Table 7
Application of different top concentrations to 38 missed chemicals (13 with minimal or some concern and 25 with negligible concern).

ID no.	Chemical name	CAS	MW	LEC (mM)	LEC (mg/mL)	Detection at different top concentration limit			
						1 mM or 0.5 mg/mL, whichever is higher	2 mM or 1 mg/mL, whichever is higher	4 mM or 2 mg/mL, whichever is lower	10 mM or 2 mg/mL, whichever is lower
13 missed chemicals with minimal or some concern									
84	Methyl acetoacetate	105-45-3	116.1	10.0	1.2	No	No	No	Yes
86	1,3-Bis(2-methylphenyl)guanidine	97-39-2	239.3	2.5	0.6	No	Yes	Yes	Yes
90	*N*-Ethylaniline	103-69-5	121.2	9.1	1.1	No	No	No	Yes
95	1,3,5-Tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)isocyanuric acid	27676-62-6	784.1	3.2	2.5	No	No	No	No
100	Ethenyltrimethoxysilane	2768-02-7	148.2	5.0	0.8	No	Yes	No	Yes
102	C.I. Fluorescent brightner 271	41267-43-0	1347.1	3.7	5.0	No	No	No	No
104	Dibutyl adipate	105-99-7	258.4	2.5	0.7	No	Yes	Yes	Yes
106	*N,N*-Dimethylbenzylamine	103-83-3	135.2	3.8	0.4	Yes	Yes	Yes	Yes
110	2-Hydroxypropanenitrile	78-97-7	71.1	10.0	0.7	No	Yes	No	Yes
111	2-Mercaptobenzimidazole	583-39-1	150.2	5.3	0.8	No	Yes	No	Yes
112	*N*-Methylaniline	100-61-8	107.2	5.5	0.6	No	Yes	No	Yes
115	Trimethoxyphosphine	121-45-9	124.1	10.0	1.2	No	No	No	Yes
116	Trimethylamine	75-50-3	59.1	6.4	0.4	Yes	Yes	No	Yes
	Number of chemicals detected among the 13 chemicals					2	8	3	11
25 missed chemicals with negligible concern									
79	3-Aminobenzenesulfonic acid	121-47-1	173.2	2.4	0.4	Yes	Yes	Yes	Yes
80	2-Amino-5-chloro-4-methylbenzenesulfonic acid	88-53-9	221.5	9.0	2.0	No	No	No	Yes
81	2-Amino-5-methylbenzenesulfonic acid	88-44-8	187.2	5.1	1.0	No	Yes	No	Yes
82	Glycerol triacetate	102-76-1	218.2	10.0	2.2	No	No	No	No
83	4-Hydroxybenzoic acid	99-96-7	138.1	5.1	0.7	No	Yes	No	Yes
85	1-Naphthylacetic acid	86-87-3	186.2	9.1	1.7	No	No	No	Yes
87	*tert*-Butyl-methacrylate	585-07-9	142.2	2.8	0.4	Yes	Yes	Yes	Yes
88	*o*-Dichlorobenzene	95-50-1	147.0	1.6	0.2	Yes	Yes	Yes	Yes
89	Dicyclohexylamine	101-83-7	181.3	3.3	0.6	No	Yes	Yes	Yes
91	2-Hydroxyethyl methacrylate	868-77-9	130.2	5.0	0.7	No	Yes	No	Yes
92	4-Methylbenzoic acid	99-94-5	136.2	8.8	1.2	No	No	No	Yes
93	Triphosphoric acid aluminium salt	13939-25-8	317.9	6.3	2.0	No	No	No	Yes
94	4,4′-Sulfonyldiphenol	80-09-1	250.3	1.6	0.4	Yes	Yes	Yes	Yes
96	1,2-Dicyanobenzene	91-15-6	128.1	2.5	0.3	Yes	Yes	Yes	Yes
97	2-(Diethylamino)ethyl methacrylate	105-16-8	185.3	3.2	0.6	No	Yes	Yes	Yes
98	Methacrylic acid, monoester with propane-1,2-diol	27813-02-1	144.2	5.0	0.7	No	Yes	No	Yes
99	(Methacryloyloxyethyl) trimethylammonium chloride	5039-78-1	207.7	10.0	2.1	No	No	No	No
101	2-Chlorophenol	95-57-8	128.6	2.0	0.3	Yes	Yes	Yes	Yes
103	1,4-Dibromobenzene	106-37-6	235.9	2.3	0.6	No	Yes	Yes	Yes
105	2-(Di-*n*-butylamino)ethanol	102-81-8	173.3	1.9	0.3	Yes	Yes	Yes	Yes
107	2,4-Dinitrophenol	51-28-5	184.1	6.5	1.2	No	No	No	Yes
108	2-Ethylbutyric acid	88-09-5	116.2	3.4	0.4	Yes	Yes	Yes	Yes
109	Ferrous sulfate heptahydrate	7782-63-0	278.0	1.8	0.5	Yes	Yes	Yes	Yes
113	*p*-Nitrophenol sodium salt	824-78-2	161.1	3.8	0.6	No	Yes	Yes	Yes
114	Sorbitan monooctadecanoate	1338-41-6	430.6	2.5	1.1	No	No	Yes	Yes
	Number of chemicals detected among the 25 chemicals					9	17	14	23

Table 8
Comparison of selection of top test concentration for chemicals with different molecular weight in 2 mM or 1 mg/mL, whichever is higher.

Molecular weight	Selection of 2 mM or 1 mg/mL (whichever is higher)		
100	2 mM (0.2 mg/mL)	<	1 mg/mL (10 mM)
300	2 mM (0.6 mg/mL)	<	1 mg/mL (3.3 mM)
500	2 mM (1 mg/mL)	=	1 mg/mL (2 mM)
800	2 mM (1.6 mg/mL)	>	1 mg/mL (1.3 mM)
1000	2 mM (2 mg/mL)	>	1 mg/mL (1 mM)

Underlines show concentration to be selected.

carcinogenicity information, so we determined the biologic relevancy of *in vitro* CA induction based on the weight of evidence approach. Results from *in vitro* CA test with CHL cells only might lead to biased conclusions. However, the strength of our study is the high reliability of the test results due to the fact that all data were generated according to national or international test guideline under GLP conditions. Therefore, our analysis would be helpful to discuss on top concentration issues. In this analysis, many "irrelevant" positives by extreme culture conditions (low pH, high toxicity, and precipitation) were also identified. Note that CHL cells are often described as among the most sensitive cells, *i.e.*, effects observed at lower concentrations as compared to the other cell lines. The recently suggested improvements in testing are important to reduce irrelevant positives, in addition to defining the top concentration. Data from *in vitro* mammalian genotoxicity tests, using the criteria defined by this paper, should be helpful in genotoxic hazard identification.

Conflict of interest

There are no conflicts of interest.

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References

[1] D. Kirkland, S. Pfuhler, D. Tweats, M. Aardema, R. Corvi, F. Darroudi, A. Elhajouji, H. Glatt, P. Hastwell, M. Hayashi, P. Kasper, S. Kirchner, A. Lynch, D. Marzin, D. Maurici, J. Meunier, L. Müller, G. Nohynek, J. Parry, E. Parry, V. Thybaud, R. Tice, J. van Benthem, P. Vanparys, P. White, How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM workshop, Mutat. Res. 628 (2007) 31–55.
[2] R.K. Elespuru, R. Agarwal, A.H. Atrakchi, C.A.H. Bigger, R.H. Heflic, D.R. Jagannath, D.D. Levy, M.M. Moore, Y. Ouyang, T.W. Robinson, R.E. Sotomayor, M.M. Cimino, K.L. Dearfield, Current and future application of genetic toxicology assays: the role and value of in vitro mammalian assays, Toxicol. Sci. 109 (2009) 172–179.
[3] J.M. Parry, E. Parry, P. Phrakonkham, R. Corvi, Analysis of published data for top concentration considerations in mammalian cell genotoxicity testing, Mutagenesis 25 (2010) 531–538.
[4] D. Kirkland, P. Fowler, Further analysis of Ames-negative rodent carcinogens that are only genotoxic in mammalian cells in vitro at concentrations exceeding 1 mM, including retesting of compounds of concern, Mutagenesis 25 (2010) 539–553.
[5] S. Galloway, E. Lorge, M.J. Aardema, D. Eastmond, M. Fellows, R. Heflich, D. Kirkland, D.D. Levy, A.M. Lynch, D. Marzin, T. Morita, M. Schuler, G. Speit, Workshop summary: top concentration for in vitro mammalian cell genotoxicity assays; and report from working group on toxicity measures and top concentration for in vitro cytogenetics assays (chromosome aberrations and micronucleus), Mutat. Res. 723 (2011) 77–83.
[6] M.M. Moore, M. Honma, J. Clements, T. Awogi, G.R. Douglas, F. van Goethem, B.B. Gollapudi, A. Kimura, W. Muster, M. O'Donovan, R. Schoeny, S. Wakuri, Suitable top concentration for tests with mammalian cells: mouse lymphoma assay workgroup, Mutat. Res. 723 (2011) 84–86.
[7] D. Kirkland, M. Aardema, L. Henderson, L. Müller, Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity, Mutat. Res. 584 (2005) 1–256.
[8] E.J. Matthews, N.L. Kruhlak, M.C. Cimino, R.D. Benz, J.F. Contrera, An analysis of genetic toxicity, reproductive and developmental toxicity and carcinogenicity data, I. Identification of carcinogens using surrogate endpoints, Regul. Toxicol. Pharmacol. 44 (2006) 83–96.
[9] E.J. Matthews, N.L. Kruhlak, M.C. Cimino, R.D. Benz, J.F. Contrera, An analysis of genetic toxicity, reproductive and developmental toxicity and carcinogenicity data, II. Identification of genotoxicants, reprotoxicants and carcinogens using in silico methods, Regul. Toxicol. Pharmacol. 44 (2006) 97–110.
[10] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), S2A Guideline, Guidance on specific aspects of regulatory genotoxicity tests for pharmaceuticals, Step 4 version, dated 19 July 1995. Available at http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step4/S2A_step_4.pdf (Accessed March 16, 2011).
[11] Organization for Economic Co-operation and Development (OECD), Guideline for the testing of chemicals, no. 473, In vitro mammalian cell chromosome aberration test, adopted May 26, 1983 (original) and July 21, 1997 (updated). Available at http://oberon.sourceoecd.org/vl=9267407/cl=19/nw=1/rpsv/cgi-bin/fulltextew.pl?prpsv=/ij/oecdjournals/1607310x/v1n4/s38/p1.idx (Accessed March 16, 2011).
[12] International Conference on Harmonization, S2(R1) Guideline, Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use, step 2 version, March 6, 2008. Available at http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step2/S2_R1_Guideline.pdf (Accessed March 16, 2011).
[13] Ministry of Health Labor and Welfare of Japan (MHLW), Toxicity Testing Reports of Environmental Chemicals, vol. 1, 1994.
[14] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 2, 1995.
[15] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 3, 1996.
[16] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 4, 1996.
[17] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 5, 1997.
[18] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 6, 1998.
[19] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 7, 1999.
[20] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 8 (1), 2001.
[21] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 8 (2), 2001.
[22] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 9, 2002.
[23] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 10, 2003.
[24] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 11, 2004.
[25] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 12, 2005.
[26] MHLW, Toxicity Testing Reports of Environmental Chemicals, vol. 13, 2006.
[27] Chromosomal aberration test in cultured mammalian cells, 49 Kikyoku no. 392, Law concerning examination and regulation of manufacture, etc. of chemical substances, 1974.
[28] M. Ishidate (Ed.), Data Book on Chromosomal Aberration Test, LIC Co., Tokyo, 1987, pp. 19–24 (revised).
[29] V.R. Williams, T.R. Naven, A.C. Marchant, A. Hirose, E. Kamata, M. Hayashi, Derek for windows assessment of chromosomal aberration effects, Toxicol. Lett. 164 (Suppl. (1)) (2006) S292.
[30] O. Mekenyan, M. Todorov, R. Serafimova, S. Stoeva, A. Aptula, R. Finking, E. Jacob, Identifying the structural requirements for chromosomal aberration by incorporating molecular flexibility and metabolic activation of chemicals, Chem. Res. Toxicol. 20 (2007) 1927–1941.
[31] O.G. Mekenyan, S.D. Dimitrov, T.S. Pavlov, G.D. Veith, A systematic approach to simulating metabolism in computational toxicology. I. The TIMES heuristic modeling framework, Curr. Pharm. Des. 10 (2004) 1273–1293.
[32] O. Mekenyan, S. Dimitrov, N. Dimitrova, G. Dimitrova, T. Pavlov, G. Chankov, S. Kotov, K. Vasilev, R. Vasilev, Metabolic activation of chemicals; *in-silico* simulation, SAR QSAR Environ. Res. 17 (2006) 107–120.
[33] R. Serafimova, M. Todorov, T. Pavlov, S. Kotov, E. Jacob, A. Aptula, O. Mekenyan, Identification of the structural requirements for mutagencitiy, by incorporating molecular flexibility and metabolic activation of chemicals. II. General Ames mutagenicity model, Chem. Res. Toxicol. 20 (2007) 662–676.
[34] OECD Screening Information Data Sets (SIDS), 2,3-Dichloronitrobenzene (3209-22-1), 1994. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/3209221.pdf (Accessed March 16, 2011).
[35] OECD SIDS, Glucidyl methacrylate (106-91-2), 2000. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/106912.pdf (Accessed March 16, 2011).
[36] OECD SIDS, 2,4-Dichloronitrobenzene (611-06-3), 1996. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/611063.pdf (Accessed March 16, 2011).
[37] OECD SIDS, Dicyclopentadiene (77-73-6), 1998. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/77736.pdf (Accessed March 16, 2011).
[38] OECD SIDS, Isocyanuric acid (108-80-5), 1999. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/108805.pdf (Accessed March 16, 2011).
[39] OECD SIDS, Trimethyl phosphate (512-56-1), 1996. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/512561.pdf (Accessed March 16, 2011).
[40] OECD SIDS, Glycerol triacetate (102-76-1), 2002. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/102761.pdf (Accessed March 16, 2011).
[41] OECD SIDS, 1,2-Dichlorobenzene (95-50-1), 2001. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/95501.pdf (Accessed March 16, 2011).

