ELSEVIER

Toxicology Letters 107 (1999) 81–87

Toxicology Letters

# Induction of mitotic cell division disturbances and mitotic arrest by pyrethroids in V79 cell cultures

Wolfgang Hadnagy [a,*], Norbert H. Seemayer [b], Karl-Heinz Kühn [a], Gabriele Leng [a], Helga Idel [a]

[a] *Institute of Hygiene, Heinrich–Heine University Düsseldorf, P.O.B. 10 10 07, 40001 Düsseldorf, Germany*
[b] *P.O.B. 10 33 08, 45127 Essen, Germany*

Accepted 31 January 1999

**Abstract**

Five pyrethroids (fenvalerate, deltamethrin, cypermethrin, permethrin, cyfluthrin) differing in their chemical purity were investigated on their cytotoxic effects, especially on their ability to induce mitotic cell division disturbances using Chinese hamster lung cells of line V79. The colony forming ability (CFA) resulted in distinct differences of the cytotoxic effect of the tested pyrethroids, whereby permethrin was found to be most toxic. With the exception of fenvalerate all tested pyrethroids gave rise to inhibition of cell cycle progression as shown by G2/M-arrest of synchronized V79 cells by flow cytometry as well as by the increase of the mitotic index as evaluated by light microscopy. The mitotic arresting activity could be attributed to the occurrence of abnormal mitotic figures such as initial and full C-metaphases. The results however indicate, that pyrethroids per se do not contribute to the cytotoxic effects but that other factors such as chemical impurities, source as well as manufacturing process and isomer composition may be responsible for the observed cytotoxic effects. 

*Keywords:* Pyrethroids; Cytotoxicity; Mitotic spindle disturbances; Mitotic arrest; V79 cells

## 1. Introduction

Besides the many-fold discussions about a neurotoxic effect of pyrethroids on humans a cytotoxic and genotoxic potential of synthetic pyrethroids must also be taken into consideration. According to their carcinogenic effects most of the pyrethroids cannot be classified and are found in group 3 by the International Agency for Research on Cancer (IARC, 1991), i.e. there exist no clear indication for or against a carcinogenic effect. Also, based on genotoxicity studies, controversial results were obtained. Pyrethroids were found to be negative in bacterial and mammalian mutagenicity test assays (Pluijmen et al., 1994). On the other hand, pyrethroids induced chromosomal aberrations, sister chromatid exchanges and micronuclei in mammalian cell cultures including

* Corresponding author.
*E-mail address:* wolfgang.hadnagy@uni-duesseldorf.de (W. Hadnagy)

0378-4274/99/$ - see front matter 
PII: S0378-4274(99)00034-X

human cells (Puig et al., 1989; Surrallés et al., 1990; Barrueco et al., 1992, 1994; Surrallés et al., 1995). However, in human in vivo studies no induction of chromosomal aberrations or sister chromatid exchanges were described after pyrethroid exposure with the exception that a complex of pesticides including pyrethroids led to the induction of chromosomal aberrations or that mixed exposure of pyrethroids with organophosphates and carbamates induced micronuclei in human lymphocytes (Páldy et al., 1987; Bolognesi et al., 1993). Based on animal experiments also an increased rate of micronuclei in the bone marrow of the rat and the mouse were found after oral or intravenous application of deltamethrin (Agarwal et al., 1994; Gandhi et al., 1995).

These results indicate, that according to a genotoxic potential of pyrethroids the induction of micronuclei might be of considerable concern. Micronuclei result on the one hand from acentric chromosome fragments which are not incorporated into the daughter nuclei during mitotic cell division and on the other hand from whole chromosomes that are left behind during mitotic cell division due to mitotic spindle disturbances. The common consequence of both mechanisms is that a small additional nucleus appears in the cytoplasm of one of the daughter cells which can be detected in the interphase. Therefore, besides a possible clastogenic effect also mitotic spindle dysfunctions must be taken into consideration. Previous studies have demonstrated an inhibition of cell cycle progression in human lymphocyte cultures and the induction of abnormal cell division in human and plant test assays, which might indicate an effect on the mitotic spindle apparatus very similar to that of the well known spindle poison colchicine (Chauhan et al., 1986; Carbonell et al., 1989; Surrallés et al., 1990, 1995).

