 88.1–95% purity) were used throughout this study, isolates were kept consistent within each assay. Water was detected in all EGCG preparations at concentrations of 5.6% or lower. Epicatechin gallate ($\leqslant$0.91%), gallocatechin gallate ($\leqslant$1.60%) and other, unidentified, catechins ($\leqslant$3.3%) were also detected, but their combined totals were below 5.3% in all EGCG preparations used here. Residual solvents from the extraction process were detected in some preparations but remained under 1350 ppm. Caffeine was present at less than 0.13% in all preparations. Although other impurities were detected (<12%), they were not identified. These impurities would occur naturally in green tea and, at the concentrations detected, would not be expected to affect the outcome of the assays.

2.2. Animals

Adult NMRI and CD-1 mice of both sexes were obtained from Charles River Laboratories (Sulzfeld, Germany and Margate, UK, respectively). Male and female SPF-bred Wistar rats 7–8 weeks in age were obtained from RCC Ltd. (Itingen, Switzerland). All animals were separated by sex, housed 3–5 per cage, and acclimatized for a minimum of 5 days prior to commencing the study. Unless otherwise specified, animals were provided with standard mouse or rat diet and drinking water ad libitum. All animal studies were conducted under the respective national ethical guidelines.

2.3. Ames tests

EGCG was evaluated for mutagenic activity in the Ames test using a standard *Salmonella typhimurium*

plate incorporation and preincubation methods (Ames et al., 1975; Maron and Ames, 1983), and in accordance with the Organization for Economic Co-operation and Development (OECD) guideline number 471 entitled "Bacterial Reverse Mutation Test" (www.oecd.org). Genotoxicity was assessed in *S. typhimurium* tester strains TA97, TA98, TA100, TA102 and TA1535 (courtesy of Dr. B.N. Ames, University of California, Berkeley) in the presence and absence of the postmitochondrial fraction of liver homogenates (S9) from male albino rats pre-treated with phenobarbitol/β-naphthoflavone (Gocke, 1996). The toxicity of EGCG (95% purity) was assessed in a preliminary toxicity assay by evaluating the growth of strain TA100 on NB and Vogel–Bonner minimal agar plates incorporating 50–5000 μg EGCG/plate. There was no apparent toxicity at any of the concentrations tested; however, a concentration-dependent brown coloration of the plates was observed after 2 days incubation.

Stock bacterial cultures were grown overnight at 37 °C in NB liquid medium. EGCG (0.1 ml) dissolved in dimethylsulfoxide, or reference chemicals, were added to 2 ml of 0.7% agar medium containing a histidine/biotin mixture, 0.1 ml of the overnight bacterial culture, and either 0.5 ml of the S9 mixture or 0.5 ml of phosphate-buffered saline. The contents of the tubes were mixed and poured immediately onto Vogel–Bonner minimal agar plates. Three replicate plates for the test compound and controls were incubated at 37 °C for 2 days prior to counting. In the modified procedure, 0.1 ml of the EGCG or reference compound was incubated with 0.1 ml of the bacterial culture and 0.5 ml of either phosphate-buffered saline or the S9 mixture, for 30 min at 37 °C prior to mixing with the agar medium containing histidine/biotin. The contents were mixed, poured onto agar plates, and incubated as for the standard assay. Three replicate plates per concentration were tested in this preincubation assay. Colonies were counted electronically using a DOMINO automatic image analysis system (Perceptive Instruments, Halstead, UK).

2.4. ML/TK assay

Mouse lymphoma L5178Y $tk^{+/-}$ cells (ATCC CRL 9518) were used to evaluate the mutagenicity of EGCG (77% purity) in a eukaryotic cell culture system (Clive et al., 1987; Cole et al., 1983) in compliance with OECD guideline number 476 entitled "In Vitro Mammalian Cell Gene Mutation Test" (Kirchner, 2000). Established cultures in suspension were maintained in RPMI 1640 medium supplemented with 10% horse serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml kanamycin. Forward mutations in the thymidine kinase locus were evaluated in cells following their exposure to EGCG, with and without S9, for 3 or 24 h. After the initial exposure time, the cells were centrifuged, washed twice and diluted to 2×10^5 cells/ml tissue culture media. Cultures were incubated in flasks for 2 days and maintained at or below 1×10^6 cells/ml during the expression period. For determination of survival, the cells were transferred to 96-well plates at a density of 1.6 cells/well and grown at 37 °C under normal culture conditions until sufficient growth occurred to allow scoring. Wells containing viable clones were determined by counting the empty wells under a microscope. For 5-trifluorothymidine (TFT) selection, the cells were transferred to 96-well plates as above, with the inclusion of 3 μg/ml TFT in the growth media, and grown at 37 °C until cultures could be scored.

Two cultures per concentration were used for both the EGCG and the positive control compound, which included 4-nitroquinoline-1-oxide, methyl methanesulfonate and benzo(α)pyrene. Plating efficiency, relative survival, adapted relative survival (ADRS), relative total growth, relative cell count and mutation frequency was determined for each experiment. The log mutant frequency of each treatment group was compared to that of control cultures using a one-sided Dunnett's test. The presence for a linear trend in the mutation frequency was determined using a one-sided χ^2 test.

