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Evaluation of the Genetic Toxicity of Synthetic Chemicals (IX)

- a Synthetic Selective Herbicide, Pretilachlor -

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합성화학물질들의 유전독성평가(IX)

- 합성 제초제 Pretilachlor -

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요 약

Pretilachlor [2-chloro-N-(2, 6-diethylphenyl)-N-(2-propoxyethyl)acetamide, $C_{17}H_{26}ClNO_2$, M.W. = 311.9, CAS No. 51218-49-6]는 제초제의 일종으로, 본 연구에서는 박테리아 복귀 돌연변이 시험과 포유동물 세포를 이용한 염색체 이상 시험 및 마우스를 이용한 *in vivo* 소핵 시험을 수행하여 pretilachlor의 유전독성을 평가하였다.

박테리아 복귀 돌연변이 시험에서 pretilachlor는 *Salmonella typhimurium* TA98, TA 100, TA1535, TA1537 균주의 대사 활성계 존재 및 부재시 313~5,000 μg/plate의 범위에서 농도의존적인 돌연변이율의 증가를 관찰할 수 없었다.

또한 포유동물 세포인 Chinese hamster lung (CHL) fibroblast를 이용한 염색체 이상 시험에서 pretilachlor는 대사 활성계 존재 및 부재시 1.56~6.24 μg/mL의 농도에서 clastogenicity를 보이지 않았고, 137.5~550.1 mg/kg의 pretilachlor를 복강 주사한 마우스의 골수세포를 이용한 *in vivo* 소핵 시험의 결과에서도 통계적으로 유의한 소핵 유발능을 관찰할 수 없었다.

Key words : Genotoxicity, Clastogenicity, Pretilachlor, *Salmonella typhimurium,* Chromosome Aberration, Micronucleus, Chinese Hamster Lung Fibroblast

INTRODUCTION

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. Despite the many toxicological researches on synthetic chemicals, there are few reports on the genotoxicity of some agrochemicals. Several assay systems having rapidity and reliability have been introduced for this purpose, such as

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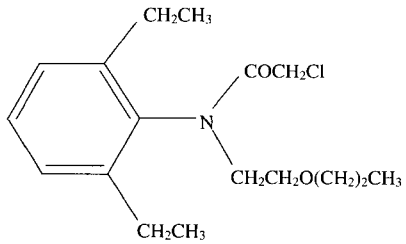

Fig. 1. The chemical structure of Pretilachlor.

reversion test with bacterial gene mutation (Ames *et al.*, 1973, 1975; Maron and Ames, 1983), chromosomal aberration assay with mammalian cells (Ishidate and Odashima, 1977), micronucleus assay with rodents (Hayashi *et al.*, 1992, 1994; Schmid, 1975). These assay systems are now well used to evaluate the genotoxicity of chemicals and also frequently adopted as methods for an index of genotoxicity worldwide. Furthermore, it was well applied as a screening probe for the detection of possible carcinogenic substances in our environment.

Pretilachlor [2-chloro-N-(2, 6-diethylphenyl)-N-(2-propoxyethyl)acetamide, $C_{17}H_{26}ClNO_2$, M.W. = 311.9, CAS No. 51218-49-6], is one of well known synthetic selective herbicide. The chemical structure of pretilachlor is illustrated in Fig. 1. It is taken up readily by the hypocotyls, mesocotyls and coleoptiles, and, to a lesser extent, by the roots of the germinating weeds. The biochemical mode of action of this chemical is cell division inhibitor and effective against main annual grasses, broad-leaved weeds and sedges in transplanted and seeded rice at 1 ~ 1.25 kg/ha. Our laboratory has great concern to evaluate the chemical hazards, and conducted the toxicity evaluations of synthetic chemicals especially in genotoxicity (Ryu *et al.*, 1993, 1994, 1996a, b, 1997, 1998a, b, c, d, 1999a, b, 2000, 2001a, b, c, d, 2002a, b, c, d, 2003a, b, c; Kim *et al.*, 2001; Heo *et al.*, 1997; Tice *et al.*, 2000).

