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The Genotoxicity Study of Molinate, an Herbicide, in Bacterial Reversion, *in vitro* and *in vivo* Mammalian System

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Abstract

The controversy on genotoxicity of molinate, an herbicide, has been reported in bacterial system, and *in vitro* and *in vivo* mammalian systems. To clarify the genotoxicity of molinate, we performed bacterial gene mutation test, *in vitro* chromosome aberration and mouse lymphoma $tk^{+/-}$ gene assay, and *in vivo* micronucleus assay using bone marrow cells and peripheral reticulocytes of mice. In bacterial gene mutation assay, no mutagenicity of molinate (12-185 μg/plate) was observed in *Salmonella typhimurium* TA 98, 100, 1535 and 1537 both in the absence and in the presence of S-9 metabolic activation system. The clastogenicity of molinate was observed in the presence (102.1-408.2 μg/mL) of metabolic activation system in mammalian cell system using Chinese hamster lung fibroblast. However, no clastogenicity was observed in the absence (13.6-54.3 μg/mL) of metabolic activation system. It is suggested that the genotoxicity of molinate was derived some metabolites by metabolic activation. Molinate was also subjected to mouse lymphoma L5178Y $tk^{+/-}$ cells using microtiter cloning technique. In the absence of S-9 mixture, mutation frequencies (MFs) were revealed 1.4-1.9 × 10^{-4} with no statistical significance. However, MFs in the presence of metabolic activation system revealed 3.2-3.4 × 10^{-4} with statistical significance ($p<0.05$). *In vivo* micronucleus (MN) assay using mouse bone marrow cells, molinate revealed genotoxic potential in the dose ranges of 100-398 mg/kg of molinate when administered orally. Molinate also subjected to acridine orange MN assay with mouse peripheral reticulocytes. The frequency of micronucleated reticulocytes (MNRETs) induced 48 hr after i.p. injection at a single dose of 91, 182 and 363 mg/kg of molinate was dose-dependently increased as10.2±4.7, 14.6±3.9 and 28.6±6.3 (mean±SD of MNRETs/2,000 reticulocytes) with statistical significance ($p<0.05$), respectively. Consequently, genotoxic potential of molinate was observed in *in vitro* mammalian mutagenicity systems only in the presence of metabolic activation system and *in vivo* MN assay using both bone marrow cells and peripheral reticulocytes in the dose ranges used in this experiment. These results suggest that metabolic activation plays a critical role to express the genotoxicity of molinate in *in vitro* and *in vivo* mammalian system.

Keywords: Molinate, Genotoxicity, Bacterial reversion, Clastogenicity, Chromosome Aberration, Mouse lymphoma $tk^{+/-}$ gene, Mouse bone marrow, Peripheral reticulocytes, Micronucleus

Molinate (S-ethyl hexahydro-1H-azepine-1-carbothioate, CAS No. 2212-67-1) is a thiocarbamate herbicide that was introduced in 1954. It is used to control broad-leafed and grassy weeds in rice and other crops. For example, in the Murrumbidgee Irrigation Area (MIA), which is just one agricultural region of NSW, Australia, 81,384 kg of the active ingredient (a.i.) of molinate is used annually[1]. Such heavy use may lead to contamination of surface and ground waters by drift, runoff, drainage and leaching. Consequently, it is important to assess the adverse impacts these chemicals may have on ecosystems particularly on non-target aquatic organisms[2].

It is toxic to germinating broad-leaved weeds and barnyard grass (*Echinochloa* spp.)[3], possibly by inhibiting cell division during mitosis[4]. It is metabolized to form the intermediate molinate sulfoxide by microsomal enzymes and is cleaved by the soluble-glutathione system[5]. There is experimental evidence indicating that molinate inhibits enzymes such as aldehyde dehydrogenase and acetyl cholinesterase[6,7]. Reproductive effects were also observed in mice and rats as reduction of fertility[5]. In a combined chronic toxicity/carcinogenicity study in rats, there was an increase in kidney tumors in males at the highest dose of molinate. Nevertheless, a recent revision by the Cancer Assessment Review Committee of the Health Effects Division (U.S. Environmental Protection Agency) concluded that there is only suggestive evidence of carcinogenicity, not sufficient to assess human carcinogenic potential for molinate[8].

The detection of synthetic chemicals, especially agrochemicals such as herbicides and insecticides that may pose a genetic hazard in our environment is subjects of great concern at present[9]. Several assay systems having rapidity and reliability have been introduced for this purpose, such as reversion test with bacterial gene mutation[10-12], chromosomal aberration assay with mammalian cells[13-15], mouse lymphoma $tk^{+/-}$ gene assay with L5178Y $tk^{+/-}$ mouse lymphoma cells[16-21], micronucleus assay with rodents[22-25]. These assay systems are now well used to evaluate the genotoxicity of chemicals and also frequently adopted as methods for an index of genotoxicity in worldwide. And also, 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.

