



***In vivo* micronucleus test of 4-butylaniline and *N*-butylaniline to classify a chemical's mutagenicity according to the globally harmonized system of classification and labelling of chemicals (GHS)**

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Abstract *In vivo* micronucleus tests were performed to investigate the mutagenic potential of 4-butylaniline and *N*-butylaniline, which are used in dye intermediates and organic intermediates respectively. Groups of 5 male ICR mice were treated with vehicle or 4-butylaniline for 2 consecutive days by oral gavage at concentrations of 0 (control), 64, 160, 400, and 1000 mg/kg. Statistically significant and dose-dependent increases were found for micronuclei frequencies in male mice ($p < 0.05$). These results suggest that 4-butylaniline can induce genetic effects in the micronuclei of male mouse bone marrow cells. Based on the positive results obtained in cytogenetic analyses of somatic cells *in vivo*, Globally Harmonized System of Classification and Labelling of Chemicals Category 2 was assigned. *N*-butylaniline was administered for 2 consecutive days by oral gavage to male ICR mice at dose of 0 (control), 64, 160, 400, and 800 mg/kg. *N*-butylaniline tested negative for micronuclei induction in mice, although *N*-butylaniline was associated with micronucleus induction at the highest dose. Based on the negative results obtained for cytogenetic analyses of somatic cells *in vivo*, “Not Classified” was assigned.

Keywords 4-butylaniline · Genotoxicity · Micronucleus assay · *N*-butylaniline

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Introduction

More than 15 million chemicals are commercially available worldwide. Approximately 60,000 to 70,000 chemicals are used regularly in everyday life, and 200 to 1,000 chemicals are produced every year in volumes exceeding 1 ton. The number and usage of chemical substances has rapidly increased with industrial advances in modern societies, such that 43,000 or more chemical substances are now circulating in Korea. Although more than 20,000 chemicals are circulating around the world, the hazards associated with these chemicals have not all been identified [1]. Since 2005, we have performed toxicology testing to assess the human health hazards of industrial chemicals. Our mission is to provide information on dangerous substances, prevent health hazards caused by exposure, assess potential hazards of chemicals, and improve the health of workers [2]. 4-Butylaniline is used in intermediates of dyes and pigments. *N*-is used in organic intermediates and is also used to make dyes. These substances are listed in both the TSCA (Toxic Substances Control Act) chemical substance inventory of the US EPA (United States Environmental Protection Agency) and the Annex III inventory of ECHA (European Chemicals Agency). Computer-aided (*in silico*) approaches were performed using QSAR Toolbox version 4.2, which is available on the OECD website (<http://www.qsartoolbox.org/>) [3,4]. 4-Butylaniline tested positive for prediction of gene mutation and carcinogenicity in read-across analyses. *N*-butylaniline tested negative for prediction of gene mutation and positive for prediction of carcinogenicity in read-across analyses. In the Derek Nexus (version 6.0.1) program, both 4-butylaniline and *N*-butylaniline tested negative for prediction. A literature review provided no data other than acute toxicity data and no genotoxic data. Therefore, in 2017, our group performed bacterial mutagenicity tests (“*Ames* test”) on two substances and determined that 4-butylaniline and *N*-butylaniline were DNA-reactive genotoxic (positive) chemicals with no safe thresholds [5]. The Globally

Harmonized System of Classification and Labelling of Chemicals (GHS) was developed to synchronize chemical regulations and standards in the United Nations. In addition to providing material safety data sheets and other information that should be included on the labelling of hazardous chemicals, the GHS includes criteria for physical hazards, health hazards, and environmental hazards [6]. GHS classification criteria for germ cell mutagens have been catalogued from around the world and have become the standard for classifying the hazards associated with chemical substances [7]. In the battery of genotoxicity tests available, rodent micronucleus assays are primarily used and recommended by regulatory agencies worldwide to assess chemical mutagenicity. When performed appropriately, this assay screens both clastogen and aneugen [8]. In *in vivo* micronucleus assays, cytogenetic assays detect not only DNA damage but also genetic toxicity by other mechanisms, such as topoisomerase inhibition, spindle poison, or excessive cytotoxicity. The objective of the present study is to demonstrate genotoxicity of 4-butylaniline and *N*-butylaniline *in vivo* using micronucleus tests.

Materials and Methods

Chemicals and reagents

4-Butylaniline (CAS No. 104-13-2) was purchased from Sigma-Aldrich (94300, St Louis, MO, USA, Lot No. MKBS5221V). Mitomycin C was used as the positive control chemical and was purchased from Sigma-Aldrich (Lot No. SLBV1185). Olive oil was used as the vehicle or negative control chemical in the oral gavage given to the mice and was purchased from Sigma-Aldrich (Lot No. BCBW5235). *N*-butylaniline (CAS No. 1126-78-9) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan, Lot No. WAQLC) (Table 1).