To elucidate the genotoxicity of pretilachlor, we performed bacterial gene mutation, *in vitro* chromosome aberration and *in vivo* micronucleus assay in this study.

MATERIALS AND METHODS

1. Materials

Pretilachlor was colourless liquid, and stock solution was prepared freshly in dimethylsulfoxide (DMSO) before use. Eagles minimum essential medium (EMEM), 0.25% trypsin-EDTA, colcemid and fetal bovine serum (FBS) were the products of Gibco BRL Life Tech. Inc. (Gaithersburg, 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 (Ames *et al.*, 1973; Maron and Ames, 1983). The S-9 fraction prepared was stored immediately at −80°C before use.

2. Ames *Salmonella* Bacterial Mutagenicity Assay

This test performed essentially as described by Ames *et al.* (1973, 1975). 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 up to 5 mg/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

Table 1. *Salmonella typhimurium* reversion assay of Pretilachlor

Compound	Dose (μg/plate)	S-9 mixture	His^+ revertants/plate (Mean±S.D.)			
			TA 98	TA 100	TA 1535	TA 1537
DMSO		-	17±2	135±7	8±2	6±1
pretilachlor	313	-	17±5	101±22	10±4	4±1
	625	-	17±3	92±13	9±1	5±1
	1,250	-	13±2	91±13	12±2	3±2
	2,500	-	17±3	100±23	7±2	3±2
	5,000	-	14±2	95±8	13±5	4±1
SA	1	-		613±222	456±58	
2-NF	0.2	-	60±9			
9-AA	80	-				662±151
DMSO		+	27±14	166±21	8±4	6±3
pretilachlor	313	+	25±8	108±13	11±1	7±4
	625	+	29±9	103±22	11±3	7±3
	1,250	+	28±9	97±25	10±4	2±1
	2,500	+	20±1	113±8	18±5	5±1
	5,000	+	20±6	111±9	14±3	3±0
2-AA	0.5	+	56±9			
2-AA	1	+		360±53		
2-AA	2	+			63±11	115±48

DMSO; dimethyl sulfoxide, SA; Sodium Azide, 2-NF; 2-Nitrofluorene, 9-AA; 9-Aminoacridine, 2-AA; 2-Aminoanthracene

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 and (2) at least a tripling of the background reversion rate for strains TA 1535 or TA 1537.

3. *In vitro* chromosomal aberration assay in CHL cells

The experiment was performed as described by OECD (1993) and Ishidate and Odashima (1977) with some minor modifications (Ryu *et al.*, 1993, 1994, 1996a, b, 1998a, b, 2001b, c, d, 2002c, d, 2003a, b) which are briefly summarized as follows. Chinese hamster lung (CHL) fibroblast 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. This cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

Test article dose levels were determined prior to the main study in a dose range-finding study performed in the absence of a rat liver S-9 activation system. CHL cells were seeded at the density of 5×10^4 cells/ml into 96 well plates after 24 hour seeding, several different doses of sample were separately added and incubated for 24 hours. And then the 50% inhibition concentration (IC_{50}) values were calculated by MTT assay (Mosmann, 1983).

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 of metabolic activation, cultures were treated for 24 hours with the test article, while in the presence of metabolic activation, cells were treated for 6 hours because of toxicity of S-9 and then maintained for 18 hours in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Cyclo-

phosphamide (CP) and mitomycin C (MMC) were used as a positive control in combination with or without S-9 mixture, respectively. After 22 hr incubation, treatment was followed by addition of colcemid at a concentration of 0.2 μg/mL. After 2 hr further incubation in the presence of colcemid, metaphase cells were harvested by centrifugation and trypsinization. The cells were swollen 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 precleaned glass microscope slides, and air dried. 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 metaphases at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS (1988). Breaks less than the width of a chromatid were designated as gaps in our criteria, and not included as chromosomal aberration. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fishers exact test (Altman, 1993) with Dunnetts adjustment and compared with results from the solvent controls. Therefore, dose-dependent responses and the statistical significance in p-value will be considered as positive results in our judgement.

