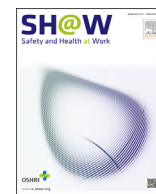


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## Review Article

# A Review on Mutagenicity Testing for Hazard Classification of Chemicals at Work: Focusing on *in vivo* Micronucleus Test for Allyl Chloride



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## ABSTRACT

Chemical mutagenicity is a major hazard that is important to workers' health. Despite the use of large amounts of allyl chloride, the available mutagenicity data for this chemical remains controversial. To clarify the mutagenicity of allyl chloride and because a micronucleus (MN) test had not yet been conducted, we screened for MN induction by using male ICR mice bone marrow cells. The test results indicated that this chemical is not mutagenic under the test conditions. In this paper, the regulatory test battery and several assay combinations used to determine the genotoxic potential of chemicals in the workplace have been described. Further application of these assays may prove useful in future development strategies of hazard evaluations of industrial chemicals. This study also should help to improve the testing of this chemical by commonly used mutagenicity testing methods and investigations on the underlying mechanisms and could be applicable for workers' health.

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## 1. Introduction

Chemicals may have various hazardous effects on human health or the environment, and chemical hazard evaluations are important for workers' health and work environments. Depending on toxicity, classified substances and their mixtures may require restricted exposure in workplaces. There is an increased need for chemical hazard assessments because the number of workers that are exposed to chemicals has risen with the development of many industries, and it is necessary to determine what these substances are and how they are regulated [1].

One chemical, allyl chloride (CAS number 107-05-1), is used in many industries, which has led to concerns about possible threats to the health of workers. Only insufficient or controversial information is available concerning the potential related hazards of allyl chloride; therefore, an *in vivo* micronucleus (MN) assay was conducted to gain additional information concerning any such hazards. Furthermore, toxicological information [e.g. the Safety Data Sheet

(SDS)] from this study could be applied for workers' rights in several industries.

Allyl chloride is used in the synthesis of allyl compounds [2]; as an intermediate for the manufacture of polymers, resins, plastics [3]; for varnishes and adhesives; and in the synthesis of pharmaceuticals and insecticides [4]. In the United States, this chemical is listed as a high production volume (HPV) chemical (65FR81686), which means that >1 million pounds was produced or imported into the United States in 1990 and/or in 1994 [5]. In workplaces where allyl chloride is produced or used, occupational exposure to allyl chloride may occur through inhalation and dermal contact [6].

Allyl chloride has already been tested for mutagenicity by the following short-term tests: *Salmonella* reversion test with strains TA1535 and TA100 (with and without activation), a forward and back mutation system in *Streptomyces coelicolor*, and two forward mutation systems in *Aspergillus nidulans*. Spot and plate incorporation assay techniques are also employed. Allyl chloride was active in *Salmonella typhimurium* and *Salmonella coelicolor* and negative in

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*A. nidulans* [7]. In our previous study [8], allyl chloride did not induce chromosomal aberrations in Chinese hamster lung (CHL/IU) cells; we therefore proceeded to perform an *in vivo* MN assay. This was necessary to improve the evaluation of the carcinogenic potential of this compound.

The purpose of the MN test is to screen for cytogenetic damage that results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. Micronuclei were first used to quantify chromosomal damage and are now recognized as one of the most successful and reliable assays for genotoxic carcinogens [9].

In the *in vivo* MN test, mammalian bone marrow cells are treated with allyl chloride. Many toxicological studies other than the MN test have been conducted, although the available genotoxic data on allyl chloride remain controversial with and without mammalian metabolic activation (S9). Therefore, to secure quality assurance, further study was necessary that was based on good laboratory practice (GLP) guidelines.

Table 1 shows physicochemical and toxicological information regarding allyl chloride. Using the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) classification, a "mutagen" is an agent that increases the occurrence of mutations in populations of cells and/or organisms. Substances and mixtures in this hazard class are assigned to one of two hazard categories. Category 1 has two subcategories (Table 2). Many studies have focused on the mutagenicity of allyl chloride (Table 3; however, with the exception of our previous study [8], no study has used GLP tests. Allyl chloride is nevertheless has category 2 germ cell mutagen notified classification and labeling, according to Classification, Labelling, and Packaging (CLP) criteria [10] on the evidence of these non-GLP dataset. The CLP regulation ensures that the hazards presented by chemicals are clearly communicated to workers and consumers in the European Union (EU) through the classification and labeling of chemicals. A public notice of the Ministry of Employment and Labor (MoEL) of Korea (Sejong, Korea) [11] has also classified it as a Category 2 germ cell mutagen following European Union Classification, Labelling, and Packaging classification (EU-CLP).