[42] OECD SIDS, 1,3-Di-o-tolylguanidine (97-39-2), 2007. Available at OECD Existing Chemicals Database, http://webnet.oecd.org/hpv/UI/Search.aspx (Accessed March 16, 2011).
[43] OECD SIDS, Dicyclohexylamine (101-83-7), 2006. Available at OECD Existing Chemicals Database, http://webnet.oecd.org/hpv/UI/Search.aspx (Accessed March 16, 2011).
[44] OECD SIDS, 2-Hydroxyethyl methacrylate (868-77-9), 2001. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/868779.pdf (Accessed March 16, 2011).
[45] OECD SIDS, p-Toluic acid (99-94-5), 2008. Available at OECD Existing Chemicals Database, http://webnet.oecd.org/hpv/UI/Search.aspx (Accessed March 16, 2011).
[46] OECD SIDS, o-Phthalodinitrile (91-15-6), 2001. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/91156.pdf (Accessed March 16, 2011).
[47] OECD SIDS Initial Assessment Profile, Iron salts category, 2007. Available at OECD Existing Chemicals Databse, http://webnet.oecd.org/hpv/UI/Search.aspx (Accessed March 16, 2011).
[48] OECD SIDS, Dibutyl adipate (105-99-7), 1996. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/105997.pdf (Accessed March 16, 2011).
[49] OECD SIDS Initial Assessment Profile, 2-Ethylbutyric acid, 2006. Available at OECD Existing Chemicals Database, http://webnet.oecd.org/hpv/UI/Search.aspx (Accessed March 16, 2011).
[50] OECD SIDS, 2-Hydroxypropanenitrile (78-97-7), 1994. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/HYDROXYPROPANE.pdf (Accessed March 16, 2011).
[51] OECD SIDS, 4-Aminotoluene-3-sulfonic acid (88-44-8), 2003. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/88448.pdf (Accessed March 16, 2011 July 7, 2011).
[52] OECD SIDS, 4-Hydroxybenzoic acid (99-96-7), 1999. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/99967.pdf (Accessed July 7, 2011).
[53] OECD SIDS, 2-Dimethylaminoethylmethacrylate (2867-47-2), 2002. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/DIMETHYLAMINO.pdf (Accessed March 16, 2011).
[54] H. Kusakabe, K. Yamakage, S. Wakuri, K. Sasaki, Y. Nakagawa, M. atanabe, M. Hayashi, T. Sofuni, H. Ono, N. Tanaka, Relevance of chemical structure and cytotoxicity to the induction of chromosome aberrations based on the testing results of 98 high production volume industrial chemicals, Mutat. Res. 517 (2002) 187–198.
[55] US National Toxicology Program (NTP) Database, Available at http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm?fuseaction=salmonella.overallresults&cas_no=111-41-1&endpointlist=SA (Accessed March 16, 2011).
[56] US NTP Database, Available at http://ntp.niehs.nih.gov/INDEX2118.HTM (Accessed July 7, 2011).
[57] US NTP Database, Available at http://ntp-apps.niehs.nih.gov/ntp_tox/index.cfm?fuseaction=salmonella.overallresults&cas_no=95-64-7&endpointlist=SA (Accessed March 16, 2011).
[58] T. Morita, Y. Watanabe, K. Takeda, K. Okumura, Effects of pH in the in vitro chromosomal aberration test, Mutat. Res. 225 (1989) 55–60.
[59] T. Morita, K. Takeda, K. Okumura, Evaluation of clastrgenicity of formic acid, acetic acid and lactic acid on cultured mammalian cells, Mutat. Res. 240 (1990) 195–202.
[60] T. Morita, T. Nagaki, I. Fukuda, K. Okumura, Clastogenicity of low pH to various cultured mammalian cells, Mutat. Res. 268 (1992) 297–305.
[61] California Environmental Protection Agency, Toxicology data review summaries, 1-Naphthaleneacetic acid, November 3, 1999. Available at http://www.cdpr.ca.gov/docs/risk/toxsums/pdfs/423.pdf (Accessed March 16, 2011).
[62] Concise International Chemical Assessment Document, No. 4, Methyl methacrylate, International Programme on Chemical Safety, WHO, 1998. Available at http://www.who.int/ipcs/publications/cicad/en/cicad04.pdf (Accessed July 7, 2011).
[63] M.J. Aardema, S. Galloway, E. Zeiger, M.C. Cimino, M. Hayashi, Guidance for understanding solubility as a limiting factor for selecting the upper test concentration in the OECD in vitro micronucleus assay test guideline no. 487, Mutat. Res. 722 (2011) 89–90.
[64] T. Sofuni (Ed.), Data Book of Chromosomal Aberration Test In Vitro, Life-Science Information Center, Tokyo, 1999 (revised edition in 1998).
[65] European Food Safety Authority (EFSA), Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request related to a 17th list of substances for food contact materials, EFSA J. 601–609 (2007) 1–24, Available at http://www.efsa.europa.eu/en/efsajournal/doc/afc_ej601-609_17thlist_en_op,1.pdf. (Accessed March 16, 2011).
[66] Australia National Industrial Chemicals Notification and Assessment Scheme (NICNAS), Full public report, Amino-functional alkoxysilane, File No: NA/648, 8 November 1999. Available at http://www.nicnas.gov.au/publications/car/new/na/nafullr/na0600fr/na648fr.pdf (Accessed March 16, 2011).
[67] IUCLID Dataset, European Chemical Bureau (ECB), 2-Chlorophenol, 2000. Available at http://ecb.jrc.ec.europa.eu/iuclid-datasheet/95578.pdf (Accessed March 16, 2011).
[68] HSDB, US NLM, 2-Chlorophenol, June 2009. Available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB (Accessed March 16, 2011).
[69] European Union (EU) Risk Assessment Report, European Commission, vol. 48, 1,4-Dichlorobenzene, 2004. Available at http://ecb.jrc.ec.europa.eu/documents/Existing-Chemicals/RISK_ASSESSMENT/REPORT/14dichlorobenzenereport001.pdf (Accessed March 16, 2011).
[70] GDCh-advisory committee on existing chemicals of environmental relevance (BUA) report 202, Monoethanolamine (2-Aminoethanol), German Chemical Society, August 1996.
[71] OECD SIDS, 2-Diethylaminoethanol (100-37-8), 2002. Available at http://www.chem.unep.ch/irptc/sids/OECDSIDS/DIETHYLAMINOETHAN.pdf (Accessed September 16, 2011).
[72] Toxikologische Bewertung, Heiderberg, Berufsgenossenschaft der chemischen Industrie, vol. 68, 1997 (in German). English abstract available at TOXNET, http://toxnet.nlm.nih.gov/index.html, Document Number: RISKLINE/1997090003 (Accessed March 16, 2011).
[73] C.A. Hilliard, M.J. Armstrong, C.I. Bradt, R.B. Hill, S.K. Greenwood, S.M. Galloway, Chromosome aberrations in vitro related to cytotoxicity of nonmutagenic chemicals and metabolic poisons, Environ. Mol. Mutagen. 31 (1998) 316–326.
[74] D.D. Scott, S.M. Galloway, R.R. Marshall, M. Ishidate Jr., D. Brusick, J. Ashby, B.C. Myhr, Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9, Mutat. Res. 257 (1991) 147–205.
[75] International Council on Mining and Metals, Health risk assessment guidance for metals, HERAG Fact sheet 05, Mutagenicity, August 2007. Available at www.icmm.com/document/265 (Accessed July 7, 2011).
[76] F. Bianchini, G. Caderni, P. Dolara, E. Tanagnelli, Nuclear aberrations and micronuclei induction in the digestive tract of mice treated with different iron salts, J. Appl. Toxcol. 8 (1988) 179–183.
[77] Toxikologische Bewertung, Heiderberg, Berufsgenossenschaft der chemischen Industrie, vol. 218, 2000 (in German). English abstract available at TOXNET, http://toxnet.nlm.nih.gov/index.html Document Number: RISKLINE/2001050011 (Accessed March 16, 2011).
[78] HSDB, US NLM, N-Methylaniline, June 2005. Available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB (Accessed March 16, 2011).
[79] EU Risk Assessment Report, European Commission, vol. 50, Aniline, 2004. Available at http://ecb.jrc.ec.europa.eu/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/anilinereport049.pdf (Accessed March 16, 2011).
[80] EU, Regulation (EC) No. 1272/2008 of the European parliament and the council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, Off. J. Eur. Union, L353/1, 31.12.2008.
[81] D.J. Tweats, D. Blakey, R.H. Heflich, A. Jacobs, S.D. Jacobsen, T. Morita, T. Nohmi, M.R. O'Donovan, Y.F. Sasaki, T. Sofuni, R. Tice, Report of the IWGT working group on strategies and interpretation of regulatory in vivo tests I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards, Mutat. Res. 627 (2007) 78–91.
[82] G. Eichenbaum, M. Johnson, D. Kirkland, P. O'Neill, S. Stellar, J. Bielawne, R. DeWire, D. Areia, S. Bryant, S. Weiner, D. Desai-Krieger, P. Guzzie-Pecka, D.C. Evans, A. Tonelli, Assessment of the genotoxic and carcinogenic risks of p-nitrophenol when it is present as an impurity in a drug product, Regul. Toxicol. Pharmacol. 55 (2009) 33–42.
[83] Hazardous Substances Data Bank (HSDB), US National Library of Medicine (NLM), Sorbitane monostearate, June 2005. Available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB (Accessed March 16, 2011).
[84] Deutsche Forschungsgemeinschaft (DFG), Occupational Toxicants, vol. 7, VCH Verlagsgesellschaft mbH, Weinhelm, 1996, pp. 75–89.

