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Evaluation of the Genetic Toxicity of Synthetic Chemicals (IV)
– *in vitro* Chromosomal Aberration Assay with 18 Chemicals in Chinese Hamster Lung Cells –

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ABSTRACT: **The detection of many synthetic chemicals used in industry that may pose a genetic hazard in our environment is of great concern at present. Since these substances are not limited to the original products, and enter the environment, they have become widespread environmental pollutants, thus leading to a variety of chemicals that possibly threaten the public health. In this respect, to regulate and to evaluate the chemical hazard will be important to environment and human health. The clastogenicity of 18 synthetic chemicals was evaluated in Chinese hamster lung fibroblast cells *in vitro*. 4-Chloro-3,5-dimethyl phenol (CAS No. 88-04-0) induced chromosomal aberrations with significance at the concentration of 15.7 μg/ml both in the presence and absence of metabolic activation system. Phenoxybenzene (CAS No. 101-84-8) which is one of the most cytotoxic chemical among 18 chemicals tested revealed no clastogenicity in the range of 0.11-0.43 μg/ml both in the presence and absence of metabolic activation system. From the results of chromosomal aberration assay with 18 synthetic chemicals in Chinese hamster lung cells *in vitro*, 4-chloro-3,5-dimethyl phenol (CAS No. 88-04-0) revealed weak positive clastogenic results in this study.**

Keywords : Genotoxicity, Clastogenicity, *in vitro* chromosome aberration, Chinese hamster lung fibroblast

Introduction

The establishment of toxicity and detection of synthetic chemicals that may pose a genetic hazard in our environment is subjects of great concern at present (WHO, 1971) because there are many synthetic chemicals used in chemical reaction processes in industry.

Several assay systems having rapidity and reliability have been introduced for this purpose, such as reversion test with bacterial gene mutation (Ames *et al.*, 1973, 1975; Maron and Ames, 1983), chromosomal aberration assay with mammalian cells (Ishidate and Odashima, 1977), micronucleus assay with rodents (Hayashi *et al.*, 1992; Schmid, 1975). These assay systems are now well used to evaluate the genotoxicity of chemicals and also frequently adopted as methods for an index of genotoxicity worldwide. Furthermore, it was well applied as a screening probe for the detection of possible carcinogenic substances in our environment. Since the tens of thousands of man-made chemicals that have been introduced into the environment in the last few decades must also be tested for their damaging effect on DNA, the agents that cause this damage must be identified.

As one of the mechanisms of carcinogenicity, induction of DNA damage was ascertained by several genotoxicity assays. Generally, the carcinogenicity of chemicals including endocrine disrupting chemicals is one of the potential toxicity that may consider for the human health. And also, it has been suggested that substances present in the environment may contribute to the development of hormone-dependent cancers and comprise reproductive capacity in humans and wildlife (Colborn *et al.*, 1993; Eubanks, 1997). One example, phthalates are often mentioned as suspected endocrine disruptors, i.e., some phthalates are blamed for causing damage to the testes and decreasing sperm production (Gray *et al.*, 1982; Hardell *et al.*, 1997) and are reported to be a potential carcinogen. The influence of phthalates on hepatocarcinogenesis was documented in animal models (Huber *et al.*, 1996; Richmond *et al.*, 1996).

Despite the many toxicological researches on synthetic chemicals, there are few reports on the genotoxicity of some chemicals especially well used in chemical reaction processes in industry. In this respect, administrative authority has great concern to regulate and to evaluate the chemical hazards, and conducted the toxicity evaluations of synthetic chemicals. Our laboratory had also been involved in toxicity evaluation, especially in genotoxicity (Ryu *et al.*, 1993a, 1994, 1996a,b, 1997, 1998a,b,c,d, 1999a,b, 2000, 2001a,b,c,d, 2002; Kim *et al.*, 2001).

In this study, we aim to elucidate the calstogenicity of 18

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synthetic chemicals used in chemical process with CHL cells *in vitro*.

Materials and Methods

The experiment was performed as described by OECD (1993) and Ishidate and Odashima (1977) with some minor modifications (Ryu *et al.*, 1993a, 1994, 1996a,b, 1998a,b, 2001b) which are briefly summarized as follows.

