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Evaluation of the Genetic Toxicity of Synthetic Chemicals (II), a Pyrethroid Insecticide, Fenpropathrin

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The detection of many synthetic chemicals used in industry that may pose a genetic hazard in our environment is subject of great concern at present. In this respect, the genetic toxicity of fenpropathrin ((RS)-α-cyano-3-phenoxybenzyl-2,2,3,3-tetramethyl cyclopropane carboxylate, CAS No.: 39515-41-8), a pyrethroid insecticide, was evaluated in bacterial gene mutation system, chromosome aberration in mammalian cell system and *in vivo* micronucleus assay with rodents. In bacterial gene mutation assay, no mutagenicity of fenpropathrin (62-5000 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. In mammalian cell system using chinese hamster lung fibroblast, no clastogenicity of fenpropathrin was also observed both in the absence and in the presence of metabolic activation system in the concentration range of 7-28 μg/ml. And also, *in vivo* micronucleus assay using mouse bone marrow cells, fenpropathrin also revealed no mutagenic potential in the dose range of 27-105 mg/kg body weight of fenpropathrin (i.p.). Consequently, no mutagenic potential of fenpropathrin was observed *in vitro* bacterial, mammalian mutagenicity systems and *in vivo* micronucleus assay in the dose ranges used in this experiment.

Key Words : Genotoxicity, Clastogenicity, Fenpropathrin, *Salmonella typhimurium*, Chromosomal aberration, Micronucleus, Chinese hamster lung fibroblast

INTRODUCTION

There are many synthetic chemicals used in chemical reaction processes in industry. The detection of synthetic chemicals that may pose a genetic hazard in our environment is subject of great concern at present (WHO, 1971).

Several assay systems having rapidity and reliability have been introduced for this purpose, such as 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; Matsuoka *et al.*, 1979; Radman *et al.*, 1982; Jenderny *et al.*, 1988; OECD, 1993), micronucleus assay with rodents (Hayashi *et al.*, 1982, 1990, 1992). 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, and 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 used in industry.

Recently, the Ministry of Environment (MOE) of Republic of Korea has great concern to regulate and to evaluate the chemical hazards to environment, and conducted the toxicity evaluations of some synthetic chemicals annually since 1988. Our laboratory has also been involved in this toxicity evaluation program, especially in genetic toxicity (MOE report, 1992, 1993, 1994, 1995). In relation to this, we already reported no clastogenicity of major trichothecene mycotoxins such as T-2, HT-2 toxin, nivalenol, deoxynivalenol (Ryu *et al.*, 1993a) and some chemicals (Ryu *et al.*, 1994a, 1994b, 1996) by using chinese hamster lung fibroblast cells *in vitro*.

Fenpropathrin ((RS)-α-cyano-3-phenoxybenzyl-2,2,3,

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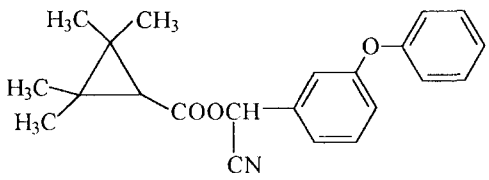

Fig. 1. The chemical structure of fenpropathrin

3-tetramethylcyclopropane carboxylate, CAS No. 39515-41-8) is one of the well known synthetic pyrethroid insecticides. The chemical structure of fenpropathrin is illustrated in Fig. 1. It is a pyrethroid with acaricidal and insecticidal activity against various species of mites and insects on cotton, ornamentals, top fruits, vine, vegetables and other field crops. So we aim to elucidate the genetic toxicity of fenpropathrin in bacterial gene mutation assay, chromosomal aberration in mammalian cells and *in vivo* micronucleus assay using mouse bone marrow.

MATERIALS AND METHODS

Ames *salmonella* bacterial mutagenicity assay

This test performed essentially as described by Ames *et al.* (1973, 1975). 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 5,000 μg/plate to 62 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 about 1×10^8 cells from an overnight growth culture of one of the tester strains with a known amount of the test substance, the S-9 mixture (when required), and top agar containing a minimal amount of histidine and then pouring the mixture onto the surface of a γ-ray sterilized 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. Negative control plates containing no added test chemical and positive control plates containing appropriate amounts of chemicals known to be active were included with each tester strain. 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 aberration assay in CHL cells

Chinese hamster lung (CHL) fibroblast cells were 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 Eagle's minimum essential medium (EMEM, Gibco, 410-1100EA) supplemented with 10% fetal bovine serum (FBS, Gibco, 26140-020). These cells were maintained at 37°C in 5% CO_2 atmosphere. Trypsin-EDTA and colcemid were the products of Gibco BRL Life Tech. Inc. (Gaithersburg, USA). 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 stored immediately at -80°C before use.