Nevertheless of many toxicological researches on synthetic chemicals, there are few reports on the genotoxicity of some chemicals especially some agrochemicals in spite of the extensive use, the possible human exposure and the lack of genotoxicity. There was some controversy on the genotoxicity of molinate. Molinate was reported as non-mutagenic in bacterial system, but mutagenic *in vitro* chromosomal aberration and *in vivo* mammalian assay system. Therefore, to clarify the genetic toxicity of molinate, it was subjected in bacterial reversion, *in vitro* chromosomal aberration assay in Chinese hamster lung (CHL) cells, mouse lymphoma $tk^{+/-}$ gene assay and *in vivo* micronucleus assay using bone marrow (p.o.) cells and peripheral reticulocytes (i.p.) in this study.

Discussion

The detection and the regulation of man-made synthetic chemicals are subjects of great concern in administrative authorities of environment because of its close correlation between environmental contamination and human health. However, there has been no attention to chemicals especially used in chemical industry. Nevertheless of the diverse uses of these chemicals in industry, there has been no attention to evaluate the toxicity for the environment and the human beings such as genetic toxicity.

It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that mutagens are carcinogens[26] and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency[27]. Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement[28] which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation of cellular cancer genes[29].

Cytogenetic studies on mammalian cells *in vivo* as well as *in vitro* have been introduced as a screening method for DNA-attacking substances. Several short term methods have been developed[10,12,30] for predicting the carcinogenicity of chemicals and also been introduced for the evaluation of genotoxicity[13,14,22-24,29,31-34] and of antimutagenicity[35,36].

The controversy on genotoxicity of molinate has been reported in bacterial system, *in vitro* and *in vivo* mammalian systems. Molinate was negative in *Salmonella typhimurium* and *Escherichia coli* assays for gene mutation, both in the absence or presence of S-9 mix[37,38], and in other short-term tests in prokaryotes[39]. Some results suggest increases in gene mutations, chromosome aberrations, and sister chromatid exchanges for mouse lymphoma cells. There are conflicting data in the mouse micronucleus assay, and the results in the dominant lethal test are negative.

To clarify the genotoxicity of molinate, we performed bacterial gene mutation test, *in vitro* chromosome aberration and mouse lymphoma $tk^{+/-}$ gene assay, and *in vivo* micronucleus assay using bone marrow cells and peripheral reticulocytes of mice.

In bacterial gene mutation assay, no mutagenicity of molinate (12-185 μg/plate) was observed in

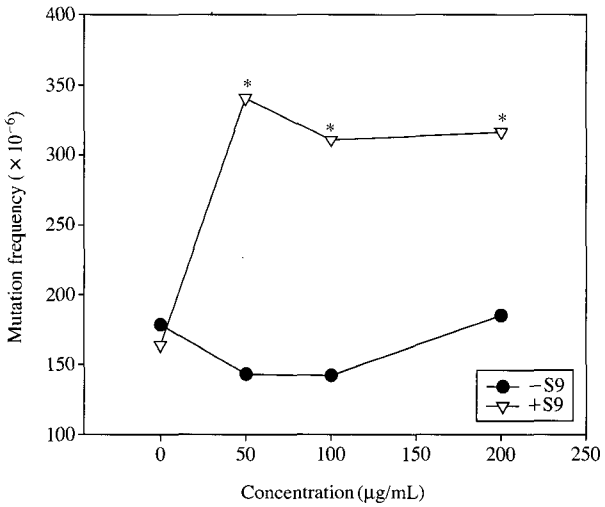

Fig. 1. Mutation frequencies by molinate in L5178Y cells in the absence and presence of metabolic activation system (3 hr treatment). Total *tk* mutantion frequency is displayed. Results are taken from one representative experiment. *statistically significant ($p < 0.05$).

Table 1. *Salmonella typhimurium* reversion assay of molinate.