Preliminary studies were conducted to determine the cytotoxicity of EGCG to the L5178Y $tk^{+/-}$ cells prior to the assay for mutagenicity. Treatment of the cells was as described for the mutation assay with EGCG tested at concentrations up to 2500 μg/ml with and without S9. Precipitates were noticed when EGCG was tested at concentrations of 1000 μg/ml or greater.

2.5. Assay for generation of hydrogen peroxide

The potential for EGCG to form hydrogen peroxide (H_2O_2) was measured in cell culture media types used for in vitro mutagenicity testing (Pähler et al., 2002). EGCG (91.2% purity) was added to RPMI 1640 or Ham F10 media, both without phenol red indicator, and assayed for H_2O_2 immediately, and following 30, 60, and 180 min incubations at 37 °C. H_2O_2 was measured using a commercially available kit (PeroXOquant™ Quantitative Assay; Pierce Biotechnology, Inc., Rockford, IL). This assay is based on the formation of Fe(III) ions from Fe(II) in the presence of H_2O_2 which yields a purple color when xylenol orange is included. The absorbance was determined at 560 nm and was proportional to the H_2O_2 concentration. A stabilized stock solution of H_2O_2 (3%; Molecular Probes, Eugene, OR) was used as a reference standard.

2.6. In vivo micronucleus tests

A single, oral dose micronucleus test was conducted in adult NMRI mice using Good Laboratory Practice regulations of the US Food and Drug Administration

(FDA). A single dose of 500, 1000, or 2000 mg/kg EGCG (91.9% purity) dissolved in physiological saline was administered by oral intubation at time 0 in a volume of 10 ml/kg (Muster and King, 1997). Negative control animals received an equal volume of saline. All test and control groups were comprised of five male and five female animals. Femoral bone marrow was obtained from each animal 24 h after dosing and smears were prepared for hematological evaluation. Marrow was also collected from a second set of animals 48 h after dosing with either saline, 2000 mg/kg EGCG or 50 mg/kg 9,10-dimethyl-1,2-benzanthracene (DMBA; positive control). Two slides were made per animal and stained by the May–Gruenwald–Giemsa method (Schmid, 1973). At least 1000 polychromatic erythrocytes (PCE) per animal were evaluated for the occurrence of micronuclei and the relative proportion of PCE and normochromatic erythrocytes (NCE), as well as the occurrence of micronuclei in NCE were determined in parallel.

In a second study, CD-1 mice were provided solid diet containing 4200, 8400 or 12,600 ppm EGCG (80% purity), delivering nominal doses of 400, 800, or 1200 mg EGCG/kg body weight/day (Pfannkuch et al., 2001). This study, and the following study conducted in rats, were in compliance with OECD guideline number 474 entitled "Mammalian Erythrocyte Micronucleus Test". Animals were maintained on the diet for 10 consecutive days; whereas, positive control animals received a single oral dose of cyclophosphamide (40 mg/kg) 24 h prior to necropsy. A negative control group consisted of animals receiving powdered diet alone for 10 consecutive days. All treatment groups consisted of six animals per sex per dose. Femoral bone marrow was collected 1 day after the final dosing and smears were fixed and stained according to the methods of Gollapudi and Kamra (1979). PCE and NCE counts as well as the presence of micronucleated cells were evaluated as above, and were considered significantly different from control at $p \leqslant 0.05$ as evaluated using a χ^2 test.

The EGCG concentration in the diet was verified by liquid chromatography–mass spectrometry methods. The oral bioavailability of EGCG was verified in an additional group of CD-1 mice. For this part of the study, five animals of each sex received a single oral administration of 1600 mg EGCG/kg by gavage. Blood was drawn from the animals 1 h after dosing and the plasma was analyzed for free EGCG.

To evaluate the potential in vivo genotoxicity of EGCG at greater plasma concentrations than was attained in the mouse model, EGCG was tested in rats following its intravenous (i.v.) administration. SPF-bred Wistar rats were administered EGCG (92.6% purity) at doses of 10, 25 and 50 mg/kg/day on two consecutive days (Pfannkuch et al., 2002). Negative control animals received physiological saline on two consecutive days; whereas, the positive controls were administered a single injection of 20 mg/kg cyclophosphamide on day 2. All treatment groups consisted of five male and five female animals. Femoral bone marrow cells were collected on day 3, prepared and analyzed according to the methods of Romagna and Staniforth (1989). At least 4000 PCE per animal were evaluated for the occurrence of micronuclei. The ratio of PCE to NCE, as well as the occurrence of micronuclei in NCE, was also determined. A preliminary single dose toxicokinetic study showed that a slow bolus i.v. administration (4 ml/min) of 50 mg/kg EGCG was well tolerated in six male and six female animals (Pfannkuch et al., 2002). Blood samples were drawn from these animals on regular intervals over a 24-h period for determination of plasma EGCG concentrations.

3. Results

3.1. Ames tests

Preliminary studies indicated there was no toxicity of EGCG towards strain TA100 at concentrations up to 5000 μg/plate. However, toxic effects, as indicated by a reduction of the background growth, were observed in the modified preincubation test in the absence of S9 for strains TA98, TA100, and TA 1535. This toxicity was observed at the two or three highest test concentrations, but revertant colony numbers were strongly reduced only at the maximal concentration screened. EGCG did not induce an increase of the number of revertant colonies for any of the five bacterial strains in either the standard or the preincubation methods (Table 1). Similarly, there was no promotion of genotoxic effects of EGCG in either assay conducted with S9 mixed function oxidase metabolism.