The controversial results of genotoxic effects by means of micronuclei induction is prominently demonstrated by the pyrethroid fenvalerate. Surrallés et al. (1995) could show that fenvalerate purchased by Ehrenstorfer (Augsburg, Germany) with a chemical purity of 97.9% and tested in the concentration range of 10–100 μg/ml has no effect on cell cycle progression and do not induce micronuclei in human lymphocyte cultures. On the other hand, the same research group described in 1990 a strong inhibition of cell cycle progression and an increased frequency of micronuclei with the same test system using fenvalerate from S.P.E. Shell (Madrid, Spain) which was considered to have a lower chemical purity (Surrallés et al., 1990). In this study 50 μg/ml fenvalerate exhibited an effect equivalent of 2 μg/ml of the cytostatic drug mitomycin C. In addition, Carbonell et al. (1989), found a strong effect of fenvalerate on the mitotic spindle apparatus resulting in the occurrence of so-called C-mitoses, whereby at the highest tested concentration of 50 μg/ml almost exclusively C-mitoses were found which corresponded to an effect equivalent of 0.01 μg/ml of demecolcin, a potent spindle poison. In accordance with the former study, fenvalerate was also obtained from S.E.P. Shell, however no indication concerning the purity was made. As already assumed by Surrallés et al. (1995) and shown by the controversial results additional factors such as the source and the purity of the pyrethroids must be taken into consideration with respect to the induction of cytotoxic and genotoxic effects.

In order to clarify the controversial findings, especially the effect on mitotic cell division, we have investigated pyrethroids on their cytotoxic and spindle disturbing effects differing in their chemical purity.

## 2. Material and methods

Pyrethroids, i.e. fenvalerate (99.5% chemical purity), deltamethrin (99.0%), cypermethrin (99.0%, 91.0%) permethrin (97.0%) and cyfluthrin (92.0%) were purchased by Ehrenstorfer (Augsburg, Germany). In addition another cypermethrin (99.0%) was obtained from Promochem (Wesel, Germany) All pyrethroids were dissolved in dimethylsulfoxide (DMSO) and tested in a concentration range of 3.1–100 μg/ml f.c. using Chinese hamster lung cells of line V79. All cell experiments were performed by using Dulbecco's modification of Eagles minimal essential medium supplemented with 10% fetal calf serum and by incubation at 37°C and 5% $CO_2$ in a humidified

atmosphere under light protection. The final concentration of DMSO in the cell cultures did not exceed 1%.

For cytotoxicity testing the colony forming ability (CFA) test was used in a slightly modified form as described previously (Hadnagy and Seemayer, 1986). Briefly, 100 logarithmically growing V79 cells per 5 ml were seeded in culture flasks. After 24 h of incubation cultures were treated with the respective pyrethroids at different concentrations. After incubation for a period of 120 h, treated cultures were washed twice with Hank's balanced salt solution, incubated for a further 24 h and then fixed and stained with 5% Giemsa.

Studies on mitotic arrest was performed by flow cytometry using synchronized V79 cells (Hader et al., 1996) and by evaluation of mitotic index, mitotic profile studies and percentage of abnormal metaphases using light microscopy (Hadnagy et al., 1997).

For flow cytometry studies $2.5 \times 10^4$ V79 cells/5 ml medium were cultivated in plastic culture flasks for 50 h. Synchronization was carried out for 16 h by addition of excess of thymidine ($2 \times 10^{-3}$ M). Cells were blocked in G1/S-phase and started synchronously to grow after elimination of thymidine and addition of desoxycytidine ($10^{-3}$ M). After 2.5 h cells entered synchronously the G2-phase and thereafter mitosis. At this check point cell cultures were treated with various concentrations of the pyrethroids and with the known spindle poison colcemid (f.c. 0.1 μg/ml). Duration of treatment lasted for 5.5 h. Then cells were fixed, treated with RNAse and stained with the fluorescence dye DAPI (4,6-diamidino-2-phenylindole). Flow cytometry was carried out with a PAS II flow cytometer (Partec). Mitotic arresting activity was evaluated from histograms corresponding to counts in the position of G2/M-phase.