Therefore, the MN assay accorded by GLP guidelines [12] was necessary to determine its mutagenicity exactly and propose it to a regulatory body such as the MoEL of Korea.

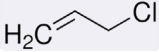
## 2. Materials and methods

### 2.1. Chemicals, animals, and experimental design

The test compound used in the *in vivo* MN test was allyl chloride (98.5%; Sigma-Aldrich, St. Louis, MO, USA; lot number MKBP7862V; cat. number 236306). Olive oil (Sigma-Aldrich; lot number BCBM3643V; cat. number O1514) was used as a solvent in accordance with the results of the solubility test. The positive control was mitomycin C (MMC; Sigma-Aldrich; lot number SLBF9516V, Cat. No. M4287).

The mouse (*Mus musculus*) bone marrow MN test was performed in accordance with the Organisation for Economic Co-operation and Development (OECD) Test TG 474 guidelines [12], and in accordance with Hayashi [13] and Heddle et al [14]. Groups of specific pathogen-free male ICR mice were treated with the test substance at three dosage levels. The highest dosage level was the estimated maximum tolerated dose or the standard limit dose for the MN test, whichever was lower. Concurrent negative group (i.e., olive oil) and positive control group (MMC, 0.5 mg/kg) were also treated. This study used 7-week old male ICR mice that were administered allyl chloride at 100 mg/kg, 200 mg/kg, and 400 mg/kg doses. At 24 hours post-treatment, six male animals that had been administered allyl chloride orally were used for each group.

**Table 1**  
Physicochemical and toxicological information of allyl chloride\*

Chemical name	Allyl chloride
CAS No.	107-05-1
Synonyms	1-Chloro-2-propene 1-Propene, 3-chloro- 2-Propenyl chloride 3-Chloropropene Chlorallylene p-Aminopropiophenon 1-Chloropropylene
Molecular formula	C <sub>3</sub> H <sub>5</sub> Cl 
Molecular weight	76.5
Melting point	-135°C
Forms	Colorless liquid
Partition coefficient	2.1
Boiling point	45°C
Water solubility	0.337 g/100 mL at 25°C
Stability & reactivity	Chemical stability: stable under recommended storage conditions Conditions to avoid: heat, flames, & sparks; temperature extremes; & direct sunlight Materials to avoid: oxidizing agents, boron trifluoride, sulfuric acid, nitric acid, & strong oxidizing agents
Toxicity	Target organs: liver, respiratory system Human LCLo inhalation, 3000 ppm Mouse LC <sub>50</sub> inhalation, 11500 mg/m <sup>3</sup> /2H Mouse LD <sub>50</sub> intraperitoneal, 155 mg/kg Mouse LD <sub>50</sub> oral, 425 mg/kg Rabbit LCLo inhalation, 22500 mg/m <sup>3</sup> /2 h Rabbit LD <sub>50</sub> skin, 2066 mg/kg Rat LC <sub>50</sub> inhalation, 11 gm/m <sup>3</sup> /2H Rat LD <sub>50</sub> oral, 460 mg/kg Guinea pig LC <sub>50</sub> inhalation, 5800 mg/m <sup>3</sup> /2 h Allyl chloride is classified as Group 3 (i.e., not classifiable regarding its carcinogenicity to humans) in IARC, A3 in ACGIH, & Carc 2 in EU-CLP
GHS classification	Flammable liquids (Category 2) Acute toxicity, oral (Category 4) Acute toxicity, inhalation (Category 3) Skin irritation (Category 2) Eye irritation (Category 2) Carcinogenicity (Category 2) in MoEL of Korea & the IARC Group 3 Germ cell mutagenicity (Category 2) in the MoEL of Korea Specific target organ toxicity-single exposure (Category 3), respiratory system

ACGIH, American Conference of Governmental Industrial Hygienists; CAS, Chemical Abstract Service; EU-CLP, European Union Classification, Labelling, and Packaging; GHS, Globally Harmonized System of Classification and Labeling of Chemicals; IARC, International Agency for Research on Cancer; LC50, median lethal concentration; LCLo, lowest lethal concentration; MoEL, Ministry of Employment and Labor.

\* The information is mostly obtained from searching ChemIDplus Advanced, U.S. National Library of Medicine (Rockville Pike, Bethesda, MD; <http://chem.sis.nlm.nih.gov/chemidplus/rn/107-05-1>), and Material Safety Data Sheet in KOSHANET (Ulsan, Korea; <http://msds.kosha.or.kr/kcic/msdsdetail.do>). The searches were conducted using keywords, the chemical name, and/or the CAS number.

The Animal Ethics Committee of OSHRI, KOSHA approved the animal studies protocol (approval number IACUC-1403) to ensure appropriate care before the animals were obtained for research.