Animals

Seven-week-old male ICR (Institute for Cancer Research in

Philadelphia) mice were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). After acclimation for one week, eight-week-old mice were used in the study. Food (a commercial diet Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C, ENVIGO RMS Inc., Indianapolis, IN, USA) and water were given to mice *ad libitum*. The mice were housed in polysulfone cages (five individuals per cage) under controlled environmental conditions (temperature, 22±3 °C; humidity, 50±20%; lights on from 08:00 to 20:00 h). Mice body weights (b.w.) were monitored and recorded before treatment (day 1) and on days 2 and 3. Male ICR mice were exposed to 4-butylaniline (0, 64, 160, 400, and 1000 mg/kg b.w.) and *N*-butylaniline (0, 64, 160, 400, and 800 mg/kg b.w.) by oral gavage, respectively. Five control mice were gavaged with vehicle control (olive oil) in the same manner. Five male ICR mice were exposed to 0.5 mg/kg b.w. mitomycin C by *i.p.* injection for the positive control. The mice exposed to 4-butylaniline and *N*-butylaniline were studied the first and second days after substances exposure (n=5 at each time point). After acute single exposure to 4-butylaniline and *N*-butylaniline, bone marrow cells were collected for micronucleus assay on days 1 and 2. The animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee/Animal Ethic Committee (IACUC/AEC) of the Occupational Safety and Health Research Institute (Approval No: IACUC1821 and IACUC1822).

Micronucleus assay

Micronucleus assays were performed as described in our previous report [9] and according to OECD Guideline 474 [10].

Twenty-four hours after the last administration for each time point, mice were euthanized by cervical dislocation, and bone-marrow cells from the right femurs were washed out with 1 mL of fetal bovine serum (FBS, Cyclone, GE Healthcare, Chicago, IL, USA). After centrifuging for 5 min at 220×g, cell pellets were resuspended with the remaining serum and spread on clean glass slides. The slides were then air-dried overnight, fixed with methanol for 5 min, and stained with acridine orange (40 µg/mL w/v in Sorensen buffer, pH 6.8). The number of micronucleated cells was recorded in each of 4,000 polychromatic erythrocytes (PCE) per animal using a fluorescence microscope (Leica, DMLB, Wetzlar, Germany). Both individual group comparisons and dose-response trends were evaluated to confirm the positive results of the micronucleus tests. A statistically significant increase in the micronucleated polychromatic erythrocyte (MNPCE) frequency at any individual concentration (compared to that of the control) or a dose-dependent increase was considered a positive result.

Statistical analysis

Micronucleus assay data were expressed as means ± SD. Statistical analyses were performed using SigmaPlot for Windows Version 11.0 (SyStat Software, Inc., San Jose, CA, USA). Comparisons between two groups were assessed using Student's *t*-tests, and

Table 1 Chemical Properties of 4-butylaniline and *N*-butylaniline

Items	4-Butylaniline Data	<i>N</i> -butylaniline Data
CAS registry number	104-13-2	1126-78-9
Molecular weight	149.23	149.23
Structural formula	C ₁₀ H ₁₅ N	C ₁₀ H ₁₅ N
Appearance	Orange-colored liquid	Yellow or orange-colored liquid
Boiling point	133-134 °C	240 °C
Water solubility	Soluble	Insoluble
Vapor density	N. A.	N. A.
Vapor pressure	N. A.	N. A.
Partition coefficient (Log <i>K</i> _{ow})	N. A.	N. A.
Occupational exposure levels (8-h TWA)	2 ppm	N. A.

values of $p < 0.05$ were considered statistically significant.

Results

4-Butylaniline, Cat. 2

Four mice demonstrated decreased locomotor activities after 4-butylalanine treatments with 400 mg/kg b.w. on day 1. Decreased locomotor activity was observed in five mice after 4-butylalanine treatments with 400 mg/kg b.w. on day 2. Decreased locomotor activity was observed in four mice after 4-butylalanine treatments with 400 mg/kg b.w. on day 3. Decreased locomotor activity was observed in five mice after 4-butylalanine treatments with 1,000 mg/kg b.w. on day 1. Decreased locomotor activity was observed in three mice after 4-butylalanine treatments with 1,000 mg/kg b.w. on day 2. In one case on day 1 and in three cases on day 2, 1,000 mg/kg b.w. of 4-butylalanine was observed to cause debilitating behavior. Decreases in body weight were evident on day 3 after dosing at 400 mg/kg b.w. Decreased body weights were also observed on day 2 after dosing at 1,000 mg/kg b.w. (Table 2). Three of the five mice treated with 1,000 mg/kg b.w. died on day 2 after dosing. Two of the two mice treated with 1,000 mg/kg b.w. died on day 3 after dosing. Statistically significant increases in micronuclei frequencies were observed when 400 mg/kg b.w. ($p < 0.05$; Table 3) was administered, indicating positive rodent micronucleus test results. No changes were observed in the polychromatic/mature erythrocytes ratios obtained for the animals treated with 4-butylaniline and negative controls. Positive results