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 density of 1×10^4 cells/0.2 ml into 96 well plates. Twenty-four hr after seeding, several different doses of sample were separately added and incubated for 24 h. And then the 50% cell growth inhibition concentration (IC_{50}) values were calculated by MTT assay (Mosmann, 1983).

For the aberration assay, 1.5×10^6 cells were seeded in 75 cm^2 flasks 24 hr prior to treatment in EMEM supplemented with 10% FBS. Three different doses, including IC_{50} values as maximum dose, were prepared and separately added to 3-day old cultures (approximately 4×10^4 cells/60 mm dish). In the absence of metabolic activation, cultures were treated for 24 hr with the test article, while in the presence of metabolic activation, cells were treated for 6 hr because of its toxicity of S-9 and then maintained for 18 hr in the fresh medium i.e. to adjust a time equivalent to about 1.5 normal cell cycle lengths. Treatment was followed by addition of medium containing 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 drops 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 scor-

ing of chromosome aberrations. The number of cells with chromosomal aberrations was recorded on 100 well-spread metaphases at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG).

The types of aberration were followed by JEMS-MMS (1988) classification. Breaks less than the width of a chromatid were designated as gaps in our criteria, and 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 Fisher's exact test (Altman, 1993) with Dunnett's adjustment and compared with results from the solvent controls. Therefore, data from count up well-spread 100 metaphase cells were expressed as percentages, and then dose-dependent response and the statistical significance in *p*-value will be considered as positive results in our judgement. When no reliable dose response was obtained, additional experiments with different dose should be performed to confirm its reproducibility.

In vivo micronucleus assay in mice

Outbred mice of strain ICR, 7-8 weeks old, were used in this study. The mice were allowed an adaptation period of about 1 week, then randomized and subjected to the study. The six animals were housed for each group. The test article was applied i. p. in three doses in volumes of 10 ml/kg body weight 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 (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 1/15M 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, 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.

RESULTS AND DISCUSSION

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 (Ames *et al.*, 1973; Maron and Ames, 1983; Mersch-Sundermann *et al.*, 1991) for predicting the carcinogenicity of chemicals and also been introduced for the evaluation of genotoxicity (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979; Radman *et al.*, 1982; Hayashi *et al.*, 1982, 1990, 1992; Ryu *et al.*, 1993a, 1994a, 1994b, 1996) and of antimutagenicity (Sato *et al.*, 1991; Ryu *et al.*, 1993b).

It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1977). Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement (Zimmermann, 1971) which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation of cellular cancer genes (Radman *et al.*, 1982).

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 environment contamination and human health. However, there has been no attention to chemicals especially used in chemical reaction process of chemical industry.

Since fenpropathrin is one of well known synthetic pyrethroid acaricide and insecticide against various species of mites and insects, it is subjected to elucidate the genetic toxicity and clastogenicity in this study.

First of all, in the dose finding assay of the bacterial Ames test, fenpropathrin revealed no cytotoxicity in *Salmonella typhimurium* TA 100. So it was decided the maximum dose applied as 5000 μg/plate. The number of His+ revertants of *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 revealed no remarkable differences compared to DMSO control both in the absence and in the presence of metabolic activation as shown in Table 1. However, the positive controls such as 9-aminoacridine (9-AA), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), 2-aminofluorene (2-AF) and 2-aminoanthracene (2-AA) revealed good positive results. So, it is suggested that fenpropathrin has no bacterial mutagenicity in Ames test.

In the cell proliferation assay using Chinese hamster lung fibroblast cells, the IC_{50} value of fenpropathrin was calculated as 28 μg/ml by MTT assay (Mosmann, 1983). Therefore, it was applied three different concentrations such as 28, 14 and 7 μg/ml in the chromosome aberration assay. No statistical significance between fenpropathrin-treated groups and DMSO control (Table 2) was observed in the dose range used in this study. The positive controls, mitomycin C (0.1 μg/ml) for the absence of metabolic activation and benzo(a)pyrene (200 μg/ml) for the presence of metabolic activation, revealed about 41 and 25 chromosomal aberrations (%) such as chromatid breakage and exchange, chromosome breakage, respectively.

Moreover, in the *in vivo* micronucleus test using mouse bone marrow cells, the positive control, MMC (2 mg/kg, ip) induced about 2.2% micronucleated polychromatic erythrocytes (MNPCE), however no statistical differences in the formations of micronuclei in the erythrocytes of mouse bone marrow were observed among three fenpropathrin administered groups compared to CMC control group (Table 3).