Compound	Dose (μg/plate)	S-9 mixture	His+ revertants/plate (Mean±S.D.)			
			TA 98	TA 100	TA 1535	TA 1537
DMSO		-	16±5	109±5	13±1	5±2
Molinate	12	-	24±9	138±10	9±2	8±5
	24	-	20±6	136±20	12±4	9±3
	47	-	19±3	109±4	10±8	6±2
	93	-	15±2	106±1	13±7	9±2
	185	-	19±1	111±16	15±2	7±1
SA	1	-	-	665±48	-	-
SA	10	-	-	-	1675±178	-
2-NF	0.2	-	85±9	-	-	-
9-AA	80	-	-	-	-	768±54
DMSO		-	15±4	105±13	11±4	18±4
Molinate	12	-	19±9	141±8	14±6	7±3
	24	-	21±9	128±33	12±1	5±3
	47	-	13±2	123±11	13±3	9±6
	93	-	11±4	128±15	13±2	7±2
	185	-	11±2	121±1	10±5	5±2
2-AA	0.5	-	202±16	-	-	-
2-AA	1	-	-	598±137	-	-
2-AA	2	-	-	-	313±81	205±72

DMSO: Dimethyl sulfoxide, SA: Sodium Azide, 2-NF: 2- Nitrofluorene, 9-AA: 9-Aminoacridine, 2-AA: 2-Amioanthracene

Table 2. Chromosome aberration assay of molinate in Chinese hamster lung fibroblast cells.

Com.	Con. (μg/mL)	Treated hr.	Without (−) or With (+) S9 Mix	Chromosome aberrations/200 cells					Extra aberrations			
				Chromatid		Chromosome		Total aberration (%)				
				Br	Ex	Br	Ex		ctg	csg	poly	nor
DMSO		6	+	1	0	0	0	0.5	2	0	1	199
CP	5	6	+	16	86	1	0	41.5	7	1	4	117
Mol	408.2	6	+	11	38	0	0	17	9	1	3	166
	204.1	6	+	8	30	1	0	11	6	1	0	178
	102.1	6	+	9	15	0	0	8	6	0	2	184
DMSO		6	−	2	0	0	0	1.0	3	0	0	195
MMC	0.1	6	−	11	34	0	0	22.5	4	0	2	149
Mol	13.58	6	−	3	0	0	0	1.5	2	0	0	195
	27.16	6	−	2	1	0	0	1.5	0	0	1	196
	54.32	6	−	1	1	0	0	1.0	1	1	0	196
DMSO		24	−	1	0	0	0	0.5	2	0	0	197
MMC	0.1	24	−	17	49	0	0	33.0	6	0	0	128
Mol	13.58	24	−	2	2	0	0	2.0	2	0	0	194
	27.16	24	−	1	2	0	0	1.5	1	1	1	194
	54.32	24	−	0	0	0	0	0.0	3	0	1	196

Com.: compound, Con.: concentration, Br: breakage, Ex: exchange, ctg: chromatid gap, csg: chromosome gap, poly: polyploid, nor: normal, DMSO : dimethylsulfoxide, MMC: mitomycin C, CP: cyclophosphamide, Mol: molinate

Salmonella typhimurium TA 98, 100, 1535 and 1537 both in the absence and in the presence of S-9 metabolic activation system (Table 1).

The clastogenicity of molinate was observed in the presence (102.1-408.2 μg/mL) of S-9 metabolic activation system in mammalian cell system using CHL fibroblast. However, no clastogenicity was observed in the absence (13.6-54.3 μg/mL) of S-9 metabolic activation system (Table 2). It is suggested that the genotoxicity of molinate was derived some metabolites by metabolic activation.

Molinate was also subjected to mouse lymphoma L5178Y cells using microtiter cloning technique, and the results were analyzed with Mutant V 2.34 program (Hazleton). In the range-finding cytotoxicity test, the concentration range was determined as 50 to

Table 3. Micronucleus assay of molinate with bone marrow of ICR male mice when administrated orally.

Test compound	Dose (mg/kg)	No. of mice tested	Sampling time (hr)	MNPCE % (Mean±SD)	PCE/PCE+NCE (Mean±SD)
0.5% CMC		6	24	0.23±0.12	0.51±0.02
MMC	2	6	24	5.70±1.14	0.47±0.06
Molinate	100	6	24	0.23±0.08	0.52±0.01
	199	6	24	0.70±0.19	0.52±0.05
	398	6	24	1.01±0.34	0.47±0.03

MNPCE: micronucleated Polychromatic Erythrocytes/1000 polychromatic erythrocytes, PCE/PCE+NCE: Polychromatic Erythocytes/1000 Erythrocytes, MMC: Mitomycin C