### 2.2. Bone marrow preparation and MN test

Twenty-four hours after the administration of allyl chloride the animals were euthanized and bone marrow cells were harvested from the mice femurs because of the cell cycle. Immature erythrocytes could be differentiated by using a variety of staining

**Table 2**  
Germ cell mutagenicity classification and standard assays in GHS classification

Category 1. Known/presumed
Known to produce heritable mutations in human germ cells
Subcategory 1A
Positive evidence from epidemiological studies
Subcategory 1B
Positive results in:
<input type="checkbox"/> <i>In vivo</i> heritable germ cell tests in mammals
<input type="checkbox"/> Human germ cell tests
<input type="checkbox"/> <i>In vivo</i> somatic mutagenicity tests, combined with some evidence of germ cell mutagenicity
Category 2. Suspected/possible
<input type="checkbox"/> May include heritable mutations in human germ cells
<input type="checkbox"/> Positive evidence from tests in mammals & somatic cell tests
<input type="checkbox"/> <i>In vivo</i> somatic genotoxicity supported by <i>in vitro</i> mutagenicity

GHS, Globally Harmonized System of Classification and Labeling of Chemicals.

Note. From: A Guide to The Globally Harmonized System of Classification and Labeling of Chemicals (GHS) [Internet]. Washington (DC):Occupational Safety and Health Administration (OSHA). 2003 [cited 2015 Jun 18]. Available from: <https://www.osha.gov/dsg/hazcom/ghs.html#3.2>. Copyright 2013, Occupational Safety and Health Administration (OSHA), Reprinted by permission.

techniques that rely on the relatively high content of the cells' residual DNA. Mature erythrocytes with a low nucleic acid content appear pink to orange when stained with 5% Giemsa, whereas immature erythrocytes stain blue. Based on the cell cycle and maturation times of the erythrocytes, the bone marrow was harvested after 24 hours.

The bone marrow was flushed from the femurs and spread onto slides. The slides were air-dried, fixed, and stained with a fluorescent DNA-specific stain that easily illuminates any micronuclei that may be present. To evaluate the cytotoxicity, the preliminary tests were performed as a limit test to determine the maximum dosage. The inhibition of proliferation in the bone marrow cells was not observed in this test. To indicate chemically induced toxicity, the percentage of polychromatic erythrocytes (PCEs) among the 500 erythrocytes in the bone marrow was recorded for each dosage group.

**Table 3**  
Summary of studies focusing on *in vitro* and *in vivo* mutagenicity and carcinogenicity of allyl chloride\*

Tests	Species	Protocol	Results	Refs
Genetic toxicity <i>in vitro</i> bacteria test (gene mutation)	<i>Salmonella typhimurium</i>	Plate inc. assay	2 tests negative, 1 positive in TA1535 with S9	Dean et al [40] McCoy et al [41]
		Spot test	2 tests positive (in TA1535 with S9) (in TA1535 with & without S9)	Neudecker & Henschler [42]
		Liquid susp. assay	Positive in TA100 without S9	Eder et al [43]
	<i>Escherichia coli</i>	Spot test	Positive with & without S9	
Nonbacterial <i>in vitro</i> test (gene mutation)	<i>Streptomyces coelicolor</i>	Plate inc. assay	Positive for both forward & reverse mutation	Bignami et al [7]
	<i>Aspergillus nidulans</i>	Spot test	Positive for both forward & reverse mutation	
	<i>Saccharomyces cerevisiae</i>	Plate inc. assay Spot test Liquid suspension assay	Negative Negative Positive both with & without S9	
Nonbacterial <i>in vitro</i> test (chromosomal aberration)	<i>A. nidulans</i>	Other	Increase in haploid segregants & diploid	Crebelli et al [44]
	Rat liver RL1	Other	nondisjunctional sectors negative	Dean et al [40]
DNA-modifying activity	Human HeLa S3	H <sub>3</sub> -thymidine incorp.	Positive UDS	Schiffmann et al [45]
	<i>E. coli</i>	Other	Positive in pol A <sub>1</sub>	McCoy et al [41]
Genetic toxicity <i>in vivo</i>	Rat/CD	Micronucleus test Dominant	Negative	McGregor [46]
	Rat/DC	lethal assay	Negative	
		Sperm abnormality		
	Mouse/B6C3F1	SLRL test	Negative	
	<i>Drosophila melanogaster</i>		Negative	

SLRL, sex-linked recessive lethal; UDS, unscheduled DNA synthesis.

\* Information is mostly obtained by searching in United Nations Environment Programme (UNEP). OECD SIDS report: chloropropene (CAS no.: 107-05-1). Nairobi (Kenya): UNEP Publications; 1996.