Cell Culture

A clonal sub-line of a chinese hamster lung (CHL) fibroblast cells was obtained from the National Institute of Health Sciences, Tokyo, Japan. The karyotype of CHL cells consisted of 25 chromosomes. The cells had been maintained by 3-4 day passages and grown in a monolayer with Eagles minimum essential medium (EMEM, Gibco, 410-1100EA) supplemented with 10% fetal bovine serum (FBS, Gibco, 26140-020). These cells were maintained at 37°C in 5% CO_2 atmosphere.

Reagents

Trypsin-EDTA and colcemid were the products of Gibco BRL Life Tech. Inc. (Gaithersburg, USA). The test chemicals were kindly donated and purchased from several companies as indicated in Table 2. The test compounds were dissolved in dimethylsulfoxide (DMSO). The preparation of rat liver S-9 fraction for metabolic activation system was previously reported (Ames *et al.*, 1973; Maron and Ames, 1983). The S-9 fraction prepared was stored immediately at −80°C before use.

Determination of the 50% growth inhibition concentration.

Test article dose levels were determined prior to the main study in a dose range-finding study performed in the absence of a rat liver S-9 activation system. For the growth inhibition assay, CHL cells were seeded at the density of 5×10^4 cells/ml into 96 well plates. Twenty-four hours after seeding, several different doses of sample were separately added and incubated for 24 hours. And then the 50% inhibition concentration (IC_{50}) values were calculated by MTT assay (Mosmann, 1983).

Chromosome aberration assay

For the aberration assay, three different doses, including the IC_{50} value as a maximum dose, were prepared and separately added to 3-day-old cultures (approximately 10^5 cells/60 mm dish). In the absence of metabolic activation, cultures were treated for 24 hours with the test article, while in the presence of metabolic activation, cells were treated for 6 hours because of toxicity of S-9 and then maintained for 18 hours in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Treatment was followed by addition of medium containing colcemid at a concentration of 0.2 μg/ml. After 2 hr further incubation in the presence of colcemid, metaphase cells were harvested by centrifugation and trypsinization. The cells were swollen with hypotonic (0.075 M) KCl solution for 20 min at 37°C, and washed three times in ice-cold fixative (methanol : glacial acetic acid = 3 : 1). After centrifugation, the fixative was removed, and cell pellet suspensions were prepared by pipetting gently. A few drop of cell pellet suspension were dropped onto pre-cleaned glass microscope slides, and air dried. Slides were stained with 5% Giemsa buffered solution at pH 6.8 for scoring of chromosome aberrations. The number of cells with chromosomal aberrations was recorded on 200 well-spread metaphases at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS (1988). Breaks less than the width of a chromatid were designated as gaps in our criteria, and not included as chromosomal aberration. The incidence of polyploid and endoreduplicated cells was also recorded when these events were observed. Solvent-treated cells served as controls in this experiment.

Evaluation

CHL cells usually have less than 3.0% cells with spontaneous chromosome aberrations. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fishers exact test (Altman, 1993) with Dunnetts adjustment and compared with results from the solvent controls. Therefore, data from count up well-spread 200 metaphase cells were expressed as percentages, and then dose-dependent responses and the statistical significance in *p*-value will be considered as positive results in our judgement.

Results and Discussion

It is well known that carcinogenicity of synthetic chemicals is the most serious problem in human health hazard. As one of the mechanisms of carcinogenicity, it has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1977). Moreover, mutagens

and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement (Zimmermann, 1971) which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of cellular cancer genes (Radman *et al.*, 1982).

Several short term methods have been developed (Ames *et al.*, 1973; Maron and Ames, 1983; Mersch-Sundermann *et al.*, 1991) for predicting the carcinogenicity of chemicals and also been introduced to the evaluation of genotoxicity (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979; Radman *et al.*, 1982; Hayashi *et al.*, 1982, 1990, 1992; Ryu *et al.*, 1993a, 1994, 1996a,b, 1997, 1998a,b,c,d, 1999a,b, 2000, 2001a,b,c,d, 2002) and of antimutagenicity (Sato *et al.*, 1991; Ryu *et al.*, 1993b, 2001c). Cytogenetic studies on mammalian cells *in vivo* (Schmid, 1975; Hayashi *et al.*, 1982, 1990, 1992, 1994; Heo *et al.*, 1997) as well as *in vitro* (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979) have also been widely used as a screening method for DNA-attacking substances.