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Original Article

Lack of chronic toxicity and carcinogenicity of dietary administrated catechin mixture in Wistar Hannover GALAS rats

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ABSTRACT — Chronic toxicity and carcinogenicity of catechin mixture were examined in Wistar Hannover GALAS rats. Administration was in the diet at concentrations of 0, 0.02, 0.3, 1 or 3%. Slight increases in relative liver weight and centrilobular hypertrophy of hepatocytes associated with induction of CYP3A2 were found at the 3% in males of both studies. However, because there were no signs indicative of hepatotoxicity on serum biochemical and histopathological examinations, the changes observed in the liver were regarded as adaptation, and not adverse effects. The slight depressions of body weights at the 3% in females of the chronic toxicity study and in both sexes of the carcinogenicity study were observed. These decreases were because the diet at the highest concentration was frangible and nominal food consumption may not have reflected the actual food consumption resulting in decrease in caloric intake, rather than toxic effects. Thus it was concluded that catechin mixture had no toxicity. In addition, tumor incidences and types were comparable between treated and control groups. Based on the results, the no observed adverse effect levels estimated from the chronic toxicity study were 3% in both sexes equal to 1922.9 in males and 2525.7 mg/kg/day in females. Catechin mixture has no carcinogenic potential in male and female rats.

Key words: Catechin mixutre, Chronic toxicity, Carcinogenicity, Rat

INTRODUCTION

Green tea is consumed as a popular beverage in Japan and throughout the world. During the past decade, epidemiological studies have shown the tea catechin intake is associated with lower risk of cardiovascular disease (Hertog *et al.*, 1993). *In vitro* biochemical studies prevent oxidation of low-density lipoprotein (LDL), which is recognized as an important step in the formation of atherosclerotic plaques and subsequent cardiovascular disease (de Whally *et al.*, 1990; Steinberg *et al.*, 1989). In addition, catechin has been known to have protective effects against some common types of cancer (Adhami *et al.*, 2003; Doss *et al.*, 2005; Stuart *et al.*, 2006; Kumar *et al.*, 2007). Epidemiological studies have indicated that Asian populations that consume tea on a regular basis have lowest incidence of many types of cancer including prostate cancer, than their western counterparts (Jian *et al.*, 2004; Sim and Cheng, 2005; Siddiqui *et al.*, 2006). The main catechin mixutre found in green tea are (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3 gallate (ECG), and EGCG. Among these, EGCG is the most abundant and has attracted the most attention with respect of anticarcinogenic activity (Doss *et al.*, 2005). There is vast amount of scientific literature which suggests that EGCG is responsible for the majority of the potential health benefits attributed to green tea consumption (Khan *et al.*, 2008). Regarding chemopreventive mechanisms, inhibition of activation of specific receptor tyrosine kinases (RTKs) (Shimizu *et al.*, 2008) and related signaling pathways (Verschoyle *et al.*, 2007) or apoptosis induction (Khan *et al.*, 2006, 2010; Yang *et al.*, 2002) have been focuses of attention.

As agents considered to be beneficial and may be expected to be ingested over prolonged period, chronic exposure information is needed. However, the safety information available for catechin is limited. In Ames tests, catechins showed positive effects with or with-

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out cytotoxic levels (Takabe, 1997, Unpublished data). On the other hand, other genotoxicity studies concluded that catechin had no potential of genotoxicities to organisms in Ames, micronucleus, and chromosomal abbreviation tests (Sofuni, 1997 Unpublished data; Kurita, 1997 Unpublished data). In a subchronic study, data suggesting slight effects of catechin mixture on the liver were obtained without any morphological changes at dietary concentration of 5% (Takami *et al.*, 2008). However, information of chronic toxicity or carcinogenicity in rodents is lacking. Although many experimental and epidemiological data supports beneficial effects on human health described above, the controversial effects for genotoxicity are concern. Therefore, the present study was conducted to clarify the chronic toxicity and/or carcinogenicity of catechin mixture with oral administration of Wistar Hannover GALAS rats for 12 and 24 months. In addition, components of catechin mixture in the prepared diet were analyzed.

MATERIALS AND METHODS

Animals

Male and female specific-pathogen-free Wistar Hannover GALAS rat (BrlHan:WIST@Jcl Clea, Tokyo, Japan) at 5 weeks of age were used after 1-week acclimatization period. This strain rat has been recently distributed for international standardization of preclinical and toxicological research. The animals had free access to basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water during the acclimatization period. They were housed three per plastic cage with sterilized soft paper chips as bedding in a barrier-maintained animal room at 24 ± 1°C and 55 ± 5% and showing no abnormalities were used at 6 weeks of age. The protocol of the experiment was reviewed and approved by the Ethical Committee of Sasaki Institute (Tokyo, Japan).

Chemicals, dose selection, preparation of diet, and analysis of chemicals.

Catechin was supplied by TAIYO Kagaku Co. Ltd. (Mie, Japan) as catechin mixture produced by 3 companies (TAIYO KAGAKU, CO., LTD., MITSUI NORIN, CO.,LTD. Tokyo, Japan and ITOEN, LTD., Tokyo, Japan). The concentration of each component in the catechin mixture was analyzed at the beginning of study (Table 1). Treated doses in the present study were based on the results of 90-day dietary subchronic study in rats (Takami *et al.*, 2008). In this study, the over 10% depression of body weights in males and effect on the liver were observed in both sexes at 5%, the highest dose. Although no related histopathological abnormalities were detected, the study indicated that the concentration of 5% might be greater than the maximum tolerance dose (MTD) in males. Therefore, we chose dietary concentrations of 0, 0.02, 0.3, 1 or 3% in the present study. The lowest dose, 0.02% was considered to be an equivalent to the exposure levels of people consuming in a bottle of green

Table 1. The concentration of components in catechin mixture, and total amounts of catechin mixture, caffeine and polyhenol in the prepared diet

Components of catechin (%)		Catechin mixture (%)	Caffeine (%)	Total polyphenol (%)
The concentration of components in catechin mixture				
GC	1.058			
EGC	11.738			
C	1.793			
EGCG	43.604			
EC	2.933	76.444	0.199	89.0
GCG	1.692			
ECG	13.424			
CG	0.202			
Total amounts of catechin mixture, caffeine and polyhenol in the prepared diet				
Dose group	Catechin 0%	0.1762 ± 0.0944	0.00011 ± 0.0010	0.4884 ± 0.0845
	Catechin 0.02%	0.1900 ± 0.0908	0.0010 ± 0.0004	0.5006 ± 0.0485
	Catechin 0.3%	0.3143 ± 0.0975	0.0010 ± 0.0007	0.6528 ± 0.0820
	Catechin 1%	0.7448 ± 0.1187	0.0023 ± 0.0003	0.8847 ± 0.1114
	Catechin 3%	1.8960 ± 0.3016	0.0055 ± 0.0022	1.5110 ± 0.5879

GC, gallocatechin; C, catechin; EGCG, (-)-epigallocatechin-3-gallate; EC, (-)-epicatechin; GCG, gallocatechin; EGC, (-)-epigallocatechin; ECG, (-)-epicatechin-3 gallate; CG, catechin gallate.

tea in Japan.

Powder basal diet (CRF-1, Oriental Yeast) mixed with catechin mixture at designated concentrations was pelleted and was given rats *ad libitum*. Analysis of dietary concentration of each catechin component in prepared diet was performed using each lot. The control animals received basal diet (CRF-1) pelleted in the same way. Stability of dietary catechin mixture and each component up to 2 months after preparation was confirmed. The analyzed concentrations and stabilities of test materials in the diets were found to be generally acceptable (data not shown). The average concentrations of catechin mixture, caffeine and total polyphenols in the control and all treated groups were analyzed at diet preparation (Table 1).

Combined chronic/carcinogenicity study

The present study was designed as a combined one-year toxicity/carcinogenicity study in accordance with guidelines for designation of food additives and for revision of standards for use of food additives, Ministry of Health Labour and Welfare, Japan (MHLW, 1996). Details of the study protocol were as follows:

Chronic toxicity study

Rats were randomly allocated to 5 groups, each consisting of 10 males and 10 females, and given the pellet diet described above for 12 months. Throughout the study, rats had free access to tap water.

The animals were observed daily for clinical signs and mortality. Body weights and food consumption were recorded every week until week 12 and every 4 weeks thereafter. After 12 months treatment, urinalysis was conducted for all animals using test paper (Pretest, Wako Pure Chemical, Ltd., Osaka, Japan), and all rats were euthanized under deep anesthesia after on overnight fast. Blood samples were collected from the abdominal aorta for hematology and serum biochemistry. Hematological examination was performed using automatic hematology analyzer, M-2000 (Toa-Medical Electronics, Hyogo, Japan), and parameters for serum biochemistry were analyzed at SRL, Inc. (Tokyo, Japan) using sera frozen after centrifugation (3,000 rpm for 10 min.) After complete macroscopic examination, the brain, pituitary, heart, spleen, liver, adrenals, kidneys, testes or ovaries and prostate or uterus were weighed. In addition, the nasal cavity, eyes, Harderian glands, spinal cords (cervical, thoracic, and lumbar portions together with corresponding vertebral bones), salivary glands, stomach, small and large intestines, pancreas, urinary bladder, skin, mammary gland, mesenteric lymph nodes, trachea, lungs, esophagus, thyroid glands, tongue, thigh muscle, sciatic nerve, epididymis, seminal vesicles, prostate, uterus, ovaries, and vagina were also removed. All organs/ tissues were fixed in 10% neutral buffered formalin except for testes fixed in Bouin's solution overnight. Bone such as the nasal cavity, spinal column with spinal cords, sternum, and femur were decalcificated. After routine processing for paraffin embedding, sectioning and staining with hematoxylin and eosin, histological assessment was performed for all tissues of all animals.

To clarify immunohistochemical distribution of cytochrome P450s in the hypertrophied hapatocytes, serial liver sections in the 0 and 3% group of both sexes were incubated with anti-rat CYP1A1, 1A2 and 3A2 antibodies (Daiichi Chemicals, Tokyo, Japan), diluted 1:100 in Tris-buffered solution (Takara Bio, Shiga, Japan) with 1% skim milk at 37°C for 1 hr. After the incubation, the sections were exposed to secondary antibodies and linked with streptavidin peroxidase using a DAKO LSAB+ kit (DAKO cytomation, California, CA, USA). The binding was visualized by 3, 3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemicals).

Carcinogenicity study

Rats were randomly allocated to 5 groups, the same doses as the chronic study, each consisting of 50 males and 50 females. The animals were housed three or four per plastic cage, and were given the prepared pellets at each dose for 24 months. Clinical observation and measurements of body weights and food consumption were conducted in the same manner as for the chronic study. After 24 month treatment, all surviving animals were euthanized under deep anesthesia, and the liver were resected and weighed. All organs and/or tissues listed above, as well as macroscopic masses and any abnormalities were examined macroscopically and microscopically as in the chronic study. Moribund or dead animals were also completely necropsied and pathologically examined to determine their cause of death.