At least 2,000 polychromatic erythrocytes (PCEs; e.g., reticulocytes, immature erythrocytes) were scored per animal with regard to the frequency of micronucleated cells in each of the six animals per dosage group. The presence of micronucleated PCEs was visually scored (at least 2,000 cells per mouse) by optical microscopy using a fluorescence microscope (Opti phot-2; Nikon, Tokyo, Japan) with a BA-2 filter. Cells were considered micronucleated if they neatly contained defined chromatin corpuscles with a diameter less than one-third the diameter of the cell nucleus, and if they stained an equal or lighter shade than the nucleus of the cell from which the micronucleated cell was developed. To reduce observer bias, only one reader was involved in scoring cells.

### 2.3. Evaluation and interpretation of the results

According to OECD TG 474 Test (i.e., Mammalian Erythrocyte Micronucleus Test) [12], the evaluation and interpretation of results were based on data that were presented as the mean number of micronucleated cells per 2,000 cells for each treatment group. The experimental and control MN frequency for each specimen within and between different mice strains were compared with a one-way analysis of variance test using SigmaStat version 3.11 software (Systat Software, Inc., San Jose, CA, USA). There is no requirement for verification of a clear positive or clear negative response.

### 2.4. Journal and book review

With particular emphasis on three topics—chemical mutagenicity, mutagenic tests associated with industrial chemicals, and the prevention of occupational diseases—we would like to discuss the prospects for developing a strategy for applying novel mutagenicity assays that are applicable for workers' health issues such as occupational cancer.

Searches were performed on the following sites: PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>), Google Scholar (<http://scholar.google.com>), and ScienceDirect ([www.sciencedirect.com](http://www.sciencedirect.com)).

The search strategy used a combination of the following Medical Subject Headings (MeSH; National Center for Biotechnology Information, Bethesda, MD) terms and keywords: “allyl chloride” and “mutagenicity test” or “workers” or “occupations.” The search results were further narrowed by reviewing titles and abstracts by two reviewers (the authors). Inclusion criteria were epidemiology, *in vitro* and *in vivo* mutagenicity and carcinogenicity studies. Additional missing case reports were identified by reviewing the references of review articles and bibliographies found on [scholar.google.com](http://scholar.google.com). Disagreements in article and conference abstract identification were resolved by mutual discussion. Based on the literature review results, our search strategy identified 632 potential articles (Fig. 1). The reviewers agreed on 31 articles (i.e., references [13,14,16–33,36–46]) and three books (i.e. [15,34,35]) that met the inclusion criteria for detailed analysis.

### 3. Results and discussion

There were no specific symptoms among the animals that were orally exposed to allyl chloride. The body weight of the animals exposed to this chemical ranged 35.74–40.20 g (Table 4). There were no environmental factors that may have affected the quality or integrity of the study results, which includes any significant behavioral changes (i.e., neurophysiological activity).

Preliminary tests were performed to determine the maximum dosage. The proliferation of bone marrow cells was not inhibited in this test. The presence of micronucleated PCEs was visually scored by optical microscopy using a fluorescence microscope (Fig. 2).

The frequency of erythrocytes with MN inductions was  $0.19 \pm 0.05\%$  in the negative control group;  $0.27 \pm 0.18\%$ ,  $0.30 \pm 0.10\%$ , and  $0.38 \pm 0.10\%$  in the 100 mg/kg, 200 mg/kg, and 400 mg/kg allyl chloride-treated groups, respectively; and  $1.19 \pm 0.22\%$  in the positive control group. The ratio of PCEs within the total number of erythrocytes was  $63.03 \pm 3.21\%$  in the negative control group;  $46.10 \pm 5.60\%$ ,  $53.89 \pm 5.98\%$ , and  $46.74 \pm 4.23\%$  in the 100 mg/kg, 200 mg/kg, and 400 mg/kg allyl chloride-treated groups, respectively; and  $45.07 \pm 9.03\%$  in the positive control group. There were no statistically significant changes in comparison to the negative control group (Table 5).

