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Evaluation of the Genetic Toxicity of Synthetic Chemicals (XIV)-*in vitro* Chromosomal Aberration Assay with 11 Chemicals in Chinese Hamster Lung Cells

Youn-Jung Kim[1] & Jae-Chun Ryu[1]

[1]Cellular and Molecular Toxicology Laboratory, Korea Institute of Science and Technology, PO Box 131, Cheongryang, Seoul 130-650, Korea
Correspondence and requests for materials should be addressed to J.-C. Ryu (ryujc@kist.re.kr)

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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 11 synthetic chemicals was evaluated in Chinese hamster lung fibroblast cells *in vitro*. 1-Chloro-3-bromopropane CAS No. 109-70-6) induced chromosomal aberrations with significance at the concentration of 185.0 μg/mL and 1,600 μg/mL both in the presence and absence of metabolic activation system, respectively. Triphenyl phosphite (CAS No. 101-02-0), which is one of the most cytotoxic chemical among 11 chemicals tested revealed no clastogenicity in the range of 95.0-4.9 μg/mL both in the presence and absence of metabolic activation system. From the results of chromosomal aberration assay with 11 synthetic chemicals in Chinese hamster lung cells *in vitro*, 1-chloro-3-bromopropane revealed a positive clastogenic result in this study.

Keywords: Genotoxicity, Clastogenicity, *in vitro* Chromosome Aberration, Chinese Hamster Lung Fibroblast

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[1] 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[2,3], chromosomal aberration assay with mammalian cells[4] and micronucleus assay with rodents[5,6]. 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[7,8]. 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[9,10] and are reported to be a potential carcinogen. The influence of phthalates on hepatocarcinogenesis was documented in animal models[11,12].

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[13-30]. In this study, we aim to elucidate the clastogenicity of 11 synthetic chemicals used in chemical process with Chinese hamster lung (CHL) cells *in vitro*.

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[31] and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency[32]. Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement[33] which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of cellular cancer genes[34].

Several short term methods have been developed[2,3,35] for predicting the carcinogenicity of chemicals and also been introduced to the evaluation of genotoxicity[4,5,13-29,34,36,37], and of antimutagenicity[27,38,39]. Cytogenetic studies on mammalian cells in vivo[5,6,36,37,40,41] as well as *in vitro*[4,36] 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 2. On looking into diverse usages of these synthetic chemicals, p-nitroanisole (CAS No. 100-17-4) is an intermediate used in the manufacture of p-Anisidine, a raw material for dyes and pharmaceuticals. Triphenyl phosphite (CAS No. 101-02-0) is used a flame-retardant plasticizer for cellulosics and a plasticizer for hot-melt adhesives. It is useful in the upholstery and roofing paper industries. Glycerol triacetate (CAS No. 102-76-1) is reported to function as a cosmetic biocide, plasticizer, and solvent in cosmetic formulations, at concentrations ranging from 0.8% to 4.0%. It is a commonly used carrier for flavors and fragrances. 2-Ethylhexyl acetate (CAS No. 103-09-3) is used as a solvent, flow improver and coalescent in paints, lacquers, coating and printing ink industry. Benzyl ether (CAS No. 103-50-4) is a plasticizer for nitrocellulose and a solvent in perfumery. Also, it is used particularly in cements based on polyvinyl acetate emulsions. Diethyl malonate (CAS No. 105-53-3) is used for manufacture of barbiturates, a pharmaceutical. Hexyl cinnamic aldehyde (CAS No. 101-86-0) is one of the most frequently used deodorants of cosmetics. 1-Chloro-3-bromopropane (CAS No. 109-70-6) is a useful reaction intermediate to prepare gamma-chloropropyl derivatives, for example, the cyanide displacement of 1-bromo-3-chloropropane forms gamma-chlorobutyronitrile, an intermediate used for the production of herbicides, pesticides, pharmaceuticals and other commercial products. n-Octyl alcohol (CAS No. 111-87-5) is used in the manufacture of perfumes and esters, in a process to extract citric acid from fermentation broths and as a solvent in resistant coatings and linings. And dioctyl phthalate (CAS No. 117-84-0) is used as a plasticizer in plastics and rubber materials and used for film, wire, cables, and adhesives. 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. 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.