Table 2 Body weight of animals

Groups	Body weight (g) at the time of		
	Administration		Termination
	1st	2nd	
Negative control	35.34±1.62	35.83±1.30	36.11±2.04
4-Butylaniline 64 mg/kg	37.18±1.83	37.49±2.06	38.01±2.10
4-Butylaniline 160 mg/kg	36.49±1.56	36.74±1.10	36.94±0.97
4-Butylaniline 400 mg/kg	36.87±1.53	36.48±2.06	34.49±2.05
4-Butylaniline 1000 mg/kg	37.45±2.20	35.39±2.74	N. A.
Mitomycin C 0.5 mg/kg	37.70±2.48	38.34±2.63	38.61±2.36

Table 3 MNPCE frequencies among total erythrocytes induced by acute single exposure to 4-butylaniline in male mice

Treatment	Mice	Immature Erythrocyte Frequency (%)	Micronuclei Frequency (%)
0 (NC)	5	52.04±6.92	0.16±0.06
64 mg/kg	5	49.52±10.56	0.21±0.05
160 mg/kg	5	51.52±8.92	0.38±0.12
400 mg/kg	5	55.60±20.08	1.35±0.64*
PC	5	52.76±7.81	1.16±0.19*

Data are presented as mean frequencies ± SD (n =5/group)
* $p < 0.05$ compared to 0 (NC)

Table 4 Body weight of animals

Groups	Body weight (g) at the time of		
	Administration		Termination
	1st	2nd	
Negative control	36.89±1.01	37.61±1.33	38.26±1.13
<i>N</i> -butylaniline 64 mg/kg	36.80±1.22	37.20±1.17	37.17±1.82
<i>N</i> -butylaniline 160 mg/kg	36.78±0.68	37.51±0.50	37.83±0.60
<i>N</i> -butylaniline 400 mg/kg	37.56±1.23	37.48±1.66	35.41±2.87
<i>N</i> -butylaniline 800 mg/kg	36.79±1.22	35.87±1.40	34.02±N. A.
Mitomycin C 0.5 mg/kg	37.30±1.06	37.93±0.96	37.77±1.68

Table 5 MNPCE frequencies among total erythrocytes induced by acute single exposure to *N*-butylaniline in male mice

Treatment	Mice	Immature Erythrocyte Frequency (%)	Micronuclei Frequency (%)
0 (NC)	5	61.68±4.22	0.21±0.05
64 mg/kg	5	59.00±6.90	0.21±0.01
160 mg/kg	5	53.80±6.80	0.23±0.06
400 mg/kg	4	49.28±2.91*	0.19±0.04
800 mg/kg	1	51.80±0.00	0.45±0.00
PC	5	50.08±6.86	1.12±0.34

Data are presented as mean frequencies ± SD (n =5/group)
* $p < 0.05$ compared to 0 (NC)

were obtained from bacterial mutagenicity tests in a previous study. Based on the positive results obtained for cytogenetic analyses in somatic cells *in vivo*, GHS Category 2 was assigned.

N-butylaniline, Not Classified

Decreased locomotor activity was observed in all subjects after treatment with 400 and 800 mg/kg b.w. on day 1. Decreased locomotor activity was observed in all (5/5) subjects after treatment with 400 mg/kg b.w. on day 2. Decreased locomotor activity was observed in three mice after treatment with 800 mg/kg b.w. on day 2. Decreased locomotor activity was observed in one mouse after treatment with 400 and 800 mg/kg b.w. on day 3, respectively. In the group that received 800 mg/kg b.w., debilitation was determined in three cases on day 2 and one case on day 3. One of the five mice treated with 400 mg/kg b.w. died on day 3 after dosing. Decreases in body weight were evident on day 3 after dosing at 400 mg/kg b.w. Decreases in body weight were evident on day 2 and day 3 after dosing at 800 mg/kg b.w. (Table 4). Two of the five mice treated with 800 mg/kg b.w. died on day 2 after dosing, and two of the three mice treated with 800 mg/kg b.w. died on day 3 after dosing. Rodent micronucleus test results were negative. Significant changes were observed in the polychromatic/mature erythrocytes ratios among the animals treated with 400 mg/kg b.w. *N*-butylaniline (Table 5). Positive bacterial mutagenicity test results were obtained in a previous study. Based on the negative results obtained for cytogenetic analyses in somatic cells *in vivo*, “Not Classified (NC)” was assigned.