Statistical analysis

In multiple comparisons continuous data were analyzed with Bartlett test. If the variance was homogeneous, the data were assessed by the Dunnett's multiple comparison test. If not, the Steel's multiple comparison test was employed. The histopathology data were employed with Fisher's exact or chi-square test. The level of significance was set at $p < 0.05$ and 0.01.

RESULTS

General condition and mortality

No treatment-related clinical signs or deaths were found throughout the feeding period in either chronic or carcinogenicity studies (Fig. 1). The mortalities at termination of the carcinogenicity study were 36, 38, 32, 26 and 22% in males, and 28, 38, 28, 30 and 30% in females of the 0, 0.02, 0.3, 1.0 or 3% group, respectively.

Body weights in the chronic and carcinogenicity studies

In the chronic toxicity study, changes in body weights were comparable among the treated and control animals in males (Fig. 2). In females, suppression or a tendency for suppression of body weight gain by approximately 15% was observed several times at 3% only after Week 25 (Fig. 2). In the carcinogenicity study, suppressions of body weights within 10% was detected in both of males and females receiving the 3% dose in the latter half of the study (Fig. 3). No changes in body weights were observed in other treated groups.

Food consumption and chemical intakes in the chronic and carcinogenicity studies

The concentrations of catechin mixture in the prepared diet showed a dose dependent increase in the 0.3% group and higher (Table 1). Their concentrations were comparable between the control and the lowest dose groups. Data for food consumption are summarized in Figs. 4 (chronic study) and 5 (carcinogenicity study) and intakes of catechin mixture is Table 2. In both studies, increases or tendencies for increased food consumption were observed in males and females at 3%, especially at beginning of the studies. Since the pellets in the 3% group were more frangible than them in other treated or control groups, there were more pellet fragments in the cages of both sexes at 3% compared to other groups. The chemical intakes were shown in Table 2.

Table 2. Food consumption and intake of catechin in the 12-month chronic or 24-month carcinogenicity study, and total amounts of catechin mixture, caffeine and polyhenol in the prepared diet

Time/Sex	Dose (%)	No. of animals examined	Food consumption (g/rat/day)	Intake of catechins Daily (mg/kg bw/day)
Food consumption and intake of catechin				
12-month chronic				
Males	Catechin 0%	10	18.8 ± 0.8	0
	Catechin 0.02%	10	19.0 ± 0.5	9.7
	Catechin 0.3%	10	19.6 ± 1.1	146.0
	Catechin 1%	10	20.6 ± 1.1	517.7
	Catechin 3%	10	23.5 ± 2.7	1922.9
Females	Catechin 0%	10	18.6 ± 2.4	0
	Catechin 0.02%	10	17.8 ± 1.7	14.2
	Catechin 0.3%	10	18.1 ± 1.2	215.7
	Catechin 1%	10	18.8 ± 1.5	765.7
	Catechin 3%	10	19.5 ± 3.6	2525.7
24-month carcinogenic				
Males	Catechin 0%	50	19.3 ± 0.7	0
	Catechin 0.02%	50	19.1 ± 0.9	8.4
	Catechin 0.3%	50	19.0 ± 0.7	122.7
	Catechin 1%	50	19.3 ± 1.5	416.4
	Catechin 3%	50	22.0 ± 1.1	1265.8
Females	Catechin 0%	50	14.7 ± 1.2	0
	Catechin 0.02%	50	14.7 ± 0.8	10.1
	Catechin 0.3%	50	14.7 ± 0.4	148.4
	Catechin 1%	50	14.8 ± 0.8	497.2
	Catechin 3%	50	15.5 ± 0.9	1539.8

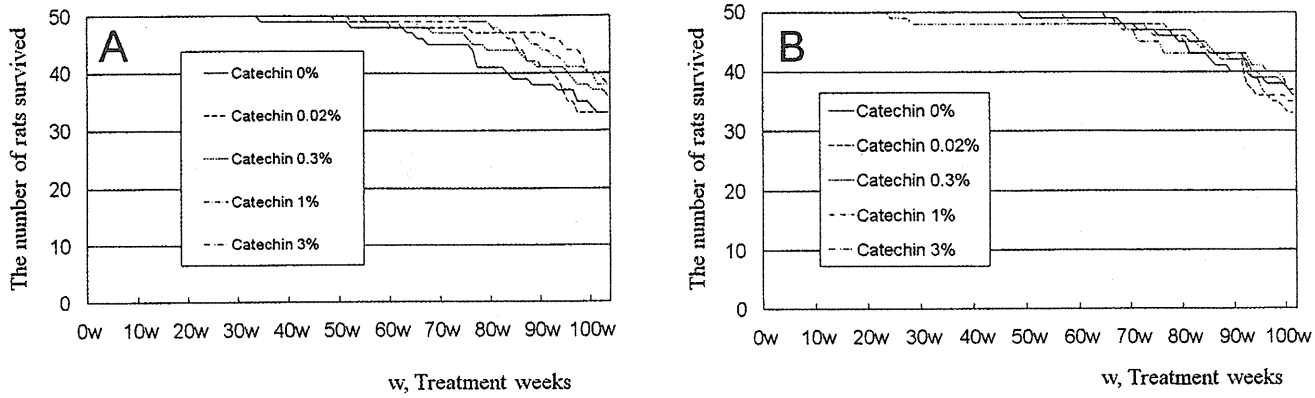

Fig. 1. Mortality of carcinogenicity study. A, males; B, females.

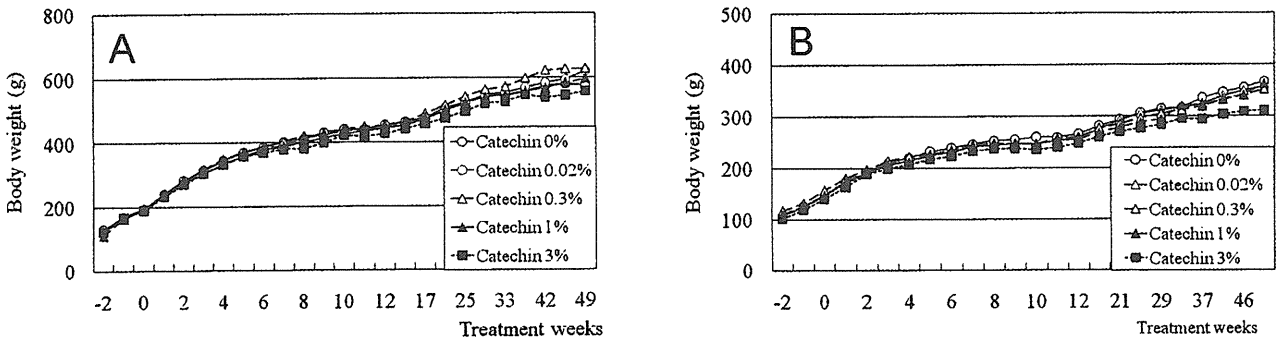

Fig. 2. Growth curve of chronic toxicity study. A, male; B, females.

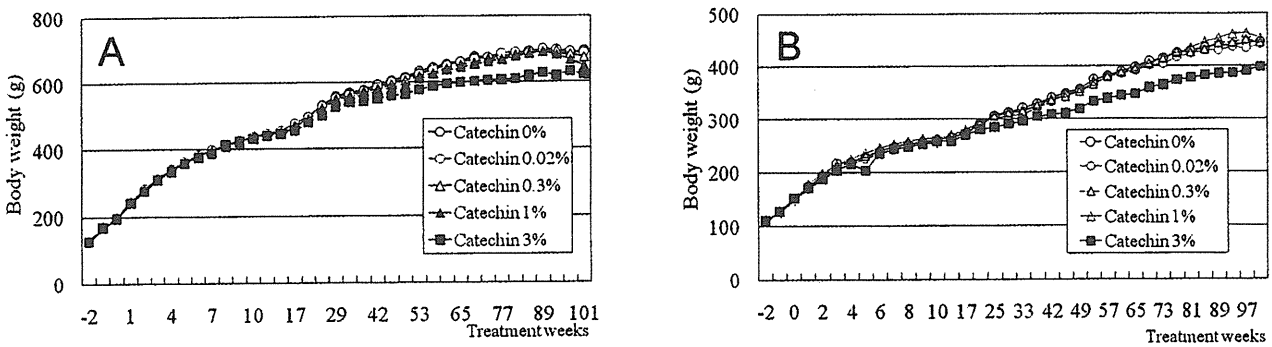

Fig. 3. Growth curve of carcinogenicity study. A, males; B, females.

Hematology and serum biochemistry

Although some significant changes were detected in hematology and blood biochemistry, no obvious dose-relation was found for any parameters among the treated groups (Tables 3 and 4).

Organ weights

In the chronic toxicity study, no significant changes were noted in the absolute and/or relative organ weights except an increase in the relative liver weight in males of the 3% group (Table 5). In carcinogenicity study, the liver weights in all treated groups were comparable to those of the control group in both sexes (Table 7).

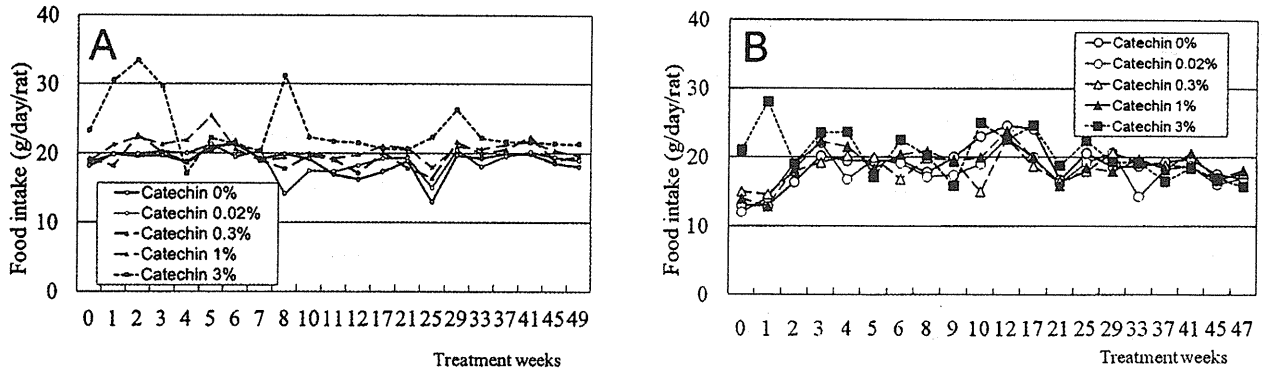

Fig. 4. Food intakes of chronic toxicity study. A, male; B, females.

Table 3A. Hematology in chronic toxicity study – Males –

Group		RBC × 10000/mm³	WBC /μl	Hb g/dl	HT %	MCV μ3	MCH pg	MCHC %
Catechin 0%	Mean	817.4	2662.5	15.1	49.4	60.5	18.4	30.5
(8)	SD	27.95	1140.1	0.38	1.27	1.77	0.52	0.53
Catechin 0.02%	Mean	820.8	3500.0	15.1	48.8	60.2	18.6	31.0
(10)	SD	36.93	1030.6	0.42	2.11	1.32	0.52	0.67
Catechin 0.3%	Mean	811.0	3500.0	15.1	48.8	60.2	18.6	31.0
(10)	SD	36.93	1030.6	0.42	2.11	1.32	0.52	0.67
Catechin 1%	Mean	820.6	3350.0	15.0	48.9	59.6	18.2	30.7
(10)	SD	32.62	861.9	0.40	1.41	2.01	0.79	0.48
Catechin 3%	Mean	793.3	3520.0	14.8	48.6	61.3	18.6	30.4
(10)	SD	25.46	853.5	0.45	1.38	1.89	0.70	1.07

( ): No. of rats examined.