Table 1. 50% Inhibition Concentration (IC_{50}) of 11 synthetic chemicals.

Chemical name	IC_{50} (μg/mL)	
	Without S9	With S9
1. p-Nitroanisole	525.0	680.0
2. Triphenyl phosphite	19.3	95.0
3. Glycerol triacetate	1,800.0	1,800.0
4. 2-Ethylhexyl acetate	660.0	3,200.0
5. Benzyl ether	650.0	544.0
6. Diethyl-malonate	4,400.0	3,600.0
7. Hexyl-cinnamic aldehyde	44.0	425.0
8. 1-Chloro-3-bromopropane	1,600.0	185.0
9. n-Hexylamine	144.9	640.9
10. n-Octyl alcohol	84.1	163.1
11. Dioctyl phthalate	>5,000.0	>5,000.0

We used CHL cells in this experiment because it was reported no differences of sensitivity between CHL and CHO (Chinese hamster ovary) cells *in vitro* chromosome aberration study[42]. 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.*[43]. The IC_{50} values of cell growth of test chemicals in CHL cells are obtained in the absence (without S9) and presence (with S9) of metabolic activation system as shown in Table 1. Triphenyl phosphite is the most cytotoxic having IC_{50} value as 19.3 μg/mL and 95.0 μg/mL in without S9 and with S9 system, respectively, among 11 chemicals tested. The concentration used and detailed data of chromosome aberration of 11 chemicals are summarized in Table 2. The DMSO negative control is revealed only 0.7%, 0.8% and 1.0% spontaneous aberrations in the absence and presence of metabolic

Table 2. Chromosome Aberration Assay of 11 chemicals in Chinese hamster lung cells.