The chemical name and CAS No. of test chemicals were listed in Table 1 and their uses in industry are diverse. Among the many synthetic chemicals used in chemical reaction processes in industry, for example, phthalates are well used as plasticizers and softeners to increase the flexibility and workability of high-molecular-weight polymers. The annual world wide production of phthalates approximates 3 million metric tons (Bauer and Herrmann, 1997). Phthalates are well used in plastic goods (e.g., in children's toys, paints, lacquers, cosmetics, as well as food wrappings) and many medical items, such as blood bags, tubes, and filtering membranes. And also, nitromethane (CAS No. 75-52-5) used as rocket fuel, solvent for zein and in coating industry. Carbazole (CAS No. 86-74-8) is important dye intermediate, and 4-chloro-3,5-dimethyl phenol (CAS No. 88-04-0) and dibenzo-1,4-thiazine (CAS No. 92-84-2) used as antibacterial and insecticide. Phenoxybenzene (CAS No. 101-84-8) also used as heat transfer medium and in organic synthesis, and dimethoxymethane (CAS No. 109-87-5) used in perfumery. Since these substances are not limited to the original products, and they may enter the environment and have become widespread environmental pollutants, thus leading to a variety of chemicals that possibly threaten the public health. Nevertheless of the diverse uses of these chemicals in industry, however, there has been no attention to evaluate the toxicity of some chemicals especially used in chemical industry. The detection and the regulation of man-made synthetic chemicals are subjects of great concern because of its close correlation between environmental contamination and human health.

We used CHL cells in this experiment because it was reported no diffrences of sensitivity between CHL and CHO (Chinese hamster ovary) cells in *in vitro* chromosome aberration study (Galloway *et al.*, 1997). It was also reported that extended harvest times are not necessary for the detection of *in vitro* clastogens in regulatory cytogenetic studies except it might help to resolve an equivocal result by Henderson *et al.* (1996). The IC_{50} values of cell growth of test articles in CHL cells are obtained in the absence of metabolic activation system as shown in Table 1. Phenoxybenzene is the most cytotoxic having IC_{50} value as 0.43 μg/ml among 18 chemicals tested. The concentration used and detailed data of chromosome aberration of 18 chemicals are

Table 1. 50% Cell Growth Inhibition concentration (IC_{50}) of 18 synthetic chemicals in Chinese hamster lung cells

Chemical Name	CAS No.	Cat. No.	IC_{50} (μg/ml)
1. Nitromethane	75-52-5	27042-3	610.0
2. Nitroethane	79-52-5	22787-0	750.0
3. 6-tert-Butyl-2,4-xylenol	1879-09-0	B0903	89.0
4. Diisobutyl phthalate	84-69-5	9669	1.7
5. Dinonyl phthalate	84-76-4	814157	1.05
6. Ditridecyl phthalate	119-06-2	047-22815	5,300.0
7. Diisodecyl phthalate	26761-40-0	3622	4.5
8. 9H-Carbazole	86-74-8	C5132	8.36
9. 4-Chloro-3,5-dimethyl phenol	88-04-0	C4394	15.7
10. Dibenzo-1,4-thiazine	92-84-2	P4889	1.0
11. 2,5-Dichloroaniline	95-82-9	10202-4	16.2
12. 2-Methyl 2-propenoic acid-2-propenyl ester	96-05-9	M01075	63.0
13. p-tert-Butylbenzoic acid	98-73-7	23975-5	178.0
14. 3-Cyanopyridine	100-54-9	C5880	1,040.0
15. Phenoxybenzene	101-84-8	820978	0.43
16. 4-Chlorophenyl isocyanate	104-12-1	15227-7	339.0
17. Propylamine	107-10-8	P5893	74.0
18. Dimethoxymethane	109-87-5	D8154	760.0