Discussion

Evaluation of micronucleus induction *in vivo* is the essential part of genotoxicity tests and is recommended by the regulatory agencies to be conducted as part of chemical risk assessment [11]. Under the Occupational Safety and Health Act, a person who intends to manufacture or import a new chemical should submit a report investigating the harmfulness or danger of the new chemical in Korea [12]. In addition, according to the Act on Registration, Evaluation, etc. of Chemicals, those who manufacture and import chemicals at least ten tons per year subject to registration should prepare and submit data for hazard review and risk assessment in Korea [13]. Micronucleus assays data using test animals correspond to mutagenicity data for human health in these reports. The micronucleus assay detects both clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to mitotic spindle dysfunction) and is designed primarily for evaluating the ability of test agents to induce structural and/or numerical chromosomal damage. Both kinds of damage are associated with the appearance and/or progression of tumors, and with adverse reproductive and developmental effects [14]. The aromatic amine, 4-butyl-aniline, is a proven and potent agent that short circuits the chromophore visual cycle in the intact mammalian retina/retinal pigment epithelium (RPE) by preventing new synthesis of 11-cis-retinal either from Vitamin A or from all-trans-retinal that results from rhodopsin bleaching [15]. In liver microsomal preparations, the *para*-hydroxylation of *N*-butylaniline was shown to be stimulated by acetone [16]. The objective of this study was to investigate whether 4-butylaniline and *N*-butylaniline caused induction of micronuclei in the *in vivo* micronucleus test. The UN GHS classifies 4-butylaniline as Category 3 for acute toxicity and Category 2 for eye and skin irritation. The UN GHS classifies *N*-butylaniline as Category 4 for acute toxicity and Category 2 for eye and skin irritation. The LD50 value reported for mouse intraperitoneal acute toxicity for 4-butylaniline is 81 mg/kg [17]. In the present study, the oral LD50 was greater than 800 mg/kg in mice. Five male mice in the 1,000 mg/kg group died. In the ICR mice administered 400 mg/kg 4-butylaniline (oral) in the present study, increases in micronucleated polychromatic erythrocytes (PCEs) were observed relative to concurrent control values. The PCE/PCE+NCE ratios in control and treated groups were compared and no significant changes were observed. The LD50 value reported for rat oral acute toxicity for *N*-butylaniline is 1,620 mg/kg [18]. In the present study, the oral LD50 was greater than 800 mg/kg in mice, four of the five male mice in the 800 mg/kg group died, and no increments of incidence of micronucleated polychromatic erythrocytes (MNPCEs) were observed relative to the negative control group. When comparing control and treated groups, statistically significant decreases were found in ratios of PCE to total erythrocytes. A statistically significant reduction in the ratio of PCEs compared with the negative control indicates bone marrow toxicity (i.e., inhibition of

cell proliferation). In the present study, mitomycin C used as a positive control, and it showed a significant increases of MNPCE ratios. This indicates that the experimental design and the results of the present study are acceptable [19]. The sensitivity, specificity and concordances of the Ames test are known to be 58.8, 73.9 or 62.5%, respectively [20]. The sensitivity, specificity or concordances of the *in vivo* micronucleus assay are known to be 41.0, 60.5 or 45.4% [21], respectively. A relatively good balance in performance has been observed for bacterial mutation assays + *in vivo* micronucleus assays compared to bacterial mutation assays + *in vitro* chromosomal aberration tests (74.3%/37.5%) [22]. The *in vivo* assays seem to be efficient for detecting rodent carcinogens. Additional *in vivo* germ cell mutagenicity assays and epidemiological studies in humans are required to further our understanding of the effects of these substances on the health of industrial workers [23]. Before conducting genotoxicity studies, referencing the results of toxicity prediction programs, which are available for free or at a fee, along with toxicity databases and literature searches, can help determine the results of genotoxicity studies. In the present study, the results of the toxicity prediction program and the micronucleus test results did not match. These results are consistent with reports that compounds with aniline moieties, such as 4-butylaniline, are skin sensitizing chemicals and are difficult to predict toxicities [24]. Classifications were based on decision logic for germ cell mutagenicity. Therefore, it is important to recognize that previous classifications can be changed based on new test data and that classifications based on outdated or limited information are unreliable. Evaluations of test results should be verified using many sources [25]. Although the decision tree for GHS classification of germ cell mutagens is a useful tool, a total-weight-of-evidence approach is needed to provide accurate conclusions. In Korea, the GHS system has been applied to the Occupational Safety and Health Act for classification and labelling of chemicals since 2008. The weight of evidence and reliable data should be assured to ensure proper classification. In conclusion, the results obtained in the current study indicate that 4-butylaniline induces micronuclei and demonstrate that *N*-butylaniline cytotoxicity via the *in vivo* micronucleus test.

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Conflicts of interest Authors declare no conflicts of interest with the contents of this article.

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