Consequently, no mutagenic potential of fen-

Table I. *Salmonella typhimurium* reversion assay of Fenpropathrin

Compound	Dose (μg/plate)	S-9 mixture	His^+ revertants/plate (Mean±S.D.)			
			TA 98	TA 100	TA 1535	TA 1537
DMSO		-	22±4	147±8	15±5	14±5
Fenpropathrin	62	-	21±5	123±13	9±4	10±1
	185	-	18±13	119±21	10±1	7±3
	556	-	16±2	101±2	10±1	12±5
	1667	-	21±5	145±10	10±4	11±2
	5000	-	22±4	134±8	16±6	8±4
9-AA	10	-	178±13	-	-	172±27
MNNG	10	-	-	2092±150	2423±74	-
DMSO		+	32±6	132±4	15±2	12±3
Fenpropathrin	62	+	35±8	156±24	15±4	9±3
	185	+	25±3	148±26	9±1	9±4
	556	+	24±2	153±9	23±4	8±1
	1667	+	22±6	143±9	10±3	9±1
	5000	+	24±5	132±17	9±2	7±2
2-AF	10	+	912±107	1409±25	78±13	-
2AA	10	+	-	-	-	109±13

9-AA; 9-aminoacridine, MNNG: N-Methyl-N'-nitro-N-nitrosoguanidine, 2-AF; 2-aminofluorene, 2AA; 2-Aminoanthracene

Table II. Chromosome aberration assay of Fenpropathrin in Chinese hamster lung fibroblast cells

Treatment	Concentration (μg/ml)	With(+) or Without(-) S9 Mix	Chromosome aberrations/100 cells					Judgement
			chromatid		chromosome		Total aberration (%)	
			breakage	exchange	breakage	exchange		
DMSO	-	-	2				2	
Fenpropathrin	28	-	4				4	
	14	-	4				4	-
	7	-	1		1		2	
MMC	0.1	-	20	18	5		41	
DMSO	-	+	2				2	
Fenpropathrin	28	+	2		1		3	
	14	+	3				3	-
	7	+	2		1		3	
B[a]P	200	+	16	5	4		25	

DMSO: Dimethylsulfoxide, MMC: Mitomycin C, B(a)P: Benzo(a)pyrene

Table III. Micronucleus assay of Fenpropathrin i.p. administered in ICR male mice

Test Compound	Dose (mg/kg)	No. of mice tested	Sampling time (hr)	MNPCE % (Mean±SD)	Ratio % PCE/PCE+NCE (Mean±SD)
CMC	-	6	24	0.28±0.09	0.49±0.06
MMC	2	6	24	2.20±0.86	0.49±0.08
Fenpropathrin	105	6	24	0.38±0.26	0.50±0.01
	53	6	24	0.40±0.26	0.50±0.01
	27	6	24	0.32±0.24	0.49±0.04

MNPCE: Micronucleated Polychromatic Erythrocytes/1000 Polychromatic Erythrocytes
PCE/PCE+NCE: Polychromatic Erythrocytes/1000 Erythrocytes
CMC: 0.5% Carboxy methyl cellulose solution (10 ml/kg)
MMC: Mitomycin C

propathrin was observed in Ames bacterial reversion test, chromosomal aberration assay in Chinese hamster lung cells and mouse bone marrow micronucleus assay *in vivo*.

Recently, several new methods for the detection of genetic damages *in vitro* and *in vivo* based on molecular biological techniques were introduced according to the rapid progress in toxicology combined with cellular and molecular biology. Among these methods, the single cell gel electrophoresis (comet assay) which can detect DNA strand breakages in cell level (Mckelvey-Martin *et al.*, 1993; Singh *et al.*, 1994), mouse lymphoma thymidine kinase gene assay (Clive *et al.*, 1983; Sawyer *et al.*, 1985), FISH (fluorescence *in situ* hybridization) (Hayashi *et al.*, 1994), PRINS (primed *in situ* hybridization) (Abbo *et al.*, 1993) and transgenic animal and cell line model as a parameter of *lac I* (Big Blue) (Kohler *et al.*, 1991) or *lac Z* (Muta Mouse) (Suzuki *et al.*, 1993) gene mutation are newly introduced based on molecular toxicological approaches. Also, *in vivo* supravital micronucleus assay by using acridine orange fluorescent staining (Hayashi *et al.*, 1990, 1992) was introduced instead of mouse bone marrow micronucleus assay. Our laboratory is now under progress these assays to evaluate and to elucidate the mechanism of genetic toxicity and/or carcinogenesis, and will be presented in near future.

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