Table 2. Chromosome aberration assay of 18 chemicals in Chinese hamster lung cells

Test chemicals (CAS No.)	Manufactured by	Concentration (μg/ml)	without (−) or with (+) S9 mix	Aberration Frequency(%)				Total aberration (%)	Extra aberrations (%)			
				Chromatid		Chromosome						
				Br	Ex	Br	Ex		ctg	csg	poly	endo
DMSO			−	1.2±0.5	0	0.3±0.1	0	1.5±0.5[a)]	0.7	0	0.1	0
			+	0.8±0.3	0	0.4±0.2	0	1.2±0.4[a)]	0.5	0	0	0
Nitromethane (75-52-5)	S	610	−	1	0	0	0	1	1	0	0	0
		305	−	0	0	0	0	0	1	0	0	0
		153	−	0	0	0	0	0	0	1	0	0
		610	+	3	0	0	0	3	0	0	0	0
		305	+	0	1	0	0	1	2	0	1	0
		153	+	0	0	0	0	0	2	0	0	0
Nitroethane (79-52-5)	A	750	−	1	0	0	0	1	2	1	0	0
		345	−	0	0	0	0	0	3	1	0	0
		173	−	0	0	0	0	0	2	0	0	0
		750	+	1	0	0	0	1	1	0	0	0
		345	+	2	0	0	0	2	0	0	0	0
		173	+	0	0	0	0	0	1	0	0	0
6-tert-Butyl-2,4-xylenol (1879-09-0)	T	89	−	1	0	0	0	1	1	0	1	0
		45	−	0	0	0	0	0	2	0	0	0
		23	−	0	1	0	0	1	0	0	0	0
		89	+	2	0	0	0	2	0	0	0	0
		45	+	2	0	0	0	2	0	0	0	0
		23	+	0	0	0	0	0	1	0	0	0
Diisobutyl phthalate (84-69-5)	M	1.74	−	3	0	0	0	3	1	0	0	0
		0.87	−	1	0	0	0	1	1	1	0	0
		0.44	−	2	0	0	0	2	1	0	0	0
		1.74	+	0	0	0	0	0	2	0	0	0
		0.87	+	1	0	0	0	1	0	1	0	0
		0.44	+	1	0	0	0	1	1	0	1	0
Dinonyl phthalate (84-76-4)	M	1.05	−	1	0	0	0	1	2	1	0	0
		0.53	−	1	0	0	0	1	1	1	0	0
		0.26	−	0	0	0	0	0	0	2	0	0
		1.05	+	0	0	0	0	0	4	2	1	0
		0.53	+	1	0	0	0	1	4	0	0	0
		0.26	+	0	0	1	0	1	4	0	0	0
Ditridecyl phthalate (119-06-2)	W	5,300	−	0	0	0	0	0	2	0	0	0
		2,650	−	1	0	0	0	1	2	0	0	0
		1,325	−	0	0	2	0	2	1	0	0	0
		5,300	+	1	0	0	0	1	1	0	0	0
		2,650	+	1	0	0	0	1	0	0	0	0
		1,325	+	1	0	0	0	1	1	0	0	0
Diisodecyl phthalate (26761-40-0)	M	4.5	−	1	0	0	0	1	2	1	0	0
		2.3	−	1	0	0	0	1	1	0	0	0
		1.2	−	0	0	0	0	0	2	0	0	0
		4.5	+	0	0	0	0	0	2	0	0	0
		2.3	+	0	0	0	0	0	1	0	0	0
		1.2	+	0	0	0	0	0	1	0	0	0
9H-Carbazole (86-74-8)	S	8.36	−	1	1	0	0	2	0	1	0	0
		4.18	−	1	0	0	0	1	1	0	0	1
		2.09	−	1	0	0	0	1	2	0	0	0
		8.36	+	2	0	0	0	2	1	0	1	0
		4.18	+	0	0	0	0	0	2	0	0	0
		2.09	+	0	0	0	0	0	2	0	0	0

