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Evaluation of the Genetic Toxicity of Synthetic Chemical (XVII) –*In vitro* Mouse Lymphoma Assay and *In vitro* Supravital Micronucleus Assay with 1, 2-Dichlorobenzene

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Abstract

Chlorobenzenes due to their acute toxicity and the capability of bioaccumulating are of great health and environmental concern. Especially, 1, 2-dichlorobenzene (CAS No. 95-50-1) is used for organic synthesis, dye manufacture, as a solvent and for other applications in chemical industry. Adverse effects of 1, 2-dichlorobenzene includes increases in liver and kidney weights and hepatotoxicity. In this study, we evaluated the genetic toxicity of 1, 2-dichlorobenzene with more advanced methods, *in vitro* mouse lymphoma assay $tk^{+/-}$ gene assay (MLA) and *in vitro* mouse supravital micronucleus (MN) assay. 1, 2-Dichlorobenzene appeared the significantly positive results and the induction of large mutant colonies only in the presence of metabolic activation system with MLA. But *in vitro* testing of 1, 2-dichlorobenzene yielded negative results with supravital MN assay. These results suggest that 1, 2-dichlorobenzene may play a mutagen rather than clastogen *in vitro* mammalian system.

Keywords: 1, 2-Dichlorobenzene, *In vitro* Mouse Lymphoma Assay, *In vitro* supravital micronucleus assay, Genotoxicity

Chlorinated hydrocarbons are important contaminants for surface and groundwaters as well as marine pollution[1-3]. Among them, chlorobenzenes due to their acute toxicity and the capability of bioaccumulating are of great health and environmental concern[3,4], many of these species being listed by US EPA as priority pollutants. Dichlorobenzene exists as three isomers [(1, 2-(*o*-); 1, 3-(*m*-); and 1, 4-(*p*-)] (Figure 1). 1, 2-dichlorobenzene (1, 2-DCB) is used for organic synthesis, dye manufacture, as a solvent and for other applications in chemical industry[5]. Occupational exposure to 1, 2-dichlorobenzene could occur during its use as an industrial cleaner in the automotive, trucking and aircraft industries. 1, 2-dichlorobenzene emissions from about 78 sites in Europe producing chlorine were estimated as 32 t yr^{-1} to air and 2.7 t yr^{-1} to water in 1997[6]. Indirect routes of entry to the environment include sewage sludge dumping at sea or disposal in soil, metabolic breakdown of lindene and the degradation of more highly chlorinated benzenes, but these are difficult to quantify.

1, 2-Dichlorobenzene is the most acutely hepatotoxic of the three isomers of dichlorobenzene (ortho, meta, and para). Although 1, 2(ortho)-dichlorobenzene exists in the environment, exposure to humans predominantly occurs at the work place[7]. The available toxicological data indicate that metabolic profiles and effects from 1, 2-dichlorobenzene exposure are similar in rats, mice and humans[8]. Animal studies with rats and mice have shown 1, 2-dichlorobenzene to induce acute hepatotoxic effects. Several oral studies of rats and mice ranging from 10 days to 2 years duration indicate that the adverse effects include increases in liver and kidney weights and hepatotoxicity.

The controversy on genotoxicity of 1, 2-dichlorobenzene has been reported in bacterial system, *in vitro* and *in vitro* mammalian systems. In several microbial organisms and mammalian systems, 1, 2-dichlorobenzene tested negative *in vitro*. However, it did induce sister chromatid exchanges in Chinese Hamster ovary cells[9] and increased mutation frequency in mouse lymphoma cells, both in the presence of metabolic activation[10]. In this study, we evaluated the genetic toxicity of 1, 2-dichlorobenzene with more advanced methods, *in vitro* mouse lymphoma assay $tk^{+/-}$ gene assay (MLA) and *in vitro* mouse supravital micronucleus (MN) assay.