Table 3B. Hematology in chronic toxicity study – Females –

Group		RBC × 10000/ mm³	WBC /μl	Hb g/dl	HT %	MCV μ3	MCH pg	MCHC %
Catechin 0%	Mean	709.2	1980.0	14.3	46.2	65.1	20.2	30.9
(10)	SD	24.04	533.90	0.50	2.66	2.59	0.60	0.97
Catechin 0.02%	Mean	693.1	1810.0	13.9	44.9	64.8	20.0	30.8
(10)	SD	22.24	438.30	0.46	1.60	1.48	0.67	0.63
Catechin 0.3%	Mean	711.0	2222.2	14.4	46.6	65.4	20.3	30.9
(9)[a]	SD	24.40	742.93	0.57	1.64	0.88	0.50	0.78
Catechin 1%	Mean	699.0	1700.0	14.2	45.7	65.4	20.2	31.0
(9)	SD	21.64	327.87	0.52	1.89	1.94	0.44	0.71
Catechin 3%	Mean	691.0	2033.3	14.1	45.9	66.4	20.4	30.7
(9)[a]	SD	19.47	259.81	0.40	1.27	1.88	0.53	0.71

( ): No. of rats examined. [a]: Female was the blood sample of one female rat could not available for analysis due to blood coagulation.

Necropsy and histopathology

In the chronic toxicity and carcinogenicity studies, no treated related macroscopic changes were detected. Microscopically, a number of non-neoplastic lesions were observed in both studies. In the chronic study, slight centrilobular hypertrophy of hepatocytes was significantly increased in males at 3% (Table 6, Fig. 6). However, this change was not observed in females at 3% or in the other treated groups. In immunohistochemistry, CYP3A2 expression was found in the hypertrophied

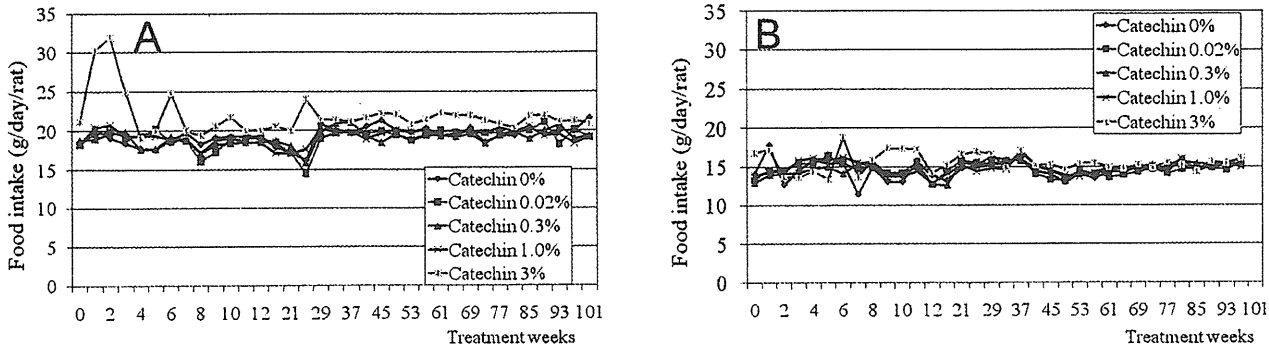

Fig. 5. Food intakes of in carcinogenicity study. A, male; B, females.

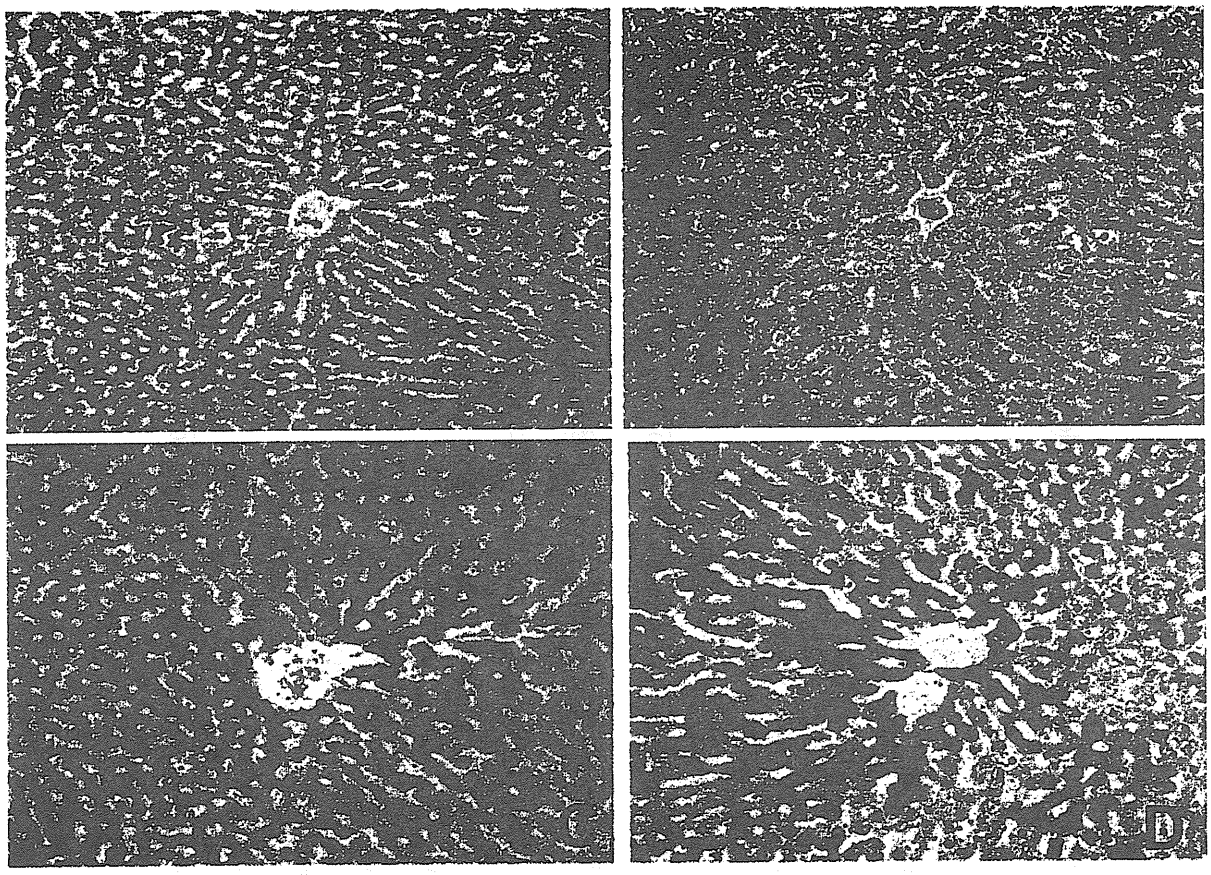

Fig. 6. Hisotpathological changes in the liver in the chronic toxicity study. A: A male in the control group (catechin 0%). No abnormalities detected. H-E stain. x 100. B: A male in the catechin 3% treated group. Slight centrilobular hypertrophy of hepatocytes. No degenerative, necrotic or inflammatory changes were detected. H-E stain. x 100. C: A male in the control group (catechin 0%). A slight positive area is apparent in the centrolobular area of hepatocytes. Immunohistochemical staining with CYP3A2 antibody. Visualized by DAB. x 100. D: A male in the catechin 3% treated group. Increased expression is apparent in the centrolobular area of hypertrophied hepatocytes, compared to that of C. Immunohistochemical staining with CYP3A2 antibodies. Visualized with DAB. x 100.

Table 4A. Serum biochemistry in chronic toxicity study – Males –

Group		TP g/dl	A/G	ALB g/dl	BIL mg/dl	TG mg/dl	TCHO mg/dl	CRE mg/dl	Na mEQ/l	K mEQ/l	Ca mg/dl	IP mg/dl	AST IU/l	ALT IU/l	ALP IU/l	γ-GTP IU/l
Catechin 0%	Mean	7.0	2.0	4.7	0.1	159.0	124.0	0.4	144.9	4.6	10.6	5.0	155.1	40.1	285.1	2 >
(8)	SD	0.17	0.34	0.29	0.02	52.80	26.49	0.04	0.64	0.31	0.23	0.76	36.05	8.84	51.75	
Catechin0.02%	Mean	7.2	1.8	4.6	0.1	189.6	149.6	0.4	144.7	4.6	10.8	5.2	148.4	42.1	267.0	2 >
(10)	SD	0.25	0.32	0.28	0.02	87.58	52.26	0.06	1.16	0.36	0.13	0.29	34.49	11.79	70.51	
Catechin 0.3%	Mean	7.3	1.8	4.6	0.1	224.0	124.3	0.3*	145.3	4.4	10.7	5.0	133.7	43.1	268.4	2 >
(10)	SD	0.31	0.31	0.18	0.03	96.76	19.65	0.03	1.49	0.32	0.19	0.42	42.75	30.90	94.59	
Catechin 1%	Mean	7.0	2.0	4.7	0.1	164.7	104.9	0.4	145.1	4.5	10.5	5.3	155.7	45.9	308.7	2 >
(10)	SD	0.28	0.19	0.21	0.02	56.19	13.40	0.04	0.88	0.28	0.28	0.48	27.13	9.99	47.66	
Catechin 3%	Mean	6.8	2.1	4.6	0.1	158.6	103.8	0.3**	145.4	4.5	10.7	5.6	137.2	54.5	347.6	2 >
(10)	SD	0.25	0.23	0.19	0.02	61.80	20.63	0.03	2.12	0.26	0.40	0.49	36.23	28.09	100.97	

( ): No. of rats examined. *, **: Significantly different from 0% ($p < 0.05$, $p < 0.01$).

Table 4B. Serum biochemistry in chronic toxicity study – Females –

Group		TP g/dl	A/G	ALB g/dl	BIL g/dl	TG mg/dl	TCHO mg/dl	CRE mg/dl	Na mEQ/l	K mEQ/l	Ca mg/dl	IP mg/dl	AST IU/l	ALT IU/l	ALP IU/l	γ-GTP IU/l
Catechin 0%	Mean	8.2	2.3	5.6	0.1	234.9	110.4	0.4	140.7	4.0	10.5	3.8	140.3	34.1	79.3	2 >
(10)	SD	0.79	0.44	0.29	0.05	164.08	30.45	0.04	2.67	0.37	0.37	0.61	31.66	5.34	25.66	
Catechin 0.02%	Mean	7.7	2.8	5.7	0.1	216.0	108.1	0.4	140.5	4.1	10.5	3.6	133.9	36.3	73.5	2 >
(10)	SD	0.53	0.46	0.44	0.05	104.58	16.87	0.04	2.68	0.37	0.32	0.41	35.26	10.86	20.97	
Catechin 0.3%	Mean	7.9	2.4	5.5	0.1	270.9	107.9	0.4	141.1	4.3	10.4	3.7	134.5	35.4	86.3	2 >
(10)	SD	0.54	0.51	0.27	0.03	148.59	28.07	0.04	3.07	0.39	0.35	0.91	30.89	9.36	41.10	
Catechin 1%	Mean	7.6	2.6	5.5	0.1	190.7	103.9	0.4	141.7	4.0	10.5	4.2	133.1	38.1	85.9	2 >
(9)	SD	0.39	0.40	0.26	0.03	63.08	32.25	0.05	3.12	0.44	0.42	0.47	27.67	13.53	37.53	
Catechin 3%	Mean	7.7	3.0**	5.7	0.1	153.3	98.5	0.3	142.5	4.3	10.4	3.6	137.9	42.7	92.4	2 >
(10)	SD	0.42	0.53	0.35	0.03	83.66	17.36	0.03	3.47	0.25	0.37	0.35	31.88	14.31	11.14	

( ): No. of rats examined. **: Significantly different from 0% ($p < 0.01$).