3.2. ML/TK assay

Preliminary cytotoxicity assays were used to determine the appropriate concentration ranges for the genotoxicity assay. Cell counts immediately after the 3 h exposure time showed the adapted relative survival (ADRS) at day 0 decreased to 28% at 50 μg EGCG/ml and to 1% at 100 μg/ml. After 24 h incubation with EGCG the ADRS decreased to 68% and 4% at 50 and 100 μg/ml, respectively. EGCG was less cytotoxic to the cells following 3 h incubation in the presence of S9 mix: under these conditions the ADRS was 17% at 500 μg/ml and 0% at 1000 μg/ml.

In the absence of an exogenous metabolic activating system there were no increases of mutation frequency compared with solvent control groups (Table 2), but some toxicity was noted at the lowest EGCG concentration tested with 24 h incubation. Although this effect may limit the sensitivity of the assay, there remained a clear absence of significant genotoxic events up to

Table 1
Salmonella typhimurium reversion mutation assay: EGCG (95% purity) was tested using the standardized plate incorporation assay (Panel A) and the modified preincubation method (Panel B)

S9 (μg/plate)	No. revertants/plate									
	TA97		TA98		TA 100		TA102		TA1535	
	−	+	−	+	−	+	−	+	−	+
Panel A										
0	185	209	11	16	90	89	402	391	16	8
50	175	198	11	19	92	77	421	501	14	7
150	163	206	15	17	86	83	436	497	19	8
500	161	179	13	16	92	91	420	435	13	6
1580	196	171	14	8	96	84	394	416	14	9
5000	197	170	15	13	81	74	304	314	10	10
Control	403[a]	3454[b]	184[c]	4552[b]	488[d]	2929[b]	1485[e]	1433[b]	208[b]	3319[b]
Panel B										
0	286	266	13	24	76	83	357	410	13	13
50	277	266	15	19	56	79	363	565	15	11
150	278	261	15	15	53	82	350	522	18	14
500	277	257	15	15	52	86	345	505	15	12
1580	309	230	10	13	33	73	310	416	11	16
5000	278	269	8	9	3	73	186	247	8	6
Control	1739[a]	2957[b]	222[c]	4506[b]	659[d]	2887[b]	2003[e]	1333[b]	1775[d]	484[b]

Mean values are derived from 3 plates per strain per condition.
Agents used for positive controls: [a]ICR 191; [b]2-aminoanthracene; [c]2-nitrofluorene; [d]sodium azide; [e]mitomycin C.

100 μg EGCG/ml. However, at the highest EGCG concentrations and 24 h exposure, statistical analysis indicated a slight positive linear trend. When S9 mix was included in the experiment, there was a statistically significant increase of 5-trifluorothymidine resistant mutants. These effects were observed starting at 125 μg/ml EGCG (approximately 210 μM) and increased by a factor of 2.5 at maximum concentration. These increases in mutant frequencies were strongly correlated with cytotoxic effects and were primarily due to the induction of small colony formation, which is indicative of clastogenic activity.

3.3. Assay for generation of hydrogen peroxide

A concentration- and time-dependent formation of H_2O_2 was found in RPMI culture media following the addition of EGCG (Fig. 2A). In the presence of 450 μM EGCG a maximum amount of H_2O_2 (600 μM) was formed within 60 min; however, it appeared that the peroxide was still being generated after 3 h in the presence of 90 and 180 μM EGCG. In contrast, H_2O_2 production in Ham F10 media was concentration dependent, but its formation was immediate, with the levels remaining comparable over time (Fig. 2B), and the amount of peroxide generated was significantly less than in RPMI culture media. No H_2O_2 was detected in either culture media in the absence of EGCG.

3.4. In vivo micronucleus tests

Following a single oral dose of 500, 1000, or 2000 mg/kg EGCG, mice showed temporary signs of reduced motility, but no fatalities. Bone marrow smears from treated animals showed a decreased ratio of PCE to NCE; however, the treatment did not induce a significant increase in the frequency of micronucleated PCE when examined 24 or 48 h post-dosing (Table 3). Both negative and positive control frequencies of micronucleated PCE agreed with historical values and with previous reports.

Inclusion of EGCG in the diet of mice to deliver nominal doses of 400, 800 or 1200 mg/kg/day for 10 days did not affect food consumption or weight gain of any dose group. Animals treated with EGCG at all doses had a mean ratio of PCE to NCE, which was similar to the values for the vehicle control group. The frequency of micronucleated PCE was not increased by EGCG treatment in this study (Table 4).

Mean EGCG plasma concentrations in CD-1 mice of 699.8 and 603.5 ng/ml (approximately 1.53 μM and 1.32 μM) were attained 1 h after the oral dosing of 1600 mg/kg EGCG to male and female mice, respectively. To evaluate the potential genotoxicity of EGCG at greater plasma concentrations, Wistar rats were intravenously administered 10, 25 and 50 mg/kg/day EGCG for two consecutive days. All animals survived the treatment without clinical signs during the course of the study. No inhibition of cellular proliferation, as measured by reductions of the ratio of PCE to NCE, was noted in the bone marrow (Table 5). EGCG did not increase the frequency of micronucleated PCE.