Test chemicals (CAS No.)	Manuf-actured by	Conce-ntration (μg/mL)	Treat-ment (hr)	without (−) or with (+) S9 Mix	Aberration Frequency (%)				Total aberration (%)	Extra aberration			
					Chromatid		Chromosome						
					Br	Ex	Br	Ex		ctg	csg	poly	endo
DMSO[a]	S		6	−	1.2±1.0	0.3±0.5	0	0	0.7±0.5	2.6±1.1	0	1.8±2.6	0
			6	+	0.8±0.9	0.8±0.8	0	0	0.8±0.3	1.7±1.4	0.2±0.4	0	0
			24	−	1.0±1.2	0.8±0.6	0	0.1±0.3	1.0±0.5	2.0±1.2	0	1.3±1.8	0
MMC[b]	S	0.1	6	−	5.9±3.1	64.4±12.0	0.1±0.3	0.5±0.5	32.9±5.0	2.9±1.6	0.1±0.3	0.5±1.2	0
		0.1	24	−	15.4±6.4	87.6±27.4	1.4±2.3	0	45.3±7.4	5.7±2.5	0.1±0.3	1.2±1.5	0
CP	S	10.0	6	+	20.6±9.9	80.5±33.2	1.0±1.4	0	43.0±16.3	5.7±1.7	0.5±0.9	1.5±2.1	0.1±0.3
p-Nitroanisole (100-17-4)	W	525.0	6	−	1	5	0	0	3.0	1	0	1	0
		262.5	6	−	0	1	0	1	1.0	3	0	0	0
		131.3	6	−	3	0	0	0	1.5	1	0	0	0
		5,000.0	6	+	3	0	0	0	1.5	0	0	0	0
		2,500.0	6	+	4	2	0	0	3.0	2	0	0	0
		1,250.0	6	+	0	0	0	0	0.0	0	0	0	0
		525.0	24	−	1	0	0	0	0.5	1	0	0	0
		262.5	24	−	0	0	0	1	0.5	1	0	0	0
		131.3	24	−	0	0	0	0	0.0	1	0	0	0
Triphenyl phosphite (101-02-0)	A	19.3	6	−	1	0	1	0	1.0	2	2	1	0
		9.7	6	−	2	0	0	0	1.0	5	4	0	0
		4.9	6	−	0	0	0	1	0.5	6	2	1	0
		95.0	6	+	4	1	0	0	2.5	6	0	3	0
		47.5	6	+	2	0	0	0	1.0	2	2	3	0
		23.8	6	+	0	0	0	0	0.0	2	0	2	0
		19.3	24	−	1	1	1	0	1.5	1	1	6	0
		9.7	24	−	0	0	0	0	0.0	2	1	2	0
		4.9	24	−	2	0	1	0	1.5	2	0	1	0
Glycerol triacetate (102-76-1)	W	1,800.0	6	−	0	0	0	0	0.0	1	0	0	0
		900.0	6	−	1	0	0	0	0.5	2	0	0	0
		450.0	6	−	0	0	0	0	0.0	1	0	1	0
		1,800.0	6	+	0	0	0	0	0.0	2	0	0	0
		900.0	6	+	0	0	0	0	0.5	4	0	0	0
		450.0	6	+	2	0	0	0	1.0	1	0	0	0
		1,800.0	24	−	0	0	0	0	0.0	1	0	0	0
		900.0	24	−	0	0	0	0	0.0	2	0	0	0
		450.0	24	−	0	1	0	0	0.5	2	0	0	0
2-Ethylhexyl acetate (103-09-3)	W	660.0	6	−	1	0	0	0	0.5	1	0	0	0
		330.0	6	−	1	0	0	0	0.5	0	0	2	0
		165.0	6	−	1	0	0	0	0.5	1	0	2	0
		3,200.0	6	+	0	0	0	0	0.0	1	0	0	1
		1,600.0	6	+	0	0	0	0	0.0	1	0	0	1

Table 2. Continued.

Test chemicals (CAS No.)	Manuf-actured by	Conce-ntration (μg/mL)	Treat-ment (hr)	without (−) or with (+) S9 Mix	Aberration Frequency (%)				Total aberration (%)	Extra aberration			
					Chromatid		Chromosome						
					Br	Ex	Br	Ex		ctg	csg	poly	endo
2-Ethylhexyl acetate (103-09-3)	W	800.0	6	+	1	0	0	0	0.5	0	0	1	0
		660.0	24	−	2	0	0	0	1.0	2	0	1	0
		330.0	24	−	1	0	0	0	0.5	1	0	2	0
		165.0	24	−	2	0	0	0	1.0	2	0	1	0
Benzyl ether (103-50-4)	W	650.0	6	−	0	0	0	1	0.5	1	1	0	0
		325.0	6	−	1	2	0	0	1.5	3	0	0	0
		162.5	6	−	0	0	0	0	0.0	3	0	0	0
		544.0	6	+	0	0	0	0	0.0	0	0	0	0
		272.0	6	+	1	0	0	0	0.5	1	0	0	0
		136.0	6	+	1	2	0	0	1.5	1	0	0	0
		650.0	24	−	3	0	0	0	1.5	3	0	0	0
		325.0	24	−	2	0	1	0	1.5	3	0	0	0
		162.5	24	−	0	0	0	0	0.0	3	0	0	0
Diethylmalonate (105-53-3)	W	4,400.0	6	−	0	1	0	0	0.5	0	0	0	0
		2,200.0	6	−	0	0	0	0	0.0	1	0	1	0
		1,100.0	6	−	0	1	0	0	0.5	0	0	0	0
		3,600.0	6	+	1	0	0	0	0.5	2	0	0	0
		1,800.0	6	+	0	0	0	0	0.0	5	0	0	0
		900.0	6	+	2	0	1	0	1.5	2	0	0	0
		4,400.0	24	−	0	1	0	0	0.5	2	0	0	0
		2,200.0	24	−	0	0	0	0	0.0	0	0	0	0
		1,100.0	24	−	0	0	0	0	0.0	1	0	0	0
Hexylcinnamic aldehyde (101-86-0)	W	44.0	6	−	1	0	0	0	0.5	0	0	0	0
		22.0	6	−	0	2	0	0	1.0	1	0	0	0
		11.0	6	−	0	0	0	0	0.0	1	0	0	0
		425.0	6	+	1	1	0	1	1.5	3	0	0	0
		213.0	6	+	1	0	0	0	0.5	3	0	0	0
		107.0	6	+	1	0	0	0	0.5	1	0	0	0
		44.0	24	−	0	0	0	0	0.0	0	0	0	0
		22.0	24	−	1	0	0	0	0.5	1	0	0	0
		11.0	24	−	1	0	0	0	0.5	0	0	0	0
1-Chloro-3-bromopropane (109-70-6)	W	1,600.0	6	−	4	9	0	0	6.5*	4	0	0	0
		800.0	6	−	2	0	0	0	1.0	0	0	0	0
		400.0	6	−	1	0	0	1	1.0	1	0	0	0
		185.0	6	+	4	9	0	0	6.5*	2	0	0	1
		92.5	6	+	2	0	0	0	1.0	2	0	1	0
		46.3	6	+	1	1	0	0	1.0	0	0	0	0
n-Hexylamine (111-26-2)	T	144.9	6	−	1	1	1	0	1.5	2	0	1	0
		72.5	6	−	3	0	1	0	2.0	2	1	3	0