Table 5A. Organ weights in chronic toxicity study – Males –

Absolute weight

Group		Final B.W. (g)	Brain (mg)	Pituitary (mg)	Heart (mg)	Spleen (mg)	Liver (mg)	Adrenals (mg)	Kidneys (mg)	Testis (mg)	Prostate (mg)
Catechin 0%	Mean	579.9	2070.6	13.8	1358.0	834.3	12971.8	60.1	2791.9	3730.6	676.8
(8)	SD	61.01	70.47	2.25	85.60	93.23	1236.93	6.85	383.37	235.53	184.03
Catechin0.02%	Mean	605.0	2148.6	11.8	1366.0	928.5	14798.1	65.2	2808.8	3938.0	653.0
(10)	SD	46.53	87.45	1.62	172.66	103.21	2667.65	13.02	417.57	313.14	141.96
Catechin 0.3%	Mean	571.0	2085.4	12.0	1393.3	1019.3	14856.8	64.8	2945.8	3460.1	668.8
(10)	SD	71.27	220.38	2.16	118.92	154.07	2504.39	7.44	316.11	574.62	138.77
Catechin 1%	Mean	586.0	2144.6	12.4	1387.7	937.9	13387.3	69.6	2921.3	4027.6	639.5
(10)	SD	52.90	82.21	1.84	164.06	153.27	1508.17	8.32	474.54	410.94	137.47
Catechin 3%	Mean	560.0	2177.5	12.1	1308.0	977.7	14289	60.0	3014.8	3884.1	611.5
(10)	SD	52.90	82.21	1.84	164.06	153.27	1508.17	8.32	474.54	410.94	137.47

Relative weight (mg/BWg × 100)

Group		Brain	Pituitary	Heart	Spleen	Liver	Adrenals	Kidneys	Testis	Prostate
Catechin 0%	Mean	360.7	2.4	235.8	145.5	2249.1	10.4	487.1	650.9	117.3
	SD	41.29	0.35	22.32	25.22	229.89	1.31	90.28	89.67	31.92
Catechin 0.02%	Mean	356.9	2.0	225.2*	153.4	2435.0	10.8*	463.0	654.5	107.5
	SD	28.72	0.29	15.04	12.19	291.61	1.92	45.34	71.21	21.07
Catechin 0.3%	Mean	343.4	2.2	227.0	168.4	2399.2	10.7	478.9	576.4	109.9
	SD	63.00	0.20	19.78	41.07	192.96	2.06	36.32	142.60	26.70
Catechin 1%	Mean	368.4	2.1	236.5	159.8	2283.6	11.9	496.4	689.4	109.1
	SD	32.35	0.31	13.92	20.49	131.24	1.28	47.07	66.34	20.67
Catechin 3%	Mean	399.1	2.2	236.6	176.4	2566.7*	10.9	541.8	707.8	110.6
	SD	64.71	0.38	29.45	27.48	269.34	2.05	42.90	108.35	21.64

( ): No. of rats examined *: Significantly different from 0% ($p < 0.05$).

Table 5B. Organ weights in chronic toxicity study – Female –

Absolute weight

Group		Final B.W. (g)	Brain (mg)	Pituitary (mg)	Heart (mg)	Spleen (mg)	Liver (mg)	Adrenals (mg)	Kidneys (mg)	Ovaries (mg)	Uterus (mg)
Catechin 0%	Mean	366.4	2011.5	25.0	978.6	673.9	7474.4	86.9	1969.5	76.9	1320.2
(10)	SD	40.12	59.24	7.07	73.76	114.77	1130.63	20.51	178.91	33.31	302.82
Catechin 0.02%	Mean	353.1	1978.7	24.2	902.6	610.8	7403.1	79.4	1954.7	87.3	1037.5
(10)	SD	56.39	107.17	7.08	97.84	98.26	915.07	16.60	171.13	27.64	469.12
Catechin 0.3%	Mean	350.0	1899.3	22.3	935.9	644.1	7468.9	85.2	1981	73.7	1059.2
(10)	SD	40.34	299.20	3.86	58.98	63.09	715.38	12.79	167.66	18.79	273.15
Catechin 1%	Mean	349.8	2024.1	20.3	905.8	664.3	7596.2	80.7	1998.8	93.9	1047.8
(9)	SD	54.47	67.51	3.32	102.43	88.48	1185.61	15.41	381.89	29.73	420.52
Catechin 3%	Mean	305.8	1970.9	18.9	876.0	667.3	6746.8	76.3	1862.2	90.3	1136.1
(10)	SD	21.58	66.46	3.28	78.92	82.44	487.23	5.44	169.20	50.44	445.64

Relative weight (mg/BWg × 100)

Group		Brain	Pituitary	Heart	Spleen	Liver	Adrenals	Kidneys	Ovaries	Uterus
Catechin 0%	Mean	554.8	6.5	269.3	185.7	2044.5	24.1	541.7	21.3	368.7
	SD	61.74	1.00	28.85	37.74	247.63	6.89	60.99	9.20	119.78
Catechin 0.02%	Mean	575.9	7.0	259.0	174.1	2114.1	22.9	565.4	24.7	301.4
	SD	114.88	2.23	29.83	20.95	178.17	5.52	94.88	6.24	139.59
Catechin 0.3%	Mean	550.2	6.5	269.7	185.7	2148.1	24.5	570.0	21.5	302.9
	SD	111.73	1.36	25.87	23.01	210.31	3.91	53.26	6.79	72.07
Catechin 1%	Mean	589.9	5.9	261.9	194.0	2176.7	23.6	573.4	26.6	314.2
	SD	83.01	1.29	32.96	42.41	155.71	6.54	78.23	8.78	161.91
Catechin 3%	Mean	646.7	6.2	286.4	219.9	2210.3	25.1	609.1	29.6	371.2
	SD	39.26	1.07	14.72	36.76	148.15	2.57	36.55	16.48	140.34

( ): No. of rats examined, *, **: Significantly different from 0% ($p < 0.05$, $p < 0.01$).

Table 6. Summary of histopathology of chronic toxicity study

Tissue	Findings	Males					Females				
		Catechin					Catechin				
	Dose (%)	0	0.02	0.3	1	3	0	0.02	0.3	1	3
Number of rats examined		10	10	10	10	10	10	10	10	10	10
Neoplastic lesions											
Pituitary	Adenoma of anterior lobe	0	0	0	0	0	1	1	0	3	0
Thyroid	C-cell adenoma	1	0	2	0	0	0	0	0	0	0
	Follicular cell adenoma	0	0	0	0	0	1	0	0	0	0
Spleen	Hemangioma	0	0	0	1	0	0	0	0	0	0
Skin	Papilloma	0	0	0	1	0	0	0	0	0	0
Testis	Leydig cell tumor	0	1	0	0	0	-	-	-	-	-
Uterus	Endometrial stromal polyp	-	-	-	-	-	3	1	1	1	0
	Duciduoma	-	-	-	-	-	1	0	0	0	0
Non-neoplastic lesions											
Liver	Centrilobular hypertrophy of hepatocytes	0	0	0	0	8*	0	0	0	0	0
	Altered foci, basophilic	0	2	2	2	0	3	1	0	4	2
	Altered foci, eosinophilic	0	0	1	0	0	0	0	0	0	0

*, Significantly different from the control value ($p < 0.05$). -, Not examined.

hepatocytes in males at 3% (Fig. 6), and any other changes except CYP3A2 were not detected. In the carcinogenicity study, similar mild hepatocellular hypertrophy was also observed in males at the same dose (Table 7). In both studies, a number of non-neoplastic changes were detected, but their incidences and severities were similar to those in the control groups, and therefore no dose-related non-neoplastic changes except liver hypertrophy were detected.

In the chronic toxicity study, several tumors were detected in all groups including the controls in both sexes. Their incidences were comparable between the control and treated groups in both sexes (Table 6). In the carcinogenicity study, a number of tumors were found in many organs and/or tissues in both sexes in all groups (Table 8). All of the incidences in the treated groups were comparable to those in controls in both sexes. There were several types of tumors showing high incidences such as C-cell adenoma in the thyroid, islet cell adenoma, endometrial stromal polyp or mammary tumor (fibroadenoma and adenocarcinoma). The most common tumor was the anterior adenoma in the pituitary, which was also major cause of death in the present study. In addition thymoma and/or malignant thymoma with lymphocyte proliferation were detected in all groups including controls with similar incidences.

DISCUSSION

The present examination of the chronic toxicity and carcinogenicity of catechin mixture, used as a food additive, in Wistar Hannover GALAS rats showed no effects at concentrations of 0, 0.02, 0.3, 1 or 3% except with regard to body weights, food consumption and hepatocellular hypertrophy at the highest dose. No changes indicating carcinogenic potential were detected.

The food consumption increased or showed a tendency of increase in both sexes of the chronic toxicity and carcinogenicity studies at 3%. In the same dose group depressed body weight gain was observed in females of chronic toxicity study, and both sexes in carcinogenicity study. As for nutritional effects, the caloric contribution of catechin mixture in feed might be low because consistent dose-dependent increase of body weights was lacking. No obvious toxicities were detected in terms of other parameters. In addition, the pellet of 3% was more frangible, but long-term effects of catechin mixture on feeding behavior with continuous dietary administration were not observed. Therefore, the body weight depressions at 3% are considered not to be a toxic effect, but rather a secondary effect resulting from decreased caloric intake. Namely, these decreases were because the diet at the highest concentration was frangible and nominal food consumption may not have reflected the actual food consumption resulting in decrease in caloric intake, rather than toxic effects. Similar suppression of body weights was also noted in males of the 5% group in the subchron-

Table 7. Final and liver weights at the termination and liver histopathology in carcinogenicity study

Group	No of rats examined (At termination)	Body weight (g)	Liver weights		Histoapthology in the liver (Incidence/number of all rats examined)		
			Absolute (mg)	Relative [a]	Hepatocellular hypertrophy	Altered foci	
						Eosinophilic	Basophilic
Male							
Catechin 0%	32	692.32 ± 93.74 [b]	21242 ± 4305.7	30.717 ± 5.1845	5/50	5/50	2/50
Catechin 0.02%	31	680.19 ± 88.480	20691 ± 4037.1	30.524 ± 5.0252	3/50		
Catechin 0.3%	34	665.94 ± 104.19	20197 ± 4470.8	30.322 ± 5.2846	4/50		
Catechin 1%	37	646.73 ± 84.77	19135 ± 4330.7	29.617 ± 5.5515	8/50		
Catechin 3%	39	627.92 ± 71.32	17957 ± 2999.2	28.762 ± 50.339	18/50*	4/50	2/50
Females							
Catechin 0%	36	441.81 ± 71.34	12884 ± 3092.5	28.334 ± 7.6226	1/50	5/50	1/50
Catechin 0%	31	438.35 ± 55.84	12252 ± 2398.6	27.981 ± 4.180			
Catechin 0.3%	36	436.86 ± 78.29	13607 ± 3511.5	31.002 ± 4.867			
Catechin 1%	35	447.91 ± 68.80	12529 ± 2492.5	28.021 ± 4.206			
Catechin 3%	35	393.8 ± 50.248	11673 ± 2022.3	29.744 ± 4.136	0/50	4/50	7/50

*, Significantly different from the control value at $p < 0.01$. [a], Liver weight (mg) / body weight (g). [b], Mean ± S.D.

ic study (Takami *et al.*, 2008).