Table 2. Continued

Test chemicals (CAS No.)	Manufactured by	Concentration (μg/ml)	without (–) or with (+) S9 mix	Aberration Frequency(%)				Total aberration (%)	Extra aberrations (%)			
				Chromatid		Chromosome						
				Br	Ex	Br	Ex		ctg	csg	poly	endo
4-Chloro-3,5-dimethyl phenol (88-04-0)	S	15.7	–	5	2	0	0	7*	4	0	0	0
		7.9	–	2	0	1	0	3	2	0	0	0
		3.9	–	2	0	1	0	3	0	0	1	0
		15.7	+	6	2	0	1	9*	3	0	0	0
		7.9	+	3	0	0	0	3	1	1	0	0
		3.9	+	2	0	0	0	2	1	1	0	0
Dibenzo-1,4-thiazine (92-84-2)	S	1	–	0	0	1	0	1	0	3	0	0
		0.5	–	0	0	1	0	1	2	0	1	0
		0.25	–	0	0	0	0	0	1	2	0	0
		1	+	0	1	1	0	2	0	1	0	0
		0.5	+	0	1	0	0	1	1	0	1	0
		0.25	+	0	1	0	0	1	0	1	0	0
2,5-dichloroaniline (95-82-9)	A	16.2	–	1	0	0	0	1	0	1	1	0
		8.1	–	1	0	0	0	1	1	1	0	0
		4.1	–	1	0	0	0	1	0	1	0	1
		16.2	+	1	0	0	1	2	1	0	0	0
		8.1	+	3	0	0	0	3	1	0	0	0
		4.1	+	0	0	1	0	1	1	0	0	0
2-Methyl-2-propenoic acid-2-propenyl ester (96-05-9)	T	63	–	1	0	1	0	2	1	0	0	0
		32	–	0	0	0	0	0	2	0	0	0
		16	–	0	0	0	0	0	1	1	0	0
		63	+	1	0	0	0	1	1	0	0	0
		32	+	1	0	0	0	1	1	0	0	0
		16	+	2	1	0	0	3	0	0	0	0
p-tert-Butylbenzoic acid (98-73-7)	A	178	–	2	0	0	0	2	2	0	0	0
		89	–	2	0	0	1	3	0	0	0	0
		45	–	1	1	0	0	2	1	1	0	0
		178	+	0	0	0	0	0	1	1	0	0
		89	+	1	0	0	0	1	1	0	0	0
		45	+	0	0	0	0	0	3	0	0	0
3-Cyanopyridine (100-54-9)	S	1,040	–	0	0	1	0	1	1	2	0	0
		520	–	0	0	0	0	0	0	2	1	0
		160	–	1	0	0	0	1	0	2	0	0
		1,040	+	0	0	0	0	0	3	0	0	0
		520	+	0	1	1	0	2	2	0	0	0
		160	+	0	0	0	0	0	2	0	0	0
Phenoxy-benzene (101-84-8)	M	0.43	–	1	0	0	0	1	1	0	0	0
		0.22	–	0	0	1	0	1	2	0	0	0
		0.11	–	1	0	0	0	1	1	0	1	0
		0.43	+	1	0	0	0	1	3	0	0	0
		0.22	+	0	0	1	0	1	2	0	0	0
		0.11	+	0	0	1	1	2	2	0	0	0
4-Chlorophenyl isocyanate (104-12-1)	A	339	–	0	0	0	0	0	3	0	0	0
		170	–	1	0	0	0	1	1	0	0	0
		85	–	1	0	0	0	1	1	0	0	0
		339	+	0	0	0	0	0	1	0	0	0
		170	+	0	0	0	0	0	1	0	0	0
		85	+	0	0	0	0	0	2	0	0	0
Propylamine (107-10-8)	S	74	–	0	0	0	1	1	1	1	1	0
		37	–	1	0	1	0	2	0	0	0	0
		18.5	–	0	0	0	0	0	1	1	0	0
		74	+	0	0	1	1	2	1	0	0	0
		37	+	0	0	0	0	0	2	2	0	0
		18.5	+	0	0	1	0	1	0	0	0	0