Following the intravenous injection of 50 mg/kg/day EGCG for two consecutive days, plasma concentrations of 19 and 44 μg/ml EGCG (approximately 41.5 μM and 96.0 μM) were attained 5 min after the second injection

Table 2
Mutation frequencies and viabilities of L5178Y $tk^{+/-}$ mouse lymphoma cells after 3 or 24 h exposure to control compounds or EGCG (77% purity) in the presence and absence of S9 metabolic activation

Incubation condition	μg/ml	RS[a] (%)		ADRS (%)		PE (%)		MF	
		I	II	I	II	I	II	I	II
3 h − S9	0	113	89	113	89	73	78	353	306
	20	102	91	110	92	89	73	333	389
	30	115	97	122	98	82	95	361	330
	40	91	102	103	104	74	78	327	325
	50	102	71	112	72	78	54	348	504
	60	51	46	55	44	82	87	378	440
NQO	0.1	83		69		72		1036*	
3 h + S9	0	101	99	105	95	98	77	154	311
	75	92	90	96	87	89	84	285	246
	150	73	66	77	61	89	73	365*	456*
	300	54	52	56	48	78	91	411*	325*
	400	43	60	41	57	77	78	453*	421*
	500	45	23	44	20	68	60	446*	578*
BP	1	68		61		61		1106*	
BP	2		21		17		60		1348*
24 h − S9	0	98	102	105	95	94	92	256	206
	10	95	84	65	69	84	106	287	192
	30	89	82	68	52	95	83	274	247
	60	75	78	42	41	88	76	249	326
	80	52	60	26	32	78	79	240	251
	100	43	44	12	14	75	82	311	290
MMS	5	55		59		67		1733*	
24 h + S9	0	96	104	96	104	82	95	223	207
	62.5	99	95	88	89	84	92	277	242
	125	88	96	83	95	98	91	323*	312*
	250	64	62	60	60	72	88	418*	335*
	500	45	39	43	37	72	74	476*	459*
	600	26	30	22	23	59	68	617*	460*
BP	1	48		41		54		1305*	
BP	2		11		8		39		2450*

Experiment I and II are replicate assays.
* Significant difference (Dunnett's test), $p < 0.05$.
[a] Abbreviations: RS, relative survival; ADRS, adapted relative survival; PE, plating efficiency; MF, mutation frequency (as mutants per 10^6 viable cells); NQO, 4-nitroquinoline-1-oxide; BP, benzo(α)pyrene; MMS, methyl methanesulfonate.

to female and male rats, respectively (Fig. 3). There was a steep decline in plasma concentrations within 2 h; however, concentrations of 51 (female) and 104 (male) ng/ml (approximately 0.11 μM and 0.23 μM) were detected 24 h post-dosing. Calculated area under the dose curves ($AUC_{0-24\,h}$) were approximately 12 and 29 μg h/ml for female and male animals, respectively. The lower plasma concentration in female animals was consistent among all six rats.

4. Discussion

The genotoxic potential of EGCG was evaluated in a series of in vitro and in vivo assays. In the *Salmonella* assay, EGCG was found to have some bactericidal activity at higher concentrations but without any mutagenicity. An antibacterial effect of EGCG towards Gram positive and negative organisms has previously been reported (Arakawa et al., 2004) and was found to correlate with its hydrogren peroxide production. However, our results with *S. typhimurium* TA102, which is more sensitive to the effects of hydrogen peroxide (Maron and Ames, 1983), did not suggest any mutagenic activity in the presence of EGCG. Chang et al. (2003) reported that Polyphenon E, a decaffeinated green tea catechin mixture containing 50% EGCG and 30% other catechins, was not mutagenic in *Salmonella* or *Escherichia coli* assays at concentrations up to 5000 μg/plate in the presence or absence of S9. The present results expand these findings, and indicate that a more purified form of EGCG (95% purity) remained non-mutagenic in