In this study, we performed *in vivo* MN tests based on the results of a dose range-finding assay. The maximum dose was estimated at 400 mg/kg, based on regulatory guidelines. Bone marrow was extracted, and at least 2,000 PCEs per animal were analyzed for the

**Table 4**

Animal body weight in micronucleus tests after oral exposure to allyl chloride

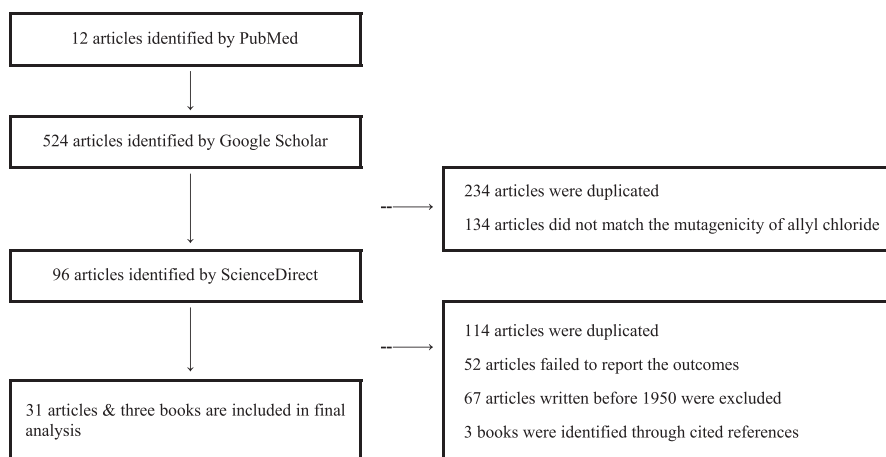
Exposure method	Concentration	No. of animals	Average body weight (g; mean $\pm$ SD)
Orally exposed to allyl chloride for 24 h	Negative control (olive oil)	6	37.97 $\pm$ 1.59
	100 mg/kg b.w.	6	37.98 $\pm$ 1.86
	200 mg/kg b.w.	6	38.08 $\pm$ 1.53
	400 mg/kg b.w.	6	37.89 $\pm$ 1.55
	Positive control (MMC, 0.5 mg/kg b.w.)	6	37.68 $\pm$ 1.86

b.w., body weight; MMC, mitomycin C.

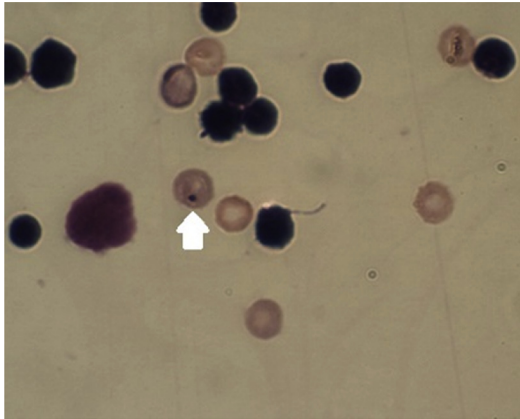
frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 erythrocytes for each animal. Allyl chloride did not induce signs of clinical toxicity in the animals treated at the highest dose level (based on regulatory guidelines). The chemical also did not induce statistically significant increases in micronucleated PCEs at any dose. It was also not cytotoxic to the bone marrow (i.e., it did not produce statistically significant decreases in the PCE:NCE ratio) at any dose.

When a bone marrow erythroblast develops into a PCE, the main nucleus is extruded. Any MN that has been formed may remain behind in an otherwise anucleated cytoplasm. Visualization of the micronuclei is facilitated in these cells because they lack a main nucleus. An increase in the frequency of MNPCEs in treated animals is an indication of induced chromosomal damage. Statistical significance should not be the only determining factor for a positive response: positive results in a MN test indicate that a substance induces micronuclei because of chromosomal damage or damage to the mitotic apparatus in the erythroblasts of the test species. We evaluated and interpreted these results according to OECD guidelines [12]. The experimental and control MN frequency for each specimen within and between different mice strains were compared. There was no requirement for verification of a clear positive or clear negative response.

Table 6 shows the *in vitro* and *in vivo* genetic toxicity assays that optimize the standard battery for genetic toxicology recommended by the International Conference on Harmonisation (ICH; Geneva, Switzerland) [15]. Allyl chloride was tested for mutagenicity in a



**Fig. 1.** Flow diagram of article identification.



**Fig. 2.** The presence of micronucleated polychromatic erythrocytes. Micronucleated mouse bone marrow cells with Giemsa staining under optical microscopy. The arrow shows a true micronucleus (magnification, 1,000 $\times$ ).