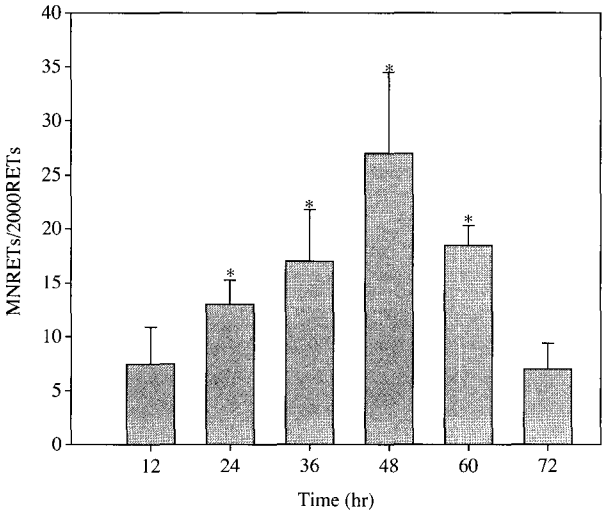

Fig. 2. Mean frequencies of micronucleated reticulocytes (MNRETs) from peripheral blood after a single intraperitoneal injection with 363 mg/kg of molinate at each sampling time. Five mice were tested and 2000 reticulocytes were analyzed per animal. *statistically significant ($p<0.05$).

200 μg/mL (>20% RS) both in the presence and absence of S-9 mixture. In the absence of S-9 mixture, mutation frequencies (MFs) were revealed 1.4-1.9 $\times 10^{-4}$ with no statistical significance. However, MFs in the presence of metabolic activation system revealed 3.2-3.4 $\times 10^{-4}$ with statistical significance ($p<0.05$) (Fig. 1).

And also, *in vivo* micronucleus assay using mouse bone marrow cells, molinate revealed genotoxic potential in the dose ranges of 100-398 mg/kg of molinate when administered orally (Table 3). Molinate 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 calculated as 726 mg/kg in our experiment. To determine the optimal sampling time, a single half dose of LD_{50}, 363 mg/kg of molinate was injected into mice, and blood samples collected at 12 hr intervals from 12 to 72 hr. Maximum frequency of micronucleated reticulocytes (MNRETs) was observed at 48 hr after treatment (Fig. 2). The frequency of MNRETs induced 48 hr after i.p. injection at a single dose of 91, 182 and 363 mg/kg of molinate was dose-dependently increased as 10.2±4.7, 14.6±3.9 and 28.6±6.3 (mean±SD of MNRETs /2000 reticulocytes) with statistical significance ($p<0.05$), respectively (Table 4).

Table 4. Frequency of MNRETs in peripheral reticulocytes of mice after a single intraperitioneal injection of molinate.

Test compound	Dose[a] (mg/kg)	# of mice	Sampling time[b] (hr)	MNRETs[c]/ 2000 RETs (Mean±SD)
Corn oil		5	48	4.8±1.3
Molinate	91	5	48	10.2±4.7*
	182	5	48	14.6±3.9*
	363	5	48	28.6±6.3*
MMC	1	5	48	44.8±2.8

[a]The highest dose of molinate, 363 mg/kg body weight of mouse, was based on half dose of LD_{50}

[b]Sampling time after intraperitoneal injection of test compound

[c]MNRETs : micronucleated reticulocytes

*$p<0.05$

Consequently, genotoxic potential of molinate was observed *in vitro* mammalian systems only in the presence of metabolic activation system and *in vivo* micronucleus assay using both bone marrow cells and peripheral reticulocytes in the dose ranges used in this experiment. These results suggest that metabolic activation plays a critical role to express the genotoxicity of molinate *in vitro* and *in vivo* mammalian system.

Methods

Materials

The test chemical (molinate) was kindly donated from Hanhwa co. ltd. Stock solution of molinate was prepared freshly in dimethylsulfoxide (DMSO) before use. Eagles minimum essential medium (EMEM),

RPMI-1640, 0.25% trypsin-EDTA, trypan blue, colcemid, fetal bovine serum (FBS) 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[10,12]. The S-9 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. 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.

Ames *Salmonella* Bacterial Mutagenicity Assay

This test performed essentially as described by Ames *et al.*[11,12]. Media and positive control chemicals were obtained from commercial sources and were the purest grades available. The dose range for test chemical was determined by performing a toxicity assay using strain *Salmonella typhimurium* TA 100 and half-log dose intervals of the test substance from 185 μg/plate to 12 μg/plate. Strain TA 100 was chosen as the representative tester strain because of its high spontaneous reversion rate. Spontaneous revertant numbers were counted and plotted against the dose of the test chemical to produce a survival curve for the his^+ genotype. The mutagenicity assay was performed by mixing one of the tester strains which was cultured overnight, with the test substance in the presence and in the absence of S-9 mixture condition, sodium phosphate buffer added instead of S-9 mixture both in negative and positive control in test tube. Then, incubating the mixture in water bath for 30 min at 37°C and after incubation, the mixture mixed with top agar containing a minimal amount of histidine and then poured onto the surface of a *r*-ray sterile Petri dish (Falcon, USA) containing 25 mL of solidified bottom agar. The finished plates were incubated for 48 hr at 37°C, and revertant colonies were counted later. Negative control plates containing no added test chemical but positive control plates containing appropriate amounts of chemicals known to be active were included with each tester strain (Table 1). All platings were done in triplicate, and the results were tabulated as the mean ± standard deviation for each condition. A response was considered to be positive in our criteria if there was a dose-dependent increase in revertants per plate resulting in (1) at least a doubling of the background reversion rate for strains TA 98 or TA 100 or (2) at least a tripling of the background reversion rate for strains TA 1535 or TA1537.