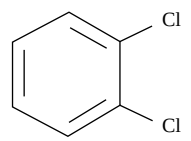
1, 2-Dichlorobenzene was Increased Mutant Frequencies in MLA Assay

The genotoxic potentials of 1, 2-dichlorobenzene (Figure 2) were assessed with various concentrations in the absence and presence of S-9 activation, respectively using MLA. Table 1 summarizes the results of the MLA after treatment of L5178Y cells with 1, 2-

dichlorobenzene at different concentrations for 3 h. DMSO was used as the negative control. The adequacy of the experimental conditions for detection of induced mutation was confirmed by employing positive control chemicals, methylmethanesulfonate (MMS) and cyclophosphamide (CP) for assays in the absence and presence of S-9, respectively. Background mutation frequencies (MF) (189.01×10^{-6} and 148.72×10^{-6}) were within the historical control range, and positive controls gave large dose-dependent increases in MF, meeting assay acceptance criteria. Treatment of cells with 1, 2-dichlorobenzene for 3 h led to a clearly increased MF at concentrations 79 μg/mL in the presence of S-9 (Table 1 and Figure 2). 1, 2-Dichlorobenzene at 79 μg/mL caused a 2.4-fold increase in the spontaneous MF. A concentration-related mutagenic effect was measured in the presence of S-9 system. However, in the absence of S-9, it did not appear significant increase of MFs at all concentrations.

1, 2-Dichlorobenzene did not Induced Micronucleus Formation in Peripheral Blood from Mice Exposed Intraperitoneally

1, 2-Dichlorobenzene also subjected to acridine orange micronucleus (MN) assay with mouse peripheral reticulocytes. The 50% lethal dose (LD_{50}) on intraperitoneal injection in male ICR mice was reported as 1,228.0 mg/kg. To determine the optimal sampling time, a single half dose of LD_{50}, 614.0 mg/kg of 1, 2-dichlorobenzene was injected into mice, and blood samples collected at 12 h intervals from 36 to 60 h. Maximum frequency of micronucleated reticulocytes (MNRETs) was observed at 48 h after treatment (Table 2). The frequency of MNRETs induced 48 h after i. p. injection at a single dose of 153.5, 307.0 and 614.0 mg/kg of 1, 2-dichlorobenzene was not dose-depen-



1, 2-Dichlorobenzene

Figure 1. Chemical structure of 1, 2-dichlorobezene.

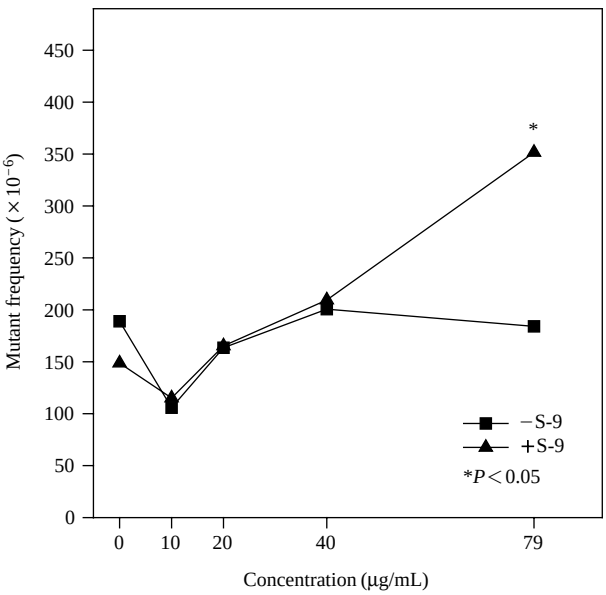


Figure 2. Mutation frequencies by 1, 2-dichlorobezene in L5178Y cells in the absence and presence of S-9 metabolic activation system (3 h treatment). Total *tk* mutation frequency is displayed. Results are taken from one representative experiment. *statistically significant ($P < 0.05$).

Table 1. Toxicity and mutagenicity of 1, 2-dichlorobenzene in L5178Y $tk^{+/-}$ mouse lymphoma cells.