battery of *in vitro* and *in vivo* assays. In the older *in vitro* assays negative results were obtained, possibly because of the vaporization of allyl chloride. Adequate mutagenicity assays with *S. typhimurium* were positive with and without metabolic activation. The mutagenicity greatly decreased in the presence of an exogenous activating system. In a spot test with *Escherichia coli* and in tests with *Streptomyces coelicolor* positive results were obtained with and without metabolic activation. Tests with *A. nidulans* were negative for allyl chloride. The substance induces gene conversions in *Saccharomyces cerevisiae* and somatic segregation in *A. nidulans*. No significant compound-related chromosome damage was observed in RL1 cells. Allyl chloride induces unscheduled DNA synthesis in human HeLa S3 cells, but not in human embryonic intestinal cells. No increase in chromosomal aberrations was observed in a cytogenetic test with rats exposed to allyl chloride by inhalation. The substance was negative in a dominant lethal assay with rats and in a sperm abnormality test with mice. Allyl chloride did not increase sex-linked recessive lethal mutations in *Drosophila melanogaster*. Based on all available mutagenicity data, it can be concluded that allyl chloride is mutagenic to bacteria and yeast and it induces unscheduled DNA synthesis in human HeLa cells but not in embryonic testicular cells. Allyl chloride did not cause chromosome aberrations *in vitro* in mammalian cells. Negative results were obtained in the available *in vivo* tests (Table 3).

A stepwise tiered approach is applied in regulatory mutagenicity testing [16]. In the first step, *in vitro* assays with a high sensitivity are used to identify test compounds that have high intrinsic genotoxic activity. In the second step, specific *in vivo* tests are performed to determine the relevance of the *in vitro* results for the *in vivo* situation. These *in vivo* mutagenicity studies are also included because some genotoxicants can only be detected *in vivo* after metabolic activation [17]. Compared to regulatory

**Table 6**

*In vitro* and *in vivo* genetic toxicity assays that optimize the standard battery for genetic toxicology recommended by the International Conference on Harmonisation

Test name *	No./Chapter No.		
	OECD	EPA-OCSP	FDA Redbook 2000
<i>In vitro</i> Bacterial reverse mutation test (Ames test)	471	870.5100	IV.C.1.a.
<i>In vitro</i> mammalian chromosome aberration assay	473	870.5375	IV.C.1.b.
<i>In vitro</i> mammalian cell micronucleus test	487	None	None
<i>In vitro</i> mammalian cell gene mutation test	476	870.5300	IV.C.1.c. (only MLA)
<i>In vivo</i> Mammalian micronucleus test	474	870.5395	IV.C.1.d. (only erythrocyte)
Mammalian bone marrow chromosome aberration test	475	870.5385	None
Unscheduled DNA synthesis (UDS) test with mammalian liver cells <i>in vivo</i>	486	None	None
Transgenic mouse mutation assay	488	None	None
<i>In vivo</i> comet assay	None	None	None
Alkaline elution assay	None	None	None
<i>In vivo</i> DNA covalent binding assay	None	None	None

EPA, Environmental Protection Agency; FDA, Food and Drug Administration; MLA, methyllycaconitine; OCSP, the Office of Chemical Safety and Pollution Prevention; OECD, Organisation for Economic Co-operation and Development.

Note. From R.D. Harbison, M.M. Bourgeois, and G.T. Johnson, *Hamilton and Hardy's Industrial Toxicology*, 6th ed, p. 1183. Copyright 2015, Hoboken (NJ): John Wiley & Sons, Inc. Adapted with permission.

\* The recommended International Conference on Harmonisation standard test battery.

carcinogenicity testing, mutagenicity testing is relatively cheap and fast. Compounds without genotoxic liability can proceed first into clinical trials in humans. The carcinogenic potential is assessed later in the full developmental phase of drug development; however, the regulatory test strategy consists of a battery of core and ancillary tests for identifying the three forms of mutagenicity (i.e., gene mutations, clastogenicity, and aneugenicity), which cannot be detected in one single test.

Genotoxicity tests are used to detect genetic damage by various mechanisms in *in vitro* and *in vivo* systems. Several regulatory guidelines have been developed to provide various assays that are conducted for testing the genotoxicity. To date, most regulatory agencies and international authorities recommend a test scheme consisting of *in vitro* and *in vivo* methods to detect genotoxicity/mutagenicity induced by substances. The ICH recommends a standard battery test for pharmaceuticals to detect their

**Table 5**

Results of the main micronucleus test with allyl chloride (for 24 hours)

Groups	PCE observed	MNPCE observed	MNPCE frequency (%)	PCE + NCE counted	PCE counted	PCE/(PCE + NCE) (%)
Negative control	2,033.83 $\pm$ 19.05	3.83 $\pm$ 0.98	0.19 $\pm$ 0.05	516.83 $\pm$ 13.11	325.83 $\pm$ 20.07	63.03 $\pm$ 3.21
100 mg/kg b.w.	2,045.50 $\pm$ 63.98	5.50 $\pm$ 3.56	0.27 $\pm$ 0.18	520.00 $\pm$ 15.49	240.33 $\pm$ 36.13	46.10 $\pm$ 5.60
200 mg/kg b.w.	2,048.33 $\pm$ 40.90	6.17 $\pm$ 1.94	0.30 $\pm$ 0.10	510.17 $\pm$ 5.81	275.17 $\pm$ 33.11	53.89 $\pm$ 5.98
400 mg/kg b.w.	2,023.33 $\pm$ 13.02	7.67 $\pm$ 2.07	0.38 $\pm$ 0.10	512.83 $\pm$ 14.72	239.50 $\pm$ 19.69	46.74 $\pm$ 4.23
Positive control (MMC, 0.5 mg/kg b.w.)	2,027.00 $\pm$ 15.84	24.17 $\pm$ 4.45	1.19 $\pm$ 0.22	517.50 $\pm$ 28.48	234.83 $\pm$ 59.44	45.07 $\pm$ 9.03