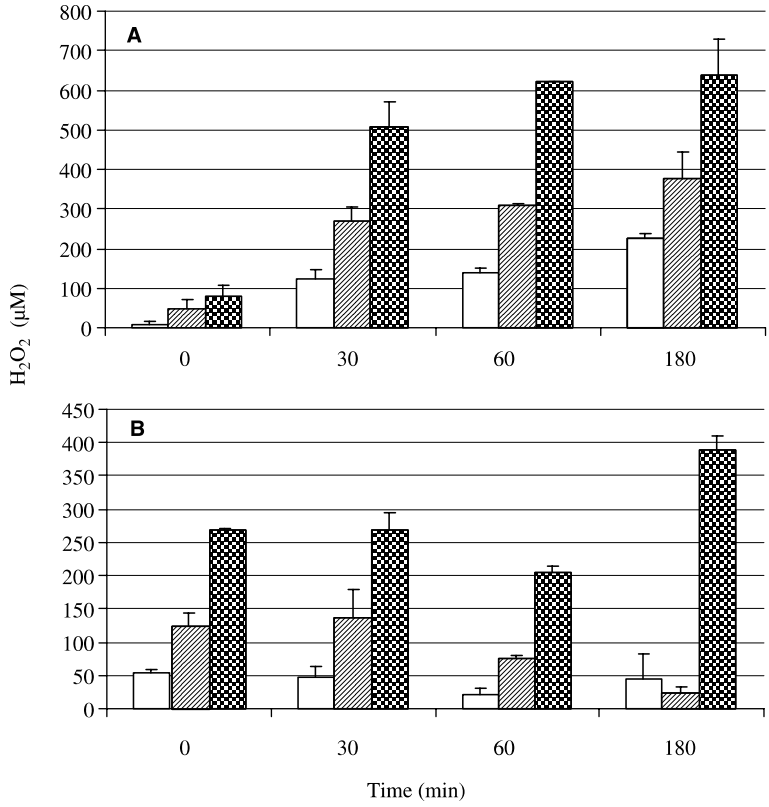

Fig. 2. Hydrogen peroxide generated in RPMI-1640 tissue culture media (A) and Ham F10 tissue culture media (B) in the presence of EGCG at 90 μM (clear bar), 180 μM (dashed bar), or 450 μM (checked bar).

Table 3
Micronucleus frequency in bone marrow cells from NMRI mice administered a single oral dose of saline (vehicle control), EGCG (91.9% purity), or 9,10-dimethyl-1,2-benzanthracene (DMBA; positive control)

Dose (mg/kg)	Sampling time (h)	Sex	No. of animals	PCE/NCE[a] ratio	Micronucleated PCE (mean per 1000 cells ± SD)
Vehicle control	24	F	5	1.62	1.54 ± 0.87
		M	5	0.95	1.77 ± 0.73
500	24	F	5	1.54	1.56 ± 0.81
		M	5	0.94	1.67 ± 0.74
1000	24	F	5	1.28	1.73 ± 0.75
		M	5	0.83	1.57 ± 0.79
2000	24	F	5	1.13	1.71 ± 0.38
		M	5	0.61	1.76 ± 0.73
Vehicle control	48	F	5	1.75	1.71 ± 0.68
		M	5	0.93	1.72 ± 0.66
2000	48	F	5	1.09	1.70 ± 0.66
		M	5	0.59	1.95 ± 0.87
DMBA	48	M	5	0.38	11.05 ± 2.13*

Bone marrow was collected 24 or 48 h after dosing.
* Significant difference from vehicle control, $p < 0.05$.
[a] Abbreviations: PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes.

bacterial cells up to 5000 μg/plate, despite a cytotoxic effect at the higher concentrations.

The in vitro clastogenic effects of EGCG to mouse lymphoma cells is in agreement with those reported by other researchers (Chang et al., 2003), and occurred at concentrations above 100 μM. In light of the high EGCG concentration, these genotoxic effects are considered an extension of peroxide formation under reducing conditions of the media. The decreased sensitivity of these cells to EGCG in the presence of rat hepatic S9 system is likely due to the presence of inherent catalase activity in the metabolic mixture and/or a sequestering of the EGCG by protein binding and not due to a metabolic de-activation of the polyphenol. The increased

Table 4
Micronucleus frequency in bone marrow cells from CD-1 mice administered a diet of rat chow containing 4200, 8400, or 12,600 ppm EGCG (80% purity) for 10 consecutive days

Nominal dose (mg/kg/day)	Sex	No. of animals	PCE/NCE[a] ratio	Micronucleated PCE (mean per 1000 cells ± SD)
0	F	6	0.94	0.67 ± 0.88
	M	6	1.01	0.50 ± 0.55
400	F	6	1.13	0.67 ± 0.52
	M	6	1.05	0.67 ± 0.26
800	F	6	0.90	0.33 ± 0.52
	M	6	1.13	0.42 ± 0.38
1200	F	6	0.99	0.25 ± 0.27
	M	6	0.93	0.58 ± 0.58
CPA	F	6	1.05	8.75 ± 4.61*
	M	6	0.96	10.50 ± 5.58*

Negative control animals received pure diet; whereas, the positive controls were fed a pure diet and administered a single oral dose of 40 mg/kg cyclophosphamide (CPA) on day 10. Bone marrow was collected on day 11.

* Significant difference from control, $p < 0.05$.

[a] Abbreviations: PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes.

Table 5
Micronucleus frequency in bone marrow cells from SPF-bred Wistar rats administered saline (vehicle control) or EGCG (92.6% purity) by intravenous injection on two consecutive days

Dose (mg/kg/day)	Sex	No. of animals	PCE/NCE[a] ratio	Micronucleated PCE (mean per 100 cells ± SD)
Vehicle control	F	5	0.88	0.10 ± 0.05
	M	5	0.35	0.10 ± 0.04
10	F	5	0.64	0.09 ± 0.06
	M	5	0.80	0.09 ± 0.06
25	F	5	0.89	0.10 ± 0.06
	M	5	0.95	0.11 ± 0.03
50	F	5	0.54	0.06 ± 0.04
	M	5	1.21	0.10 ± 0.05
CPA	F	5	0.55	0.96 ± 0.44*
	M	5	0.60	1.14 ± 0.77*

Positive control animals were administered a single intravenous dose of 20 mg/kg cyclophosphamide (CPA) 24 h prior to the collection of bone marrow cells.

* Significant difference from vehicle control, $p < 0.05$.