4. *In vivo* bone marrow micronucleus assay in mice after intraperitoneal administration

Outbred mice of strain ICR, 7~8 weeks old, were used in this study. The mice were allowed an adaptation period for 1 week, then randomized and subjected to the study. The six animals were housed for each group. The test article was applied intraperitoneally in three doses in volumes of 10 mL/kg. The test substance was given once, and then 24 hr interval, they 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 (1975). 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, separately in normochromatic erythrocytes. The rate of micronucleated cells, expressed in percentage, were 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.

RESULTS AND DISCUSSION

It is well known that carcinogenicity of synthetic chemicals is the most serious problem in human

health hazard. As one of the mechanisms of carcinogenicity, it has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1991). The detection and the regulation of man-made synthetic chemicals are subjects of great concern because of its close correlation between environmental contamination and human health. Nevertheless of the extensive use of agrochemicals, however, there has been few attention to evaluate the genotoxicity. Since these substances are not limited to the original products, and they may enter the environment and have become widespread environmental pollutants, thus leading to a variety of chemicals that possibly threaten the public health.

Since pretilachlor is one of well known synthetic herbicide, it is subjected to elucidate the genotoxicity and clastogenicity in this study. The biochemical mode of action of this chemical is known as cell division inhibitor and effective against main annual grasses, broad-leaved weeds and sedges in transplanted and seeded rice at 1-1.25 kg/ha. It is taken up readily by the hypocotyls, mesocotyls and coleoptiles, and, to a lesser extent, by the roots of the germinating weeds. In animals, the major metabolic routes for pretilachlor involve substitution of the chlotine atom for glutathione to form a conjugate and cleavage of the ether bond to yield an ethyl alcohol derivatives. (Tomlin, 2000)

1. Ames reverse mutation assay

The mutagenic potential of pretilachlor was investigated in the *Salmonella typhimurium* microsomal activation assay. This assay detects materials that cause specific point mutations such as base-pair substitution and frameshift mutation in different *S. typhimurium* strains (TA98, TA100, TA1535 and TA1537), in the presence and in the absence of S-9 mixture. Positive controls specific to each of the four tester strains resulted in the expected increases in the number of histidine revertants (Table 1). In observation of the background lawns of treated bacteria, pretilachlor was no cytotoxic at doses 5,000 μg/plate in the absence and presence of S-9 mixture, and so, we determined as optimal maximum concentrations of pretilachlor for this assay. As summarized in Table 1, pretilachlor revealed no dose-dependent mutagenic potential in 313~5,000 μg/plate of Salmonella typhimurium TA 98, TA 100, TA1535 and TA 1537 both in the absence and presence of S-9 metabolic activation system.

2. Cytotoxicity of pretilachlor in CHL cells

Relative survival of CHL cells following exposure to a range of concentrations of pretilachlor was determined by MTT assay. The survival percentage relative to solvent control (DMSO) was determined as a percentage of the number of cells survived after treatment without metabolic activation system. The 50% cell growth inhibition concentration (IC_{50}) of pretilachlor was determined in the absence of metabolic activation system as 6.24 μg/mL in CHL fibroblast cells (data not shown)

3. *In vitro* chromosomal aberration assay in the CHL cells

The clastogenicity of pretilachlor was assessed by its ability to cause chromosomal aberrations in cultured CHL cells. The types and frequencies of chromosomal aberrations seen in treated and control cultures for 6 hr and 24 hr pretilachlor treatment in the presence and in the absence of S-9 metabolic activation, respectively, are listed in Table 2. The concentrations for the assay was determined as 6.24, 3.12 and 1.56 μg/mL in the absence and the presence of S-9 mixture. The solvent (DMSO)-treated control was revealed only 2% of spontaneous chromosomal aberrations in 200 metaphase cells. Cyclophosphamide (50 μg/mL) used as an indirect-acting mutagen that requires metabolic activation and mitomycin (0.1 μg/mL) as a direct-acting mutagen, induced remark-

Table 2. Chromosome Aberration Assay of Pretilachlor with Chinese hamster lung fibroblast cells