Table 2. Continued.

Test chemicals (CAS No.)	Manuf-actured by	Conce-ntration (μg/mL)	Treat-ment (hr)	without (−) or with (+) S9 Mix	Aberration Frequency (%)				Total aberration (%)	Extra aberration			
					Chromatid		Chromosome						
					Br	Ex	Br	Ex		ctg	csg	poly	endo
n-Hexylamine (111-26-2)	T	36.3	6	−	3	0	0	0	1.5	1	1	2	0
		640.9	6	+	3	3	0	0	3.0	4	1	9	0
		320.5	6	+	2	0	0	0	1.0	4	0	2	0
		160.3	6	+	2	0	1	0	1.5	3	3	2	0
		144.9	24	−	3	0	0	0	1.5	2	0	3	0
		72.5	24	−	3	1	0	0	2.0	4	0	4	0
		36.3	24	−	4	0	1	0	2.5	7	1	3	0
n-Octyl alcohol (111-87-5)	W	84.1	6	−	1	0	0	0	0.5	2	0	8	0
		42.1	6	−	2	0	1	0	1.5	4	0	2	0
		21.0	6	−	1	1	0	0	1.0	2	1	3	0
		163.1	6	+	1	0	1	0	1.0	3	1	1	0
		81.6	6	+	7	0	0	0	3.5	5	1	4	0
		40.8	6	+	2	1	0	0	1.5	4	2	3	0
		84.1	24	−	3	0	0	0	1.5	2	0	8	0
		42.1	24	−	2	0	0	0	1.0	2	0	2	0
		21.0	24	−	2	0	0	0	1.0	3	1	3	0
Dioctyl phthalate (117-84-0)	W	5,000.0	6	−	2	1	0	0	1.5	1	1	3	0
		2,500.0	6	−	4	0	1	0	2.5	2	0	1	0
		1,250.0	6	−	1	1	0	0	1.0	2	0	1	0
		5,000.0	6	+	1	0	0	0	0.5	4	1	1	0
		2,500.0	6	+	3	1	0	0	2.0	1	1	2	0
		1,250.0	6	+	3	0	1	0	2.0	7	0	3	0
		5,000.0	24	−	1	0	0	0	0.5	6	2	0	0
		2,500.0	24	−	2	0	1	0	1.5	3	3	5	0
		1,250.0	24	−	1	1	0	0	1.0	5	0	1	0