[a] Abbreviations: PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes.

mutation frequency and cytotoxicity observed at higher EGCG concentrations would be indicative of either the H_2O_2 production exceeding the rate of catalase activity or for secondary biological activities of EGCG itself. Roy et al. (2003) also reported that EGCG at concentrations above 100 μM significantly increased the apoptotic index of cultured K562 human lymphoma cells. Similarly, Nakagawa et al. (2004) reported that EGCG increased H_2O_2 levels in cultures of human leukemia Jurkat cells as well as in cell-free culture medium and sodium phosphate buffer. This effect was accompanied by concentration-dependent growth inhibition, an increase in caspase-3 activity and induction of nuclear fragmentation. Normal human lymphocytes and Nalm6 human leukemia cells also showed concentration-dependent DNA damage following exposure at 20–100 μM EGCG (Bertram et al., 2003), but this was not observed in cultured Chinese hamster lung cells (Tanaka, 2000). Oxidative damage to isolated cellular DNA in the presence of EGCG has been linked to the production of H_2O_2, which itself was dependent on the reducing capacity of the media (Furukawa et al., 2003). Similarly, Long et al. (2000) demonstrated the generation of peroxide in commonly used tissue culture media formulations upon the addition of EGCG or related polyphenolic compounds, and that the quantity of peroxide produced was influenced by the media type used. We have reported similar differences with almost half of the H_2O_2 being generated in Ham F10 media than in RPMI 1640. Although the transition metal content is similar between these culture media, their availability and antioxidant capacity are likely to account for the differences found. Other additives, such as serum, are also expected to have a strong influence on EGCG-promoted H_2O_2 production. In light of these findings, it seems prudent for researchers to take into consideration the culture conditions in which their experiments are conducted, especially when comparing results from cell culture systems using different media formulations.

Although several animal studies have illustrated that the in vitro anti-genotoxic effects of EGCG are comparable when using in vivo systems, only one other study is known to have evaluated EGCG for its genotoxic potential in animal models. Our results show no promotion of bone marrow micronuclei formation following a single oral dosing of EGCG to mice or when administered in feed for 10 consecutive days. Exposure of the bone marrow cells was evident from the reduced PCE/NCE ratio in the highest single-dosed group (2000 mg/kg), suggesting a toxic effect on these cells. Chang et al. (2003) reported no suppression of PCE/RBC ratio following the oral administration of 2000 mg/kg Polyphenon E to mice. However, these authors did report a small, but statistically significant increase in micronucleated PCE at two intermediate doses but no dose-related trend was found. This same study also reported no organ-specific mutations when Polyphenon E was administered at 500 or 1000 mg/kg/day for 28 consecutive days to $B6C3F_1$ Big Blue® *lacI*/*cII* transgenic mice.

In a separate experiment, we found that mouse plasma EGCG concentrations following a single oral dose of 1600 mg/kg EGCG were comparable to the reported human plasma concentrations following the oral administration of 400–800 mg EGCG (Chow et al., 2001, 2003; Ullmann et al., 2003, 2004), and much greater than following a dose of 2 mg/kg (Lee et al.,

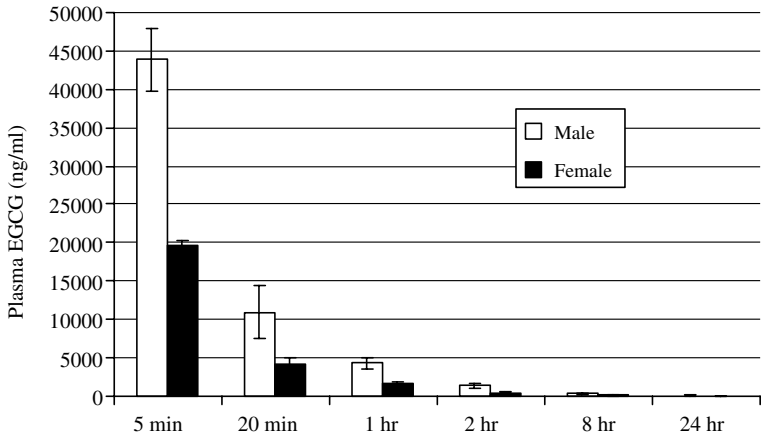

Fig. 3. Plasma EGCG concentration after the administration of the second of two consecutive doses of 50 mg/kg/day EGCG (92.6% purity) to SPF-bred Wistar rats. Bars represent the mean from 6 animals ± standard deviation.

2002). However, the plasma concentrations attained in these mice (1.3–1.5 μM) is still far below the minimum concentration found to induce any genotoxic effect in mouse lymphoma cells (210 μM). The in vitro no-effect level of 62.5 μg/ml was approximately 90-fold higher than the maximum plasma concentrations observed in human volunteers administered 400 mg EGCG as Teavigo™ (Ullmann et al., 2003, 2004). Because it was felt that the highest oral dose did not provide a reasonable margin of safety in terms of the plasma concentrations when compared to that found in humans, another study was conducted using rats administered EGCG intravenously. Two doses, 24 h apart, of 50 mg EGCG/kg/day produced plasma EGCG levels up to 44 μg/ml (approximately 96 μM)—a plasma concentration 18–45 times greater than that observed in humans given an oral dose of 800 mg EGCG as Teavigo™ (Ullmann et al., 2003, 2004), and within a concentration range which is known to induce genotoxic events in cell culture systems. Even at this greatly elevated dose there was neither a reduction of the PCE/NCE ratio nor an induction of micronucleated PCE.