Treatment			S9 Mix	Chromosome aberrations/100 cells									
				Chromatid type		Chromosome type		Total aberration (%)	Extra aberration				
Compound	Con.(μg/mL)	hr		Br	Ex	Br	Ex		ctg	csg	poly	endo	nor
Saline	−	6	+	0	0	0	0	0	2	0	0	0	98
CP	50	6	+	5	24	0	1	30	5	0	0	0	65
Pretilachlor	6.24	6	+	1	0	0	0	1	1	0	0	0	98
	3.12	6	+	0	0	0	0	0	1	0	0	0	99
	1.56	6	+	0	0	0	0	0	0	0	0	0	100
Saline	−	6	−	0	0	0	0	0	1	0	0	0	99
MMC	0.1	6	−	2	15	0	2	19	6	0	0	0	75
Pretilachlor	6.24	6	−	1	1	0	0	2	2	0	0	0	96
	3.12	6	−	0	0	0	0	0	0	0	0	0	100
	1.56	6	−	0	0	0	0	0	0	0	0	0	100

Com. : compound, Con. : concentration, Br : breakage, Ex : exchange, ctg : chromatid gap, csg : chromosome gap, poly : polyploid, endo : endoreduplicate, nor : normal, MMC : mitomycin C, CP : cyclophosphamide
Total aberration (%) : Number of chromosome aberrations/total cells (100)*100

Table 3. Micronucleus Assay of Pretilachlor with ICR male mice (i.p.).

Test compound	Dose (mg/kg)	No. of mice tested	Sampling time (hr)	MNPCE % (Mean±SD)	PCE/PCE+NCE (Mean±SD)
Corn oil	*	6	24	0.10±0.11	0.49±0.02
MMC	2	6	24	4.50±1.26	0.50±0.02
Pretilachlor	137.5	6	24	0.10±0.11	0.51±0.02
	275.1	6	24	0.10±0.06	0.52±0.02
	550.1	6	24	0.07±0.08	0.48±0.02

MNPCE : Micronucleated Polychromatic Erythrocytes/1000 Polychromatic Erythrocytes; PCE/PCE+NCE : Polychromatic Erythrocytes/1000 Erythrocytes; MMC : Mitomycin C

able chromosomal aberrations (about 20~30%) in CHL fibroblasts. Low frequencies of breaks and fragments were seen in treated cultures and solvent controls, both with and without S-9 mixture. No statistically significant increases in the mean percentage of aberrant cells in the types of aberrations noted between treated and solvent-control were seen both with or without S-9 mixture (Table 2). No unusual types or distributions of aberrations were evident. The mean percentage of aberrant cells both with and without S-9 mixture ranged from 1.0 to 2.0%, compared with a mean solvent control value of 2.0%. From this result, 6 hr treatment of pretilachlor was not revealed clastogenicity both in the presence and absence of S-9 mixture in this assay. Consequently, no clastogenicity of pretilachlor was observed both in the absence and presence of S-9 mixture in the concentration range of 1.56~6.24 μg/mL.

4. *In vivo* bone marrow micronucleus assay in mice after intraperitoneal administration

The 50% lethal dose (LD_{50}) after oral administration in rats was reported as 6,099 mg/kg (Tomlin, 2000) and determined with intraperitoneal administration in mice as 1,100.2 mg/kg in our laboratory, so we adopted half LD_{50} value as maximum dose. Each group was consisted of 6 mice and sampling time was 24 hour in this experiment. The positive control, mitomycin C (2 mg/kg) revealed remarkable induction of MNPCE (micronucleated polychromatic ery-

throcytes/1000 polychromatic erythrocytes). However, *in vivo* micronucleus assay using mouse bone marrow, pretilachlor revealed no clastogenic potential in the dose range of 137.5~550.1 mg/kg body weight of pretilachlor when administered intraperitoneally compared to control level (Table 3).

Consequently, Ames bacterial gene mutation with *Salmonella typhimurium*, *in vitro* chromosome aberration with mammalian cells and *in vivo* bone marrow micronucleus assay revealed no clastogenic potential of pretilachlor.

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