Table 2. Continued

Test chemicals (CAS No.)	Manufactured by	Concentration (μg/ml)	without (−) or with (+) S9 mix	Aberration Frequency (%)				Total aberration (%)	Extra aberrations (%)			
				Chromatid		Chromosome						
				Br	Ex	Br	Ex		ctg	csg	poly	endo
Dimethoxymethane (109-87-5)	S	760	−	1	0	0	0	1	2	0	0	0
		380	−	0	1	0	0	1	3	0	0	0
		190	−	0	0	0	0	0	3	0	0	0
		760	+	0	0	0	0	0	1	0	0	0
		380	+	0	0	0	0	0	2	0	1	0
		190	+	0	0	0	0	0	1	0	0	0
MMC	S	0.1	−	13.3±4.6	16.4±3.4	8.2±3.1	2.0±0.5	32.9±5.5[a)]	5.2	1.5	0.2	0
B(a)P	S	200	+	9.2±4.1	5.5±3.8	5.4±3.0	1.5±1.0	18.9±5.5[a)]	4.6	0.5	0	0

*significant at $p<0.05$.

Br: Breakage, Ex: Exchange, ctg: chromatid gap, csg: chromosome gap, poly: polyploid, endo: endoreduplicate, DMSO: dimethlysulfoxide, MMC: mitomycin C, B(a)P: benzo(a)pyrene.

The values of solvent and positive controls are expressed as mean ±S.D.

A: Aldrich Chemical Co. Inc., WI., USA, M: Merck AG., Darmstadt, Germany, S: Sigma Chemical Co. Ltd., Seoul, Korea, T: Tokyo Casei Inc., Japan , W: Wako Pure Chemical Co., Osaka, Japan.

summarized in Table 2. The DMSO negative control is revealed only 1.5% and 1.2% spontaneous aberrations in the absence and presence of metabolic activation system in 200 metaphase of CHL cells, respectively. However, the positive controls, benzo(a)pyrene (200 μg/ml) as an indirect mutagen that require metabolic activation and mitomycin C (0.1 μg/ml) as a direct-acting mutagen, induced remarkable chromosome aberrations (18.9-32.9%) in CHL cells as shown in Table 2.

Phenoxybenzene, the most cytotoxic compound among 18 chemicals tested, revealed no clastogenicity in the concentration range of 0.11-0.43 μg/ml both in the presence and absence of metabolic activation system. However, 4-chloro-3,5-dimethyl phenol (CAS No. 88-04-0) induced chromosomal aberrations with significance at the concentration of 15.7 μg/ml both in the presence and absence of metabolic activation system.

From the results of chromosomal aberration assay with 18 synthetic chemicals in Chinese hamster lung cells, 4-chloro-3,5-dimethyl phenol (CAS No. 88-04-0) revealed weak positive clastogenic results in this study.

Recently, several next generation battery of genotoxicity for the detection of genetic damages *in vitro* and *in vivo* were introduced according to the rapid progress in toxicology combined with cellular and molecular biology. Among these methods, the single cell gel electrophoresis (comet assay) which can be detected DNA damages in cell level (Singh *et al.*, 1994; Ryu *et al.*, 1997, 2001a,d; Tice *et al.*, 2000), mouse lymphoma thymidine kinase gene assay (Clive *et al.*, 1983; Sawyer *et al.*, 1985; Ryu *et al.*, 1999a), FISH (fluorescence in situ hybridization) (Hayashi *et al.*, 1994), PRINS (primed in situ hybridization) (Abbo *et al.*, 1993) and transgenic animal and cell line model as a parameter of *lac I* (Big Blue) (Kohler *et al.*, 1991; Ryu *et al.*, 1998c,d, 1999b, 2000, 2002) or *lac Z* (Muta Mouse) (Suzuki *et al.*, 1993) gene mutation are newly introduced based on cellular and molecular toxicological approaches. Also, *in vivo* supravital micronucleus assay with peripheral reticulocytes by using acridine orange fluorescent staining (Hayashi *et al.*, 1990, 1992; Ryu *et al.*, 1998b) was introduced instead of mouse bone marrow micronucleus assay. Our laboratory is now under pro-gress these assays to evaluate and to elucidate the mechanism of genetic toxicity and/or carcinogenesis, and will be presented in near future.

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