Dats are presented as mean  $\pm$  the standard deviation.

b.w., body weight; MMC, mitomycin C; MNPCE, micronucleated polychromatic erythrocyte; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte.

genotoxicity. The ICH guidance optimizes the standard battery test for genetic toxicology and provides guidelines on the interpretation of results (Table 6) [15]. These guidelines help improve risk characterization for carcinogenic effects. In the following sections, some regulatory agencies or organizations are briefly described with regard to their own guidelines.

The Ames assay has a relatively high specificity, compared to other *in vitro* mutagenicity tests (Table 7). The sensitivity, specificity, and predictivity of the Ames assay calculated by Kirkland et al [18] were 58.8%, 73.9%, and 62.5%, respectively.

The chromosome aberration test is performed *in vitro* in cultured mammalian cells. It is also performed in the presence and in the absence of the S9 mixture [19,20]. Scoring the test requires specialized training and experience. The sensitivity and predictivity of carcinogenicity for this test are 65.6% and 59.8%, respectively. The specificity of this test is low (44.9%).

The sensitivity and predictivity of the mouse lymphoma thymidine kinase assay is 73.1% and 62.9%, respectively. As for the chromosome aberration assay, the specificity of this assay is low (39%).

The fourth regulatory mutagenicity assay is the MN test. The sensitivity, specificity, and predictivity of the *in vitro* MN assay are 78.7%, 30.8%, and 67.8%, respectively. The specificity of the *in vivo* MN assay in bone marrow is much higher (75%). The sensitivity of the *in vivo* test is lower (40%) and the predictivity is 48% [21,22].

In this paper, a regulatory test battery to determine the genotoxic potential of industrial chemicals has been described. The validation data for these higher throughput assays show that bacterial mutagenicity (i.e., gene mutations) and mammalian mutagenicity (i.e., chromosome damage) can be predicted early. To develop a strategy for applying the novel mutagenicity assays in the lead optimization phase, several combinations of assays must be evaluated. Further application of these assays may prove useful in future development strategies of chemicals.

The toxicological relevance of the MN test is well defined: it is a multitarget genotoxic endpoint; it assesses clastogenic and aneugenic events; and it assesses some epigenetic effects, which is simple to score, accurate, and applicable in different cell types. In addition, it is predictive for cancer, amenable for automation, and allows good extrapolation for potential limits of exposure or thresholds. It is easily measured in experimental *in vitro* and *in vivo* systems. Implementation of *in vitro* micronucleus (IVMN) assays in the battery of tests for hazard and risk assessment of potential mutagens/carcinogens is therefore fully justified. The final draft of the OECD guideline is available for this test [20].

The presence of MN in cultured human cells was reported as early as the 1960s [21] and 1970s [22]. The *in vitro* micronucleus test (IVMNT) has evolved into a robust quantitative assay of chromosome damage by the development of the cytokinesis-block technique that eliminated the confounding effects on MN

expression by the cytostatic effects caused by poor culture conditions, treatment effects, cell senescence, and variability in mitogen response in the lymphocyte test system [23].