3 hr									
	−S-9					+S-9			
Treatment (μg/mL)	%RS	RTG	Mutation Frequency ($\times 10^{-6}$)		Treatment (μg/mL)	%RS	RTG	Mutation Frequency ($\times 10^{-6}$)	
0	100.00	1.00	189.01		0	100.00	1.00	148.72	
9.9	95.83	1.46	105.83	NS	9.9	73.03	1.04	115.09	NS
19.8	91.84	1.11	163.56	NS	19.8	90.56	0.61	165.23	NS
39.5	150.22	1.04	200.68	NS	39.5	124.07	0.63	209.49	NS
79.0	145.37	0.68	184.09	NS	79.0	135.72	0.44	351.59	*
Linear trend			NS		Linear trend			**	
MMS					CP				
10	102.90	0.77	608.24	*	3	108.91	0.81	540.18	*

NS: Not significant
*, **: Significant at 5% and 1% level, respectively
MMS: methylmethanesulfonate, CP: cyclophosphamide

Table 2. Frequencies of MNRETs in peripheral blood of ICR mice after a single intraperitoneal administration of 1, 2-dichlorobenzene.

Chemical	Route of administration	Dose (mg/kg)	No. of mouse	Sampling time (h)	%MNRET[a] (Mean ± S.D.)	*P* value
Solvent[b] control	i.p.	–	6	48	0.25 ± 0.04	
MMC	i.p.	1	6	48	3.30 ± 1.31	< 0.05
1, 2-Dichlorobenznene	i.p.	614.0	6	36	0.23 ± 0.05	> 0.05
		614.0	6	48	0.26 ± 0.09	> 0.05
		614.0	6	60	0.23 ± 0.08	> 0.05
		307.0	6	48	0.25 ± 0.08	> 0.05
		153.5	6	48	0.28 ± 0.09	> 0.05

[a]2000 Reticulocytes were counted in each mouse
[b]Solvent control group was injected i.p. with corn oil
MNRET: micronucleated reticulocyte
MMC: mitomycin C

dently increased (Table 2).

Discussion

We investigated whether 1, 2-dichlorobenzene induce the base-pair as well as frameshift mutations or small deletions in L5178Y cells using MLA and it induce the clastogenic potential *in vitro* using supravital micronucleus (MN) assay.

MLA using the thymidine kinase (*tk*) locus has been widely used to detect the ability of chemicals to induce genetic damage in cultured mammalian cells. A large body of information demonstrates the capability of the MLA *tk* assay to detect a broad spectrum of mutational events[11]. Therefore, the International Committee for Harmonization (ICH), in selecting a recommended test battery, discussed the MLA and the *in vitro* mammalian cytogenetic assays as possible alternatives. The rodent MN assay has been widely applied as an *in vitro* assay for detecting genotoxic agents. The assay became a standard test system for genotoxicity evaluations in regulatory agencies in several countries[12-15].

In MLA assay, 1, 2-dichlorobenzene induced the mutation frequency only in the presence of S-9 mix, which related to the increases in large mutant colonies but not in small colonies. Generally, large colony mutants reflected the induction of point mutation. Genotoxicity testing with several microbial species produced negative results with the exception of one recombination assay with *Bacillus subtilis*[16]. 1, 2-Dichlorobenzene was previously found to be non-genotoxic in a bacterial reverse mutation assay (Ames test) and an *in vitro* chromosomal aberration assay and HGPRT assay using Chinese hamster ovary cells[17]. In other study, two sister chromatid exchange assays performed with CHO cells[9,18] were positive in the presence of, and negative in the absence of, metabolic activation. A DNA synthesis inhibition assay performed with human lymphocytes was positive without metabolic activation and negative with metabolic activation[19]. Following accidental exposure to 1, 2-dichlorobenzene vapour, estimated by the study authors to be no greater than 100 ppm (602 mg/m^3), the mean value of chromosomal aberrations in peripheral blood leukocytes from exposed individuals was 8.92% compared to 2.02% for a control group[20]. Due to the relatively low number of cells examined, little confidence can be attributed to findings of this study.

And *in vitro* testing of 1, 2-dichlorobenzene yielded negative results with supravital MN assay. There are some *in vitro* data similar with this data. In the Drosophila sex-linked recessive mutation (BUA, 1990) and eye mosaic assays[21], 1, 2-dichlorobenzene did not appear positive results. Chromosomal aberration assays with rat bone marrow[17,22] and DNA damage studies in rats were negative[23]. Also, positive micronucleus assay in mouse bone marrow[24] was not confirmed in a more recent, well-conducted study[25].