*significant at $p < 0.05$, [a] : solvent, [b] : positive control

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

Br: Breakage, Ex: Exchange, ctg: chromatid gap, csg: chromosome gap, poly: polyploid, endo: endoreduplicate, DMSO: Dimethylsulfoxide, CMC: Carboxylmethyl cellulose, CP: Cyclophosphamide, MMC: Mitomycin C, W: Waco Pure Chemical Industries, Ltd. Osaka, Japan, S: Sigma-Aldrich Korea, Seoul, Korea, T: Tokyo Chemical Industry Co.

activation system in the case of 6 hr treatment and 24 hr treatment in the absence of metabolic activation system in 200 CHL cells on metaphase, respectively. However, the positive controls, cyclophosphamide (10 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 (45.3-32.9%) in CHL cells as shown in Table 2.

Triphenyl phosphite, the most cytotoxic compound among 11 chemicals tested, was observed no clastogenicity in the concentration range of 95.0-4.9 μg/mL both in the presence and absence of metabolic activation system for 6 hr and 24 hr. And most of tested chemicals was not induced a significant chromosomal aberration (Table 2). However, 1-chloro-3-bromopropane (CAS No. 109-70-6) induced chromosomal aberrations (6.5% and 6.5%) with significance at the concentration of 185.0 μg/mL with S9 and 1,600.0 μg/mL without S9 for 6 hr, respectively. This data is similar with data obtained in Japan chemical industry ecology-toxicology and information center, that this chemical induced chromosomal structural changes in CHL cells with S9 and without S9 for 6 hr and 24 hr[44].

From the results of chromosomal aberration assay with 11 synthetic chemicals in CHL cells, we suggested that 1-chloro-3-bromopropane (CAS No. 109-70-6) was revealed a positive clastogenicity in mammalian cell.

Methods

Materials

The test chemicals were kindly donated and purchased from several companies as indicated in Table 2. The test compounds were dissolved in dimethylsulfoxide (DMSO) before use. Eagles minimum essential medium (EMEM), 0.25% trypsin-EDTA, trypan blue, colcemid and fetal bovine serum (FBS) were the products of GIBCO® (California, USA). All other chemicals used were of analytical grade or the highest grade available. The preparation of rat liver S9 fraction for metabolic activation system was previously reported[2,3]. The S9 fraction prepared was stored immediately at −80°C before use.

Cell Lines and 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 EMEM supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. These cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

Cytotoxicity (Cell Growth Inhibition)

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 S9 activation system. For the growth inhibition assay, CHL cells were seeded at the density of 5×10^4 cells/mL into 96 well plates. 24 hr after seeding, several different doses of sample were separately added and incubated for 6 hr in the presence of S9 activation system and 24 hr in the absence of S9 system. And then the 50% inhibition concentration (IC_{50}) values were calculated by MTT assay[45].

In vitro Chromosomal Aberrations Assay in CHL Cells

The clastogenicity of 11 synthetic chemicals were evaluated for their ability to induce chromosomal aberrations in CHL cells. The experiment was performed as described by OECD[46] and Ishidate and Odashima[4] with some minor modifications[13-16,18,19,26], which are briefly summarized as follows. Concentration selection for this assay was based on the determination of cytotoxicity. 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 and in the presence of S9 mixture, cultures were treated for 6 hr with chemicals and then maintained for 18 h in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths or for 24 hr. Cyclophosphamide (CP) and mitomycin C (MMC) were used as a positive control in combination with or without S9 mixture, respectively. After 22 hr incubation, the treatment was followed by addition of medium containing colcemid at a concentration of 0.2 μg/mL. Then, 2 hr further incubated in the presence of colcemid, metaphase cells were harvested by trypsinization and centrifugation. The cells were swollen by adding 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-dried in the air. 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 metaphase cells at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS[47]. Breaks less than the width of a chromatid were designated as gaps in our criteria, and it was 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[48] 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.

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