In the cytokinesis-block micronucleus (CBMN) assay, the scoring of MN discriminates between the accumulation of once-divided cells that appear binucleated and mononucleated cells that did not divide during the *in vitro* culturing period [24]. In recent years, the IVMNT has become an attractive tool for mutagenicity testing because of its capacity to detect clastogenic and aneugenic events and some epigenetic effects and because of the simplicity of scoring; its accuracy, wide applicability in different cell types; and its amenability to automation. More recently, the final draft of the OECD guideline 478 has become available [24]. The initial recommendations for this guideline came from two workshops [by the International Workshops on Genotoxicity Testing (IWGT)], which proposed an internationally harmonized protocol designed for human primary lymphocytes and for cell lines [25,26]. The European Centre for the Validation of Alternative Methods (ECVAM) validated the methodology by retrospectively examining the existing published data on the IVMNT [27–31] using the modular approach for validation [32]. The ECVAM confirmed that the IVMNT is reliable, reproducible, transferable, and predictive [33], and ECVAM Scientific Advisory Committee endorsed the IVMNT [34,35]. The final step before acceptance by the OECD consists of an interlaboratory exercise to evaluate different measures of cytotoxicity/cytostasis that can be applied when the IVMNT is performed in the absence of cytochalasin B [34]. The use of the IVMNT within a battery of tests will be defined by various regulatory bodies responsible for developing such test strategies. The advantages of the IVMNT are well defined and discussed by Bonassi et al [36], Decordier et al [37], and Elhajouji et al [38]: it is a multitarget genotoxic endpoint and predictive for cancer [39], it is amenable for automation [38], and it allows good extrapolation for potential limits of exposure or thresholds [38]. In addition, the MN can be scored and easily measured in a variety of *in vitro* and *in vivo* systems [39]. Implementation of IVMN assays in the battery of tests for hazard and risk assessment of potential mutagens/carcinogens is therefore fully justified.

Many findings have been used to determine the mutagenicity of allyl chloride; however, no GLP tests have been performed, except for our previous study [8]. It is nevertheless classified as a Category 2 germ cell notified mutagen and labeled according to CLP criteria on the evidence of these non-GLP dataset. A Public Notice of ministry of employment and labor (MoEL), Korea was also classified it as a category 2 germ cell mutagen in accordance with the classification of the European Union Classification, Labelling, and Packaging (EU-CLP). Therefore, the MN assay accorded by GLP guidelines [12] was needed to determine exactly its mutagenicity and propose it to a regulatory body such as the MoEL of Korea. Based on these results and discussion, allyl chloride did not induce micronuclei, as reflected by the MN test of the bone marrow cells of mice. Allyl chloride should be categorized as “not classified” as to its mutagenicity, according to GHS.

Nevertheless, it is anticipated that allyl chloride will trigger health problems of workers as occupational diseases. To predict risks for workers' health, especially occupational cancers, and to improve the assessment of hazardous effects, we recommend additional studies that focus on lung exposure and the long-term effects of these low-level contaminants with inhalation. Allyl chloride is practically regulated by Occupational Safety and Health Act (OSHA) in the MoEL of Korea with its carcinogenicity (Category 2; Table 1).

Despite the use of large amounts of allyl chloride, the available data for the mutagenicity of this chemical remains controversial. We believe this is the first study to involve an *in vivo* MN assay with

**Table 7**

The sensitivity, specificity, and predictivity of the assays of the standard regulatory test battery for the assessment of carcinogenicity [18,19]

Assay	Sensitivity (%)	Specificity (%)	Predictivity (%)
Ames test	58.8	73.9	62.5
Chromosome aberration test	65.6	44.9	59.8
Mouse lymphoma TK test	73.1	39.0	62.9
Micronucleus test <i>in vitro</i>	78.7	30.8	67.8
Micronucleus test <i>in vivo</i>	40.0	75.0	48.0

TK, thymidine kinase.

Note. From P. Steinberg (editor), *High-throughput Screening Methods in Toxicity Testing*, p. 213–69, Copyright 2013, Hoboken (NJ): John Wiley & Sons, Inc., Adapted with permission.

a mouse bone marrow cells. In conclusion, the test substance allyl chloride did not show any evidence of inducing MN under the conditions of this study.

In this paper, a regulatory test battery has been described with a medium or high throughput to determine the genotoxic potential of chemicals at work. The validation data for these higher throughput assays show that an early prediction is possible for bacterial mutagenicity (i.e., gene mutations) and mammalian mutagenicity (i.e., chromosomal damage). Several combinations of assays must be evaluated to develop a strategy to apply the novel mutagenicity assays in the lead optimization phase. Further application of these assays may prove useful in future development strategies of hazard evaluations of industrial chemicals.

Many findings have been used to determine the mutagenicity of allyl chloride; however, no GLP tests have been performed, except in our previous study [8]. Allyl chloride is nevertheless classified as a Category 2 germ cell notified mutagen and labeled according to EU-CLP criteria on the evidence of these non-GLP dataset. The public notice of the MoEL of Korea also classified it as a category 2 germ cell mutagen in accordance with the classification of the EU-CLP. However, based on these results and discussion, the mutagenicity of allyl chloride should be “not classified” according to GHS.

This study was performed with GLP guidelines to determine exactly its mutagenicity and propose it to a regulatory body such as the MoEL of Korea. This study also should help to improve the testing of this chemical by commonly used mutagenicity testing methods and investigations on the underlying mechanisms and could be applicable for workers' health issues, which include occupational cancers.

### Conflicts of interest

The authors have no potential conflicts of interest to report relevant to this article.

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