Consequently, genotoxic potential of 1, 2-dichlorobenzene was observed not *in vitro* micronucleus assay using peripheral reticulocytes but MLA assay only in the presence of metabolic activation system. These results suggest that 1, 2-dichlorobenzene may play a mutagen rather than clastogen *in vitro* mammalian system.

Methods

Materials

1, 2-Dichlorobenzene (1, 2-DCB; CAS No. 95-50-1) was purchased from Junsei Chem. (Japan). Stock

solutions of used chemicals were prepared freshly in medium before use. RPMI-1640, pluronic solution, antibiotics and horse serum 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 S-9 fraction for metabolic activation system was previously reported[26]. The S-9 fraction prepared was stored immediately at −80°C before use.

Cell Line and Culture

The mouse lymphoma L5178Y cell line ($tk^{+/-}$ 3.7.2c) was cultivated in 90% RPMI-1640 with 1 mM sodium pyruvate, 0.1% pluronic supplemented with 10% heat-inactivated horse serum and antibiotics. These cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

L5178Y *thymidine kinase* ($tk^{+/-}$)-3.7.2C Mouse Lymphoma Assay (MLA)

To prepare working stocks for gene mutation experiments, cultures were purged of $tk^{+/-}$ mutants by exposure for 1 day to THMG medium (culture medium containing 3 μg/mL thymidine, 5 μg/mL hypoxanthine, 0.1 μg/mL methotrexate and 7.5 μg/mL glycine) and then the cells were transferred to THG medium (THMG but without methotrexate) for 2 days. The purged cultures were checked for low background $tk^{+/-}$ mutants and stored in liquid nitrogen. Each experiment started with working stock. The cells were usually used on day 3 or 4 after thawing and during logarithmic growth. A single lot of post-mitochondrial supernatant fractions of rat liver homogenates (S-9) for exogenous metabolic activation had been made from the liver of phenobarbital- and 5, 6-benzoflavone-pretreated Sprague Dawley rats. S-9 mixture was prepared just prior to use by combining 4 mL S-9 with 2 mL each 180 mg/mL glucose-6-phosphate, 25 mg/mL NADP and 150 mM KCl. The concentration of S-9 mixture was 5% during treatment and the final concentration of S-9 was 2%. For treatment, cells were centrifuged and suspended at a concentration of 0.5×10^6 cells in 10 mL of medium in 15 mL polystyrene tubes. 1, 2-Dichlorobenzene was tested with and without S-9 mixture. 1, 2-Dichlorobenzene at each concentration was added and these tubes were gassed with 5% CO_2 in air and sealed. The cell culture tubes were placed on a roller drum and incubated at 37°C for 3 h. At the end of the treatment period, the cell cultures were centrifuged and washed twice with fresh medium and resuspended in fresh medium. We conducted preliminary experiments to determine the solubility and cytotoxicity of 1, 2-dichlorobenzene. Cytotoxicity was determined by relative survival (RS) and relative total growth (RTG) following 3 h treatments at concentrations up to 5 mg/mL, usually regardless of solubility. The recommended highest concentration was one with a 10-20% RS and/or RTG. Mutant selection was performed using the modified microwell version of the assay as described by Clements *et al.*[27]. Simply, the treated cells in medium containing 3 μg TFT/mL for selection or without TFT for cloning efficiency were distributed at 200 μL/well into 96-well flat-bottom microtiter plates. For mutant selection, two plates were seeded with ~2,000 cells/well. For cloning efficiency, two plates were seeded with ~1 cell/well. All plates were incubated in 5% CO_2 in air in a humidified incubator at 37°C. After 11-13 days incubation, clones were counted and the colony size distribution was determined. Mutant frequencies were calculated using a statistical package (MutantTM: UKEMS, York, UK) in accordance with the UKEMS guidelines[28].