In vitro Chromosomal Aberrations Assay in CHL Cells

The clastogenicity of molinate was evaluated for its ability to induce chromosomal aberrations in CHL cells. The experiment was performed as described by OECD[15] and Ishidate and Odashima[13] with some minor modifications[31-34,40-42], which are briefly summarized as follows.

Test article dose levels were determined prior to the main study in a dose range-finding study performed both in the presence and in the absence of a rat liver S-9 activation system. For the growth inhibition assay, CHL cells were seeded at the densities of 1×10^4 cells/0.2 mL into 96 well plates. 24 hr after seeding, several different doses of sample were separately added and incubated for 24 hr. And then the 50% inhibition concentration (IC_{50}) values were calculated by MTT assay[43]. For the aberration assay, three different doses, including the IC_{50} value as a maximum dose, were prepared and separately added to 3-day-old cultures (approximately 10^5 cells/60 mm dish). In the absence and in the presence of S-9 mixture, cultures were treated for 6 hr with derivatives and then maintained for 18 hr in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Cyclophosphamide (CP) and mitomycin C (MMC) were used as a positive control in combination with or without S-9 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[44]. 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. 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[45] 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.

L5178Y *Tk*$^{+/-}$ 3.7.2C Mouse Lymphoma Assay (MOLY)

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. All chemicals were tested with and without S-9 mixture. Molinate 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 hr. 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 the test chemicals. Cytotoxicity was determined by relative survival (RS) and relative total growth (RTG) following 3 hr 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.*[46]. 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 ~2000 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 (Mutant^{TM}; UKEMS, York, UK) in accordance with the UKEMS guidelines[47].

In vivo Micronucleus Assay in Mice

a. Using bone marrow cells after oral administration. 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 test substance was given twice with a 24 hr interval and killed by cervical dislocation. Normally, the tested dose range included the span from no effect up to complete halt of bone marrow proliferation. Preparation of bone marrow and staining were carried out according to the method worked out Schmid[25]. From the freshly killed animal both femora were removed *in toto*, which means that one was cutting through pelvis and tibia. The bones were then freed from muscle by the use of gauze and fingers. With the needle of appropriate size mounted, about 1 mL of serum was pulled from the tube into a disposable plastic syringe. Then the needle (24 gauge) was inserted a few mm into the proximal part of marrow canal to flush the marrow cells. After centrifugation, the supernatant was removed, and cell pellet suspension of bone marrow cells was dropped onto glass slides, and then air dried. After fixation in methanol, slides were stained with 4% Giemsa in 1/15 M sodium phosphate buffered saline (PBS, pH 6.8) for 30 min, washed with PBS, and then air dried for microscopic observation. In scoring the preparations, micronuclei were counted in polychromatic and, sepa-

rately in normochromatic, erythrocytes. The rate of micronucleated cells, expressed in percentage, was based on the total of polychromatic erythrocytes present in the scored optic fields. This mode of scoring, which must always be followed where the test substance markedly influences the proliferation rate in the bone marrow, prevents a distortion of the results by the influx of peripheral blood into the damaged marrow. The scoring of micronucleated normocytes not only serves to recognize the presence of artifacts (which is rare in preparations from mouse) but provides additional interesting information on the mode of action of the test substance. Generally, an incidence of more than 1 micronucleated normocyte per thousand polychromatic erythrocytes indicates an effect on cell stages past the S-phase.

b. Using peripheral reticulocytes after intraperitoneal administration. The micronucleus test with mouse peripheral blood reticulocytes using acridine orange-supravital staining method was performed essentially as described by Hayashi *et al.*[23]. To determine the mutagenicity of molinate, molinate dissolved in corn oil was administrated intraperitoneally (i.p.) at either 91, 182, or 363 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 hr intervals from 36 hr to 60 hr 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[47] 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 pair-wise test for statistical differences between the control and specific treatment groups.

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