Histopathologically slight centrilobular hypertrophy of hepatocytes associated with induction of CYP3A2 was found at 3% in males of both studies, and relative weights of the liver increased were limited to the chronic toxicity study. However, no signs indicative of hepatotoxicity were seen in the serum biochemical and histopathological examinations in either study. Therefore, the changes observed in the liver were considered to be treatment-related but not adverse in nature. They might be regarded as adaptation. A joint FAO and WHO meeting of pesticides, residues concluded that in the absence of histopathological damage and relevant clinical chemistry changes at a dose inducing only hepatocellular hypertrophy and/or liver size/weight changes, hypertrophy should not be identified as an adverse effects as a general principle in the final assessment of the liver hypertrophy (JMPR, 2006). Large doses of many dietary supplements including green tea are reported to induce cytochrome P450s in rats (Jang *et al.*, 2004). In an earlier study administration of 2% green tea supplement in drinking water for 3 days induced CYP1A2 but not 3A2 (Jang *et al.*, 2004). Although the reasons for the discrepancy with the present study are not clear, the ingredients of the supplement in the previous study might have differed from those in the present study, because details were not given (Jang *et al.*, 2004).

In the present carcinogenicity study, none of the treated groups showed a significant increase in the incidence of any specific tumors compares with the corresponding control ones, and also there were no positive trend in both sexes, clearly indicating no carcinogenic potential of long-term treatment with catechin mixture in rats. All the tumors observed in the present study are known to occur spontaneously in aged rats.

Green tea has been consumed as a popular beverage in Japan and throughout the world for many years. During the past decade, epidemiological studies have shown tea catechin intake to be associated with lower risk of cardiovascular disease (Hertog *et al.*, 1993), or protective effects against some common types of cancer including in the prostate (Adhami *et al.*, 2003; Doss *et al.*, 2005; Jian *et al.*, 2004; Kumar *et al.*, 2007; Siddiqui *et al.*, 2006; Sim and Cheng, 2005; Stuart *et al.*, 2006). These experimental and epidemiological data might indicate that intake of tea catechin is favorable to human health under appropriate consumption conditions. Many studies reported that EGCG is responsible for the majority of the potential health benefits attributed to green tea consumption (Khan *et al.*, 2008) and this was in fact contained as a major component in the test material in the present study.

Based on the results in the present study, we conclude that catechin mixture has no appreciable toxicity in Wistar Hannover GALAS rats. The no observed adverse effect levels estimated form the chronic toxicity study were 3% in both sexes equal to 1922.9 and 2525.7 mg/kg/day in males and females, respectively. No carcinogenic potential was found in male and female rats fed catechin mixture for 2 years.

Table 8. Incidence of neoplastic lesions in the carcinogenicity study

		Male Catechin					Female Catechin				
Organ	Findings	0%	0.02%	0.3%	1%	3%	0%	0.02%	0.3%	1%	3%
	No. of rats examined	50	50	50	50	50	50	50	50	50	50
Brain	(B) Astrocytoma	0	0	1	0	1	0	0	0	0	0
	(M) Malignant meningioma	0	0	0	0	0	0	0	0	1	0
Pituitary	(M) Anterior adenocarcinoma	0	4	3	3	0	1	7	4	1	3
	(B) Anterior adenoma	12	21	16	19	12	22	27	25	31	16
	(B) Medium lobe adenoma	0	1	0	0	0	0	0	0	0	0
Zymbal gland	(M) Squamous cell carcinoma	0	1	0	0	0	0	0	0	0	0
Eye	(M) Malignant Schwannoma	0	0	1	0	0	0	0	0	0	0
Thyroid	(B) C-cell adenoma	3	6	2	2	6	3	1	4	4	3
	(B) Follicular cell adenoma	3	5	2	1	1	1	2	2	1	0
	(M) Follicular cell adenocarcinoma	0	1	1	1	0	0	0	0	0	0
Parotid gland	(M) Adenocarcinoma	0	0	0	0	0	0	0	0	0	1
Salivary gland	(M) Mixed tumor, malignant	0	0	1	0	0	0	0	0	0	0
Thymus	(B) Thymoma	0	0	2	0	2	1	5	1	2	2
	(M) Malignant thymoma	1	0	1	0	1	2	2	3	2	1
Lung	(B) Alveolar/broncholar adenoma	0	0	1	0	0	0	0	0	0	0
	(M) Alveolar/broncholar adenocarcinoma	0	0	0	1	0	0	0	0	0	0
Heart	(B) Endocardial schwannoma	0	1	0	0	2	0	0	0	0	0
Liver	(M) Angiosarcoma	1	0	0	0	0	0	0	0	0	0
	(B) Adenoma	0	0	1	0	0	1	0	2	1	0
Kidney	(B) Papilloma	0	1	0	0	0	0	0	0	0	0
	(M) Liposarcoma	1	0	0	0	0	0	0	0	0	0
	(M) Renal cell carcinoma	0	0	1	0	0	0	0	0	0	0
Pancreas	(M) Islet cell adenocarcinoma	2	1	0	0	3	1	0	0	0	0
	(B) Islet cell adenoma	1	3	7	4	2	1	1	1	0	1
	(B) Acinar cell adenoma	0	0	1	1	1	0	0	0	0	0
Adrenal	(B) Pheochromocytoma	2	3	3	3	0	1	2	0	0	1
	(B) Adenoma of cortex	0	0	1	0	0	0	1	1	1	1
	(B) Ganglioneuroma	0	0	0	0	0	1	0	0	0	0
	(M) Malignant pheochromocytoma	1	0	1	0	0	0	0	0	0	0
	(M) Pheochromocytoma complex	0	0	2	0	0	0	0	0	0	0
Lymph node	(M) Malignant lymphoma, systermic	0	1	0	0	1	0	0	0	1	0
	(B) Hemangioma	1	0	1	0	1	0	0	0	1	0
Spleen	(B) Hemangioma	0	0	0	0	0	0	0	0	1	0
	(M) Histiocytic sarcoma	0	0	0	0	0	0	0	0	1	0
Stomach	(M) Fibrosarcoma in forestomach	1	0	0	0	0	0	0	0	0	0
Intestine	(B) Fibroadenoma	0	0	0	0	0	1	0	0	0	0
	(B) Fibroma in muscle layer	0	0	0	0	0	1	0	0	0	0
	(B) Leiomyosarcoma	0	1	0	0	0	0	0	0	0	0
	(M) Mucinous carcinoma	0	0	1	0	0	0	0	0	0	0
Muscle	(B) Granular cell tumor	0	0	0	0	1	0	0	0	0	0
Urinay bladder	(B) Papilloma	0	1	0	0	0	0	0	0	0	0

Table 8. (Continued).

Organ	Findings	Male Catechin 0%	0.02%	0.3%	1%	3%	Female Catechin 0%	0.02%	0.3%	1%	3%
Testis	(B) Leydig cell tumor	0	4	3	3	2					
Prostate	(B) Adenoma of ventral lobe	1	1	1	2	0					
	(M) Squamous cell carcinoma	0	1	0	0	0					
Seminal vesicle	(B) Adenoma	0	0	0	2	0					
Ovary	(B) Granulosa-theca cell tumor						1	0	0	0	1
	(B) Granulosa cell tumor						0	0	1	0	0
	(B) Luteoma						0	0	0	0	1
	(B) Cystadenoma						0	1	0	0	0
Uterus	(B) Endmetrial stromal polyp						7	4	6	11	5
	(M) Stromal sarcoma						1	0	1	0	0
	(M) Endmetrial adenocarcinoma						0	1	0	1	1
Preputial gland	(B) Adenoma	1	0	0	0	0					
	(M) Adenocarcinoma	0	0	0	1	0					
	(M) Squamous cell carcinoma	0	1	0	0	0					
Nasal cavity	(B) Adenoma	0	0	0	1	0	0	0	0	0	0
	(B) Chondroma	0	0	0	0	0	0	0	0	1	0
	(M) Osteosarcoma	1	0	0	0	0	0	0	0	0	0
Oral cavity	(M) Squamous cell carcinoma	0	0	0	1	0	0	0	0	0	0
Abdominal cavity	(M) Angiosarcoma	1	0	0	0	0	0	0	0	0	0
	(B) Paraganlioma	0	1	0	0	0	0	0	0	0	0
Spinal cord	(B) Osteoma	0	0	0	0	0	1	0	0	0	0
Skin	(B) Keratoacanthoma	0	0	0	0	1	0	0	0	0	0
	(B) Trichoepithelioma	0	0	0	1	0	0	0	0	0	0
	(B) Papilloma	0	0	0	0	0	0	1	0	0	0
	(M) Squamous cell carcinoma	0	0	1	4	0	1	0	0	0	0
	(M) Basal cell carcinoma	0	0	0	0	0	0	0	0	1	0
	(M) Fibrosarcoma	0	1	0	0	0	0	0	0	0	0
Mammary gland	(B) Adenoma	0	0	0	0	0	5	6	1	3	1
	(B) Fibroadenoma	1	0	0	1	0	9	10	17	7	4
	(M) Adenocarcinoma	0	0	0	0	0	5	1	5	5	2
Subcutis	(B) Fibroma	2	3	2	1	0	0	1	0	2	0
	(B) Lipoma	0	0	0	0	0	1	0	0	0	0
	(B) Hemangioma	0	0	0	2	0	0	0	0	0	0
	(M) Fibrosarcoma	0	0	1	0	0	0	0	0	0	0
	(M) Leiomyosarcoma	0	0	1	0	0	0	0	0	0	0
	(M) Malignant Schwannoma	0	1	0	0	1	0	0	0	0	0
	(M) Angiosarcoma	0	1	1	0	0	0	0	0	0	0
	(M) Liposarcoma	0	0	0	0	0	0	0	0	1	0
	(M) Histiocytic sarcoma	0	0	0	0	1	0	0	0	0	0
	(M) Sarcoma NOS	0	0	0	1	0	0	0	0	0	0
Scrotum/abdomen	(M) Mesothelioma	1	1	0	1	1	0	0	0	0	0
Bone	(B) Schwannoma	0	0	0	1	0	0	0	0	0	0

(B), Benign; (M), Malignant

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REFERENCES

Adhami, V.M., Ahmad, N., Mukhtar, H. and Ahmad, N. (2003): Molecular targets for green tea in prostate cancer prevention. J. Nutr., **133**, 2417S-2424S.

de Whalley, C.V., Rankin, S.M., Hoult, J.R., Jessup, W. and Leake, D.S. (1990): Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. Biochem. Pharmacol., **39**, 1743-1750.

Doss, M.X., Potta, S.P., Hescheler, J. and Sachinidis, A. (2005): Trapping of growth factors by catechins: a possible therapeutical target for prevention of proliferative diseases. J. Nutr. Biochem., **16**, 259-266.

Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B. and Kromhout, D. (1993): Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet, **342**, 1007-1011.

Jang, E., Park, Y. and Chung, W.G. (2004): Effects of dietary supplements on induction and inhibition of cytochrome P450s protein expression in rats. Food Chem. Toxicol., **42**, 1749-1756.

Jian, L., Xie, L.P., Lee, A.H. and Binns, C.W. (2004): Protective effect of green tea against prostate cancer: a case-control study in southeast China. Int. J. Cancer, **108**, 130-135.

Joint FAO/WHO Meeting of Pesticide Residues (JMPR) (2006): Guidance on the interpretation of hepatocellular hypertrophy. Summary report.

Khan, N., Adhami, V.M. and Mukhar, H. (2010): Apoptosis by dietary agents for prevention and treatment of prostate cancer. Endocr. Relat. Cancer, **17**, R39-R52.

Khan, N., Afaq, F. and Mukhtar, H. (2008): Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid. Redox Signal., **10**, 475-510.

Khan, N., Afaq, F., Saleem, M., Ahmad, N. and Mukhtar, H. (2006): Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate. Cancer Res., **66**, 2500-2505.