In vitro Supravital Micronucleus Assay in Mice

Outbred mice of strain ICR, 7-8 weeks old, were used in this study. The mice were allowed an adaptation period of about 1 week, then randomized and subjected to the study. The six animals were housed for each group. The test article was applied orally in three doses in volumes of 10 mL/kg. The micronucleus test with mouse peripheral blood reticulocytes using acridine orange-supravital staining method was performed essentially as described by Hayashi *et al.*[29]. To determine the clastogenicity, 1, 2-dichlorobenzene dissolved in corn oil was administrated intraperitoneally (i.p.) at either 153.5, 307.0, or 614.0 mg/kg. A single dose of MMC at 1 mg/kg was injected i.p. as a positive control, and corn oil was administrated i.p. with 0.1 mL/kg as a solvent control. Peripheral bloods were collected from mouse tail vein at 12 h intervals from 36 h to 60 h after administration. The 10 μL of 1 mg/mL acridine orange (AO) dissolved in distilled water was placed on a glass slide pre-heated at about 70°C, spread out, and dried at room temperature. This glass slides were stored in a dark and dry location at room temperature until used. Peripheral blood was taken by piercing a tail blood vessel. Five μL of blood was obtained directly without anticoagulant from a tail, and placed on an AO-coated glass slide. Glass slide was covered with coverslip, and allowed to be supravitally stained. To score and data analysis, two thousand reticulocytes (RETs) of type I, II, and III per animal were observed[30] and RETs with micronucleated reticulocytes (MNRETs) were recorded under the fluorescent microscopy which had the combination of a blue excitation and a yellow to

orange barrier filter. The data was analyzed by pairwise test for statistical differences between the control and specific treatment groups.