It is clear from these animal studies that the potential clastogenic effects of EGCG observed in cultured eukaryotic cell systems is not translated to the in vivo condition, even when administered to rats at sufficiently high doses to yield plasma concentrations approaching 100 μM. In general, cell culture conditions, which are highly sensitive to oxidants, do not reflect the true physiological situation where the ubiquitous presence of catalase, glutathione peroxidase and other in vivo antioxidant systems makes the EGCG-catalyzed formation of H_2O_2 biologically negligible. Given the history of green tea consumption, data published in the literature regarding its potential anti-genotoxic effects, and from the studies presented here, we conclude that EGCG is non-genotoxic, even when administered to animals at doses which are significantly higher than those intended for humans.

Acknowledgements

We wish to thank E. Gocke, W. Muster, S. Kirchner, and A. Pähler from F. Hoffmann-La Roche Pharma Division for their expert help during the course of the genotoxicity testing studies.

References

Adhami, V.M., Ahmad, N., Mukhtar, H., 2003. Molecular targets for green tea in prostate cancer prevention. Journal of Nutrition 133, 2417S–2424S.

Ames, B.N., McCann, J., Yamasaki, E., 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31, 347–364.

Arakawa, H., Maeda, M., Okubo, S., Shimamura, T., 2004. Role of hydrogen peroxide in bactericidal action of catechin. Biological and Pharmaceutical Bulletin 27, 277–281.

Arimoto-Kobayashi, S., Inada, N., Sato, Y., Sugiyama, C., Okamoto, K., Hayatsu, H., Negishi, T., 2003. Inhibitory effects of (−)-epigallocatechin gallate on the mutation, DNA strand cleavage, and DNA adduct formation by heterocyclic amines. Journal of Agricultural and Food Chemistry 51, 5150–5153.

Bertram, B., Bollow, U., Rajaee-Behbahani, N., Burkle, A., Schmezer, P., 2003. Induction of poly(ADP-ribosyl)ation and DNA damage in human peripheral lymphocytes after treatment with (−)-epigallocatechin-gallate. Mutation Research 534, 77–84.

Chang, P.Y., Mirsalis, J., Riccio, E.S., Bakke, J.P., Lee, P.S., Shimon, J., Phillips, S., Fairchild, D., Hara, Y., Crowell, J.A., 2003. Genotoxicity and toxicity of the potential cancer-preventive agent polyphenon E. Environmental and Molecular Mutagenesis 41, 43–54.

Chow, H.H., Cai, Y., Alberts, D.S., Hakim, I., Dorr, R., Shahi, F., Crowell, J.A., Yang, C.S., Hara, Y., 2001. Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. Cancer Epidemiology, Biomarkers and Prevention 10, 53–58.

Chow, H.-H.S., Cai, Y., Hakim, I.A., Crowell, J.A., Shahi, F., Brooks, C.A., Dorr, R.T., Hara, Y., Alberts, D.S., 2003. Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. Clinical Cancer Research 9, 3312–3319.

Clive, D., Caspary, W., Kirby, P.E., Krehl, R., Moore, M., Mayo, J., Oberly, T.J., 1987. Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity. Mutation Research 189, 143–156.

Cole, J., Arlett, C.F., Green, M.H., Lowe, J., Muriel, W., 1983. A comparison of the agar cloning and microtitration techniques for assaying cell survival and mutation frequency in L5178Y mouse lymphoma cells. Mutation Research 111, 371–386.

Fujita, Y., Yamane, T., Tanaka, M., Kuwata, K., Okuzumi, J., Takahashi, T., Fujiki, H., Okuda, T., 1989. Inhibitory effect of (−)-epigallocatechin gallate on carcinogenesis with *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine in mouse duodenum. Japanese Journal of Cancer Research 80, 503–505.

Furukawa, A., Oikawa, S., Murata, M., Hiraku, Y., Kawanishi, S., 2003. (−)-Epigallocatechin gallate causes oxidative damage to isolated and cellular DNA. Biochemical Pharmacology 66, 1769–1778.

Gocke, E. 1996. Evaluation of the mutagenic potential of Ro 26-7624/000, (−)-epigallocatechin gallate in the Ames test (Study No.: 017M96). Roche Vitamins Ltd., Report No. B-163'294.

Gollapudi, B., Kamra, O.P., 1979. Application of a simple Giemsa-staining method in the micronucleus test. Mutation Research 64, 45–46.

Gupta, S., Saha, B., Giri, A.K., 2002. Comparative antimutagenic and anticlastogenic effects of green tea and black tea: a review. Mutation Research 512, 37–65.

Higdon, J.V., Frei, B., 2003. Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. Critical Reviews in Food Science and Nutrition 43, 89–143.

Hirose, M., Hoshiya, T., Akagi, K., Takahashi, S., Hara, Y., Ito, N., 1993. Effects of green tea catechins in a rat multi-organ carcinogenesis model. Carcinogenesis 14, 1549–1553.

Hou, Z., Lambert, J.D., Chin, K.V., Yang, C.S., 2004. Effects of tea polyphenols on signal transduction pathways related to cancer chemoprevention. Mutation Research 555, 3–19.

Hour, T.C., Liang, Y.C., Chu, I.S., Lin, J.K., 1999. Inhibition of eleven mutagens by various tea extracts, (−)epigallocatechin-3-gallate, gallic acid and caffeine. Food and Chemical Toxicology 37, 569–579.