Kumar, N., Shibata, D., Helm, J., Coppola, D. and Malafa, M. (2007): Green tea polyphenols in the prevention of colon cancer. Front. Biosci., **12**, 2309-2315.

MHLW (Ministry of Health Labor Welfare of Japan) (1996): Guidelines for Designation of food additives and for revision of Standard for Use of Food Additives. Japan Food Additive Association.

Shimizu, M., Shirakami, Y. and Moriwaki, H. (2008): Targeting receptor tyrosine kinases for chemoprevention by green tea catechin, EGCG. Int. J. Mol. Sci., **9**, 1034-1049.

Siddiqui, I.A., Adhami, V.M., Saleem, M. and Mukhtar, H. (2006): Beneficial effects of tea and its polyphenols against prostate cancer. Mol. Nutr. Food Res., **50**, 130-143.

Sim, H.G. and Cheng, C.W. (2005): Changing demography of prostate cancer in Asia. Eur. J. Cancer, **41**. 834-845.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989): Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med., **320**, 915-924.

Stuart, E.C., Scandyln, M.J. and Rosengren, R.J. (2006): Role of epigallocatechin gallate (EGCG) in the treatment of breast and prostate cancer. Life Sci., **79**, 2329-2336.

Takami, S., Imai, T., Hasumura, M., Cho, Y.M. and Hirose, M. (2008): Evaluation of toxicity of green tea catechins with 90-day dietary administration to F344 rats. Food Chem. Toxicol., **46**, 2224-2229.

Verschovle, R.D., Steward, W.P. and Gescher, A.J. (2007): Putative cancer chemopreventive agents of dietary origin - how safe are they? Nutr. Cnacer, **59**, 152-162.

Verschovle, R.D., Steward, W.P. and Gescher, A.J. (2007): Putative cancer chemopreventive agents of dietary origin - how safe are they? Nutr. Cnacer, 59, 152-162.Yang, C.S., Maliakal, P. and Meng, X. (2002): Inhibition of carcinogenesis by tea. Annu. Rev. Pharmacol. Toxicol., **42**, 25-54.

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Validation of the (Q)SAR combination approach for mutagenicity prediction of flavor chemicals

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ABSTRACT

Most exposure levels of flavor in food are considered to be extremely low. If at all, genotoxic properties should be taken into account in safety evaluations. We have recently established a (quantitative) structure–activity relationship, (Q)SAR, combination system, which is composed of three individual models of mutagenicity prediction for industrial chemicals. A decision on mutagenicity is defined as the combination of predictive results from the three models. To validate the utility of our (Q)SAR system for flavor evaluation, we assessed 367 flavor chemicals that had been evaluated mainly by JECFA and for which Ames test results were available. When two or more models gave a positive evaluation, the sensitivity was low (19.4%). In contrast, when one or more models gave a positive evaluation, the sensitivity increased to 47.2%. The contribution of this increased sensitivity was mainly due to the result of the prediction by Derek for Windows, which is a knowledge-based model. Structural analysis of false negatives indicated some common sub-structures. The approach of improving sub-structural alerts could effectively contribute to increasing the predictability of the mutagenicity of flavors, because many flavors possess categorically similar functional sub-structures or are composed of a series of derivatives.

1. Introduction

Many flavor chemicals in current food use have been evaluated under the Threshold of Toxicological Concern concept at the FAO/WHO Joint Expert Committee on Food Additives (JECFA). Most exposure levels of flavor in food are considered to be extremely low. In such cases, genotoxic properties should be taken account in safety evaluations in addition to the empirical threshold. Recently, (quantitative) structure–activity relationship ((Q)SAR) systems have been used to quickly assess the human hazards of chemicals for regulatory purposes (Cronin et al., 2003).

We had developed (Q)SAR models for assessment of chemical genotoxicity, which was optimized for application to industrial chemicals using three commercially available (Q)SAR systems, Derek for Windows and MultiCase, which are used widely by regulatory agencies, and ADMEWorks, which we customized. The results of previous evaluations of our (Q)SAR models using industrial chemical sets independent of the chemicals used for the model development indicated that the sensitivity, specificity and concordance rates were increased when we combined the three (Q)SAR systems to make a definitive decision on mutagenicity. Accordingly, we concluded that the (Q)SAR evaluation could be optimized by combining the evaluations from different systems (Hayashi et al., 2005).

Currently, about 3000 synthetic flavors are distributed commercially in Japan. About 900 of these originate from Japan and have not yet been assessed for their effect on human health. The Japan Flavor and Fragrance Materials Association (JFFMA) has been revaluating these flavor compounds, based on the safety assessment processes of the JECFA; however, for a number of these compounds there is insufficient information on their genotoxicity to be able to follow the JECFA process. It is not realistic for all of the flavor chemicals already used widely in Japan to be examined for genotoxicity because they are so numerous. Therefore, if we could make a reliable prediction of their genotoxicity (the results of the Ames

Abbreviations: JECFA, FAO/WHO Joint Expert Committee on Food Additives; (Q)SAR, (quantitative) structure–activity relationship; JFFMA, Japan Flavor and Fragrance Materials Association; FAS, WHO Food Additives Series; JFSC, Japan Food Safety Commission.

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Table 1
Performances of each (Q)SAR model.

	Ames results	(Q)SAR		
		+	–	Total
Derek for Windows	Positive	10	14	24
	Equivocal	4	8	12
	Negative	22	309	331
	Total	36	331	367
MultiCASE	Positive	6	18	24
	Equivocal	3	9	12
	Negative	19	312	331
	Total	28	339	367
ADMEWorks	Positive	4	20	24
	Equivocal	1	11	12
	Negative	28	303	331
	Total	33	334	367

test or chromosomal aberration test) based on their chemical structures *in silico*, it would be useful in the assessment of flavor chemicals originally used in Japan.

The purpose of our study was to develop an *in silico* system in order to define the priorities for conducting genotoxicity studies of many existing flavors unevaluated and/or flavors newly synthesized; furthermore, in future, to enable exemption from actual genotoxicity studies for evaluating specific chemical groups. In the present study, we applied our previously developed (Q)SAR combination system for predicting the Ames test results of flavors, which we selected from the series of JECFA reports. The prediction performance was not so high, because our system had been customized for industrial chemicals, but the results of this study indicated that our system is capable of improving the predictability of Ames test results for flavors.

2. Materials and methods

2.1. Set of chemicals for validation

The WHO Food Additives Series (FAS) from 1965 to 2008 and evaluation reports published by the Japan Food Safety Commission (JFSC) were used to select a set of flavor chemicals with information from the Ames test.

There were 367 flavor items with information on their activity in the Ames assay. We considered optical and geometrical isomers to be the same compound because sometimes isomers are not distinguished in Ames tests. The 2D structures of chemicals prepared by JFFMA were used for *in silico* evaluation. Moreover, because the results of the (Q)SAR models were not considered by the differences in strains, and with or without S9 mix in Ames tests on a training set, we did not consider their differences in the validation set of 367 compounds.

In the current study, flavors were defined as positive if at least one positive result had been reported. In order to confirm the positive results, we reviewed the corresponding reports in detail, and justified the positive results according to the following criteria. In the case of results obtained by standard methods, a positive result was assigned when a revertant count that exceeded twice the background revertant count was obtained. However, for results by typical methods that were slightly greater than twice or, in the case of positive results obtained by atypical methods, experts reviewed the data of a report, did not consider the report to have clear positive data and judged the report equivocal. If a flavor has reports only with Ames-equivocal results other than Ames-negative results, we considered that flavor to be equivocal. As a result, the judgment consists of "positive," "equivocal," and "negative." Among these 367 flavors, 24 were positive, 12 were equivocal, and 331 were negative compounds in the Ames assay. Overall, 367 flavoring compounds evaluated, and their Ames test and (Q)SAR results are shown in Appendix A. To move closer to our current purpose, we put weight on the findings of Ames-positive alerts, and then considered Ames-equivocal flavors to be positive.

2.2. (Q)SAR programs and in silico definition of positive and negative responses

In silico evaluation of potential mutagenicity was carried out using three commercially available (Q)SAR programs. Derek for Windows (version 10.0.2; Lhasa Ltd., UK) is a specialized or toxic chemical sub-structure rules-based system (Greene et al., 1999). When the system gave an evaluation as "certain", "probable", "plausible" or "equivocal", we considered this as "positive", and when the system gave "doubted", "improbable", "impossible" or "no alert", we considered this as "negative." MultiCase (version 1.90; Multicase Co. Ltd., Japan) is a hybrid system of 2D chemical descriptors based (Q)SAR and known toxic sub-structure identification (Rosenkranz et al., 1999). When the system showed "active", "borderline" or "probably inactive", we considered this as positive, and only when the system showed "inactive" did we consider this as negative. ADMEWorks (version 4.0; Fujitsu Kyushu Systems Ltd., Japan) is a system based mainly on 2D (sometimes 3D) descriptors, such as topological, topographical, physicochemical, and sub-structural parameters. When the system showed "positive," we considered this as "positive", and when the system showed "negative" we considered this as "negative". We selected these systems for the combined prediction system because of their different modes of analysis. In this study, *in silico* prediction of the mutagenicity of 367 flavor chemicals was performed using prediction models developed in our previous study (Hayashi et al., 2005), and compared with the reported experimental results.

2.3. Definitions in (Q)SAR models

We calculated sensitivity, specificity, concordance, applicability, false positive, and false negative as follows:

$$\text{Sensitivity (\%)} = N_{AS+}/N_{A+} \times 100,\ \text{Specificity (\%)} = N_{AS-}/N_{A-} \times 100,$$

$$\text{Concordance (\%)} = (N_{AS+} + N_{AS-})/N_{eval} \times 100,\ \text{Applicability (\%)} = N_{eval}/N_{all} \times 100,$$

$$\text{False positive (\%)} = (N_{A-} - N_{AS-})/N_{S+} \times 100,\ \text{False negative (\%)} = (N_{A+} - N_{AS+})/N_{S-} \times 100,$$

where N_{A+} is the number of chemicals that are positive in an *in vitro* assay (Ames test); N_{A-} is the number of chemicals negative in an *in vitro* assay (Ames test); N_{AS+} is the number of chemicals positive by both the Ames test and (Q)SAR evaluation; N_{AS-} is the number of chemicals negative in both the Ames test and (Q)SAR evaluation; N_{all} is the total number of chemicals analyzed; N_{eval} is the number of chemicals evaluated; N_{S+} is the number of chemicals positive in (Q)SAR evaluation; and N_{S-} is the number of chemicals negative in (Q)SAR evaluation.

3. Results

The predictions were performed by the single (Q)SAR model, the performances of each (Q)SAR model are shown in Table 1 and the results of their evaluations are summarized in Table 2. The predictions were performed also by combined evaluation of the three (Q)SAR models in three different ways: combination-1, -2 and -3. In combination-1, *in silico* mutagenicity evaluated using (Q)SAR systems was considered to be positive (or negative) only when all three models gave unanimous evaluations. In combination-2, *in silico* mutagenicity was considered to be positive (or negative) when two or more models gave the same evaluations. In combination-3, *in silico* mutagenicity was considered to be positive when one or more models gave a positive evaluation and to be negative when all three models gave negative evaluations. Performances of each combination of three (Q)SAR modes are shown in Table 3 and results of their evaluations are summarized in Table 4.

Table 2
Results of evaluation of each (Q)SAR model.

	Sensitivity (%)	Specificity (%)	Concordance (%)	Applicability (%)	False positive (%)	False negative (%)
Derek for Windows	38.9	93.4	88.0	100.0	61.1	6.6
MultiCase	25.0	94.3	87.5	100.0	67.9	8.0
ADMEWorks	13.9	91.5	83.9	100.0	84.8	9.3