References

1. Martí, I., Lloret, R., Martín-Alonso, J. & Ventura, F. Determination of chlorinated toluenes in raw and treated water samples from the Llobregat river by closed loop stripping analysis and gas chromatography-mass spectrometry detection. *J. Chromatogr.* **1077**(1):68-73 (2005).
2. Huybrechts, T., Dewulf, J. & Van Langenhove, H. Priority volatile organic compounds in surface waters of the southern North Sea. *Environ. Pollut.* **133**(2): 255-264 (2005).
3. Amaral, O. C., Otero, R. & Grimalt, J. O. Volatile and semi-volatile organochlorine compounds in tap and riverine waters in the area of influence of a chlorinated organic solvent factory. *Water Res.* **30**(8): 1876-1884 (1996).
4. Heidrich, S. *et al*. Regionally contaminated aquifers-toxicological relevance and remediation options (Bitterfield case study). *Toxicology* **205**(3):143-155 (2004).
5. US Department of Commerce. US exports, schedule E commodity by country (FT410/December 1980), Bureau of Census, Washington DC, pp. 2-74 (1981).
6. ECETOC. 'Risk assessment in marine environments'. *Technical Report No*. 82, Brussels, pp. 141, ISSN-0773-8072-82 (2001).
7. Kumagai, S. & Matsunaga, I. Identification of urinary metabolites of human subjects exposed to *o*-dichlorobenzene. *Int. Arch. Occup. Environ. Health* **67**:207-209 (1995).
8. OECD. SIDS Initial Assessment Report For SIAM 13 1, 2-DICHLOROBENZENE Bern, Switzerland, 6-9 November. *UNEP PUBLICATIONS* (2001).
9. Loveday, K. S., Anderson, B. E., Resnick, M. A. & Zeiger, E. Chromosome aberration and sister chromatid exchange tests in Chinese hamster ovary cells *in vitro*. V: Results with 46 chemicals. *Environ. Mol. Mutagen.* **16**:272-303 (1990).
10. Myhr, B. C. & Caspary, W. J. Chemical mutagenesis at the thymidine kinase locus in L5178Y mouse lymphoma cells: results for 31 coded compounds in the National Toxicology Program. *Environ. Mol. Mutagen.* **18**:51-83 (1991).
11. Applegate, M. L. *et al*. Molecular dissection of mutations at the heterozygous thymidine kinase locus in mouse lymphoma cells. *Proc. Natl. Acad. Sci. USA* **87**:51-55 (1990).
12. Sofuni, T. Japanese guidelines for mutagenicity testing. *Environ. Mol. Mutagen*. **21**:2-7 (1993).
13. Kirkland, D. J. Genetic toxicology testing requirements: official and unofficial views from Europe. *Environ. Mol. Mutagen*. **2**:8-14 (1993).
14. Auletta, A. E., Dearfield, K. L. & Cimino, M. C. Mutagenicity test schemes and guidelines: US EPA office of pollution prevention and office of pesticide programs. *Environ. Mol. Mutagen*. **21**:38-45 (1993).
15. Health Protection Branch Genotoxicity Committee. The assessment of mutagenicity: health protection branch mutagenicity guidelines. *Environ. Mol. Mutagen.* **21**:15-37 (1993).
16. Matsui, S., Yamamoto, R. & Yamada, H. The Bacillus subtilis/microsome ret-assay for the detection of DNA damaging substances which may occur in chlorinated and ozonated waters. *Wat. Sci. Tech.* **21**:875-887 (1989).
17. BUA Report No. 53, VCH, September (1990).
18. Tennant, R. W. *et al*. Prediction of chemical carcinogenicity in rodents from *in vitro* genetic toxicity assays. *Science* **236**:933-941 (1987).
19. Perocco, P., Bolognesi, S. & Alberghini, W. Toxic activity of seventeen industrial solvents and halogenated compounds on human lymphocytes cultured *in vitro*. *Toxicol. Lett.* **16**:69-75 (1983).
20. Zapata-Gayon, C., Zapata-Gayon, N. & Gonzalez-Angulo, A. Clastogenic chromosomal aberrations in 26 individuals accidentally exposed to ortho dichlorobenzene vapors in the National Medical Center in Mexico City. *Arch. Environ. Health* **37**:231-235 (1982).
21. Vogel, E. W. & Nivard, M. J. Performance of 181 chemicals in a Drosophila assay predominantly monitoring interchromosomal mitotic recombination. *Mutagenesis* **8**:57-81 (1993).
22. Reustle, J. A. & Scribner, H. E. *o*-Dichlorobenzene; Myelotoxicity and cytogenetic study in rats. Report of the Toxicology Department, Rohm and Haas Company, Spring House, Pennsylvania. *EPA/OTS Doc.* No. 878212182, 1-71 (1979).
23. Kitchin, K. T., Brown, J. L. & Kulkarni, A. P. Predictive assay for rodent carcinogenicity using *in vitro* biochemical parameters: operational characteristics and complementarity. *Mutation Res.* **266**:253-272 (1992).
24. Mohtashamipur, E., Triebel, R., Straeter, H. & Norpoth, K. The bone marrow clastogenicity of eight halogenated benzenes in male NMRI mice. *Mutagenesis* **2**:111-113 (1987).
25. Shelby, M. D., Erexson, G. L., Hook, G. J. & Tice, R. R. Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals. *Environ. Mol. Mutagen*. **21**:160-179 (1993)
26. Ames, B. N., Durston, W. E., Yamasaki, E. & Lee, F. D. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl. Acad. Sci. USA* **70**: 2281-2285 (1973).
27. Clements, J. Gene mutation assays in mammalian cells, In S. O'Hare and C. K. Atterwill (Ed.), *Methods in Molecular Biology*, Vol. 43: *in vitro* Toxicity Testing Protocols. Humana Press Inc. Totowa, NJ. 277-286 (1990).

28. Robinson, W. D. *et al*. Statistical evaluation of bacterial/mammalian fluctuation tests, in *Statistical Evaluation of Mutagenicity Test Data* (Kirkland, D. J., ed.). Cambridge University Press, Cambridge, UK, 102-140 (1990).
29. Hayashi, M. *et al*. The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. *Mutation Res*. **245**:245-249 (1990).
30. Hayashi, M. *et al*. Isolation of micronuclei from mouse blood and fluorescence in situ hybridization with a mouse centromeric DNA probe. *Mutation Res.* **307**:245-251 (1994).