Kirchner, S. 2000. Ro 26-7624/000: Mouse lymphoma cell mutation test (ML/TK) Study No. 214M00. Roche Vitamins Ltd., Report No. 1002914.

Lee, M.J., Maliakal, P., Chen, L., Meng, X., Bondoc, F.Y., Prabhu, S., Lambert, G., Mohr, S., Yang, C.S., 2002. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. Cancer Epidemiology, Biomarkers and Prevention 11, 1025–1032.

Liao, S., Kao, Y.H., Hiipakka, R.A., 2001. Green tea: biochemical and biological basis for health benefits. Vitamins and Hormones 62, 1–94.

Long, L.H., Clement, M.V., Halliwell, B., 2000. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochemical and Biophysical Research Communications 273, 50–53.

Maron, D.M., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. Mutation Research 113, 173–215.

Mimoto, J., Kiura, K., Matsuo, K., Yoshino, T., Takata, I., Ueoka, H., Kataoka, M., Harada, M., 2000. Epigallocatechin gallate can prevent cisplatin-induced lung tumorigenesis in A/J mice. Carcinogenesis 21, 915–919.

Muster, W., King, M.T. 1997. Ro 26-7624/000: Micronucleus test (MNT) in mouse bone marrow. Roche Vitamins Ltd., Report No. B-165'533.

Muto, S., Yokoi, T., Gondo, Y., Katsuki, M., Shioyama, Y., Fujita, K., Kamataki, T., 1999. Inhibition of benzo[*a*]pyrene-induced mutagenesis by (−)-epigallocatechin gallate in the lung of *rpsL* transgenic mice. Carcinogenesis 20, 421–424.

Nakagawa, H., Hasumi, K., Woo, J.T., Nagai, K., Wachi, M., 2004. Generation of hydrogen peroxide primarily contributes to the induction of Fe(II)-dependent apoptosis in Jurkat cells by (−)-epigallocatechin gallate. Carcinogenesis 25, 1567–1574.

Pähler, A., Klammers, F., Gocke, E. 2002. Generation of hydrogen peroxide from epigallocatechin gallate in cell culture media. Roche Vitamins Ltd., Report No. 1007579.

Pfannkuch, F., Wolz, E., Marshall, R., Bachmann, M. 2001. Ro 26-7624/000 (EGCG): Induction of micronuclei in the bone marrow of treated mice. Roche Vitamins Ltd., Report No. 1004250.

Pfannkuch, F., Wolz, E., Rosner, E., Chetelat, A.A., Bachmann, M. 2002. Ro 26-7624/000 (EGCG): Micronucleus assay in bone marrow cells of the rat after intravenous administration. Roche Vitamins Ltd, Report No. 1009357.

Romagna, F., Staniforth, C.D., 1989. The automated bone marrow micronucleus test. Mutation Research 213, 91–104.

Roy, M., Chakrabarty, S., Sinha, D., Bhattacharya, R.K., Siddiqi, M., 2003. Anticlastogenic, antigenotoxic and apoptotic activity of epigallocatechin gallate: a green tea polyphenol. Mutation Research 523–524, 33–41.

Sakagami, H., Arakawa, H., Maeda, M., Satoh, K., Kadofuku, T., Fukuchi, K., Gomi, K., 2001. Production of hydrogen peroxide and methionine sulfoxide by epigallocatechin gallate and antioxidants. Anticancer Research 21, 2633–2641.

Schmid, W., 1973. Chemical mutagen testing on in vivo somatic mammalian cells. Agents and Actions 3/2, 77–85.

Sugisawa, A., Umegaki, K., 2002. Physiological concentrations of (−)-epigallocatechin-3-*O*-gallate (EGCg) prevent chromosomal damage induced by reactive oxygen species in WIL2-NS cells. Journal of Nutrition 132, 1836–1839.

Tanaka, R., 2000. Protective effects of (−)-epigallocatechin gallate and (+)-catechin on paraquat-induced genotoxicity in cultured cells. Journal of Toxicological Sciences 25, 199–204.

Ullmann, U., Haller, J., Decourt, J.P., Girault, N., Girault, J., Richard-Caudron, A.S., Pineau, B., Weber, P., 2003. A single ascending dose study of epigallocatechin gallate in healthy volunteers. Journal of International Medical Research 31, 88–101.

Ullmann, U., Haller, J., Decourt, J.D., Girault, J., Spitzer, V., Weber, P., 2004. Plasma-kinetic characteristics of purified and isolated green tea catechin epigallocatechin gallate (EGCG) after 10 days repeated dosing in healthy volunteers. International Journal for Vitamin and Nutrition Research 74, 269–278.

Yamane, T., Takahashi, T., Kuwata, K., Oya, K., Inagake, M., Kitao, Y., Suganuma, M., Fujiki, H., 1995. Inhibition of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced carcinogenesis by (−)-epigallocatechin gallate in the rat glandular stomach. Cancer Research 55, 2081–2084.

Yamane, T., Nakatani, H., Kikuoka, N., Matsumoto, H., Iwata, Y., Kitao, Y., Oya, K., Takahashi, T., 1996. Inhibitory effects and toxicity of green tea polyphenols for gastrointestinal carcinogenesis. Cancer 77, 1662–1667.