For light microscopical examination V79 cells were seeded on sterile glass slides placed in plastic dishes (Quadriperm, Heraeus) at a density of $2.5 \times 10^4$ cells/5 ml medium. After incubation for 24 h cells were treated with various concentrations of pyrethroids. Treated cell cultures were further incubated for 16 h (covering 1 cell cycle). Then cells were immediately fixed with methanol-acetic acid (3:1), air dried and thereafter stained with 5% Giemsa in Sørensen buffer (pH 6.8). As a parameter of mitotic arresting activity mitotic index was determined by scoring 1000 cells/slide. Normal and abnormal cell division stages were evaluated from 100 mitotic cells/slide. Abnormal mitotic figures were classified as initial C-metaphases and full C-metaphases according to Schmid et al. (1989). Initial C-metaphases were characterized by chromosomes dislocated from the metaphase plate and by chromosomes which were arranged in groups or located in the center of the cell exhibiting so-called ball-metaphases. Full C-metaphases were identified by a complete scattering of contracted chromosomes in the cytoplasma similar to those induced by the known spindle poison colchicine. As an additional important index of mitotic arresting activity the proportion of anaphases plus telophases to metaphases (AT/M ratio) was evaluated.

With the exception of the flow cytometry studies, all results are presented as mean values from four replicative experiments.

## 3. Results and discussion

The colony forming ability (CFA) resulted in distinct differences of the cytotoxic effects between the tested pyrethroids (Fig. 1). A dose-dependent strong effect was found for permethrin, showing 100% reduction of CFA already at 25 μg/ml as compared to the control. Only moderate effects were found for deltamethrin and fenvalerate at the highest tested concentration of 100 μg/ml, whereas CFA of cypermethrin was reduced to 31.3% and cyfluthrin to 0%. The described effect on CFA for fenvalerate, delthamethrin, cypermethrin and permethrin is in good agreement with another cytotoxic index, the cytokinesis block proliferation index (CBPI) as evaluated in human lymphocyte cultures (Surrallés et al., 1995). In both of these investigations pyrethroids from the same source (Ehrenstorfer, Augsburg) were used exhibiting similar toxicity in different cytotoxicity test systems and reflecting inhibition of cell cycle progression.

Reduction of CFA might be the result of mitotic arrest as could be demonstrated by flow

cytometry (Fig. 2). The lowest arrest was found for fenvalerate, the highest arrest for permethrin. The mitotic arresting effect of the other pyrethroids are rather similar and can be found between the effect of fenvalerate and permethrin. Comparing mitotic arresting activity of the spindle poison colcemid being about 60% at a concentration of 0.1 μg/ml, permethrin at a concentration of 12.5 μg/ml reached 40%. On the other hand G2/M-arrest of the tested pyrethroids is in good agreement with their cytotoxic activity as shown by CFA.

Although cell synchronisation in combination with flow cytometry offers a reliable method for the detection of substances with mitotic arresting activity with advantages as selective treatment of cells in G2/M phase of the cell cycle and higher sensitivity as compared to asynchroneous growing cell culture systems, the only disadvantage is that flow cytometry cannot distinguish between cells in the G2 or M-phase, that means between premitotic and mitotic cells (Hader et al., 1996). This differentiation is only possible by analyzing cells

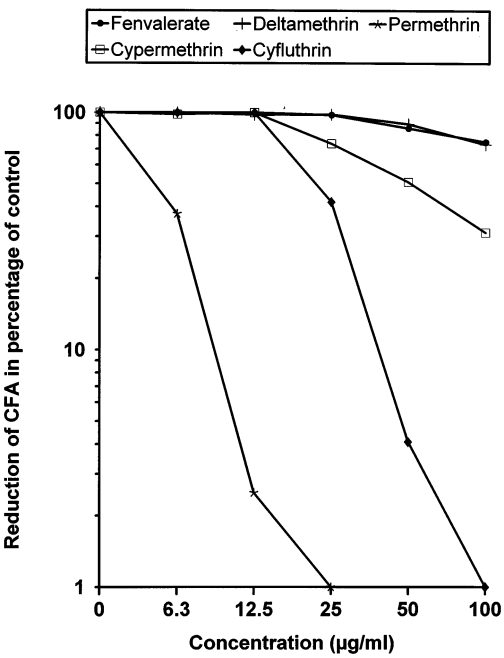


Fig. 1. Reduction of CFA of V79 cells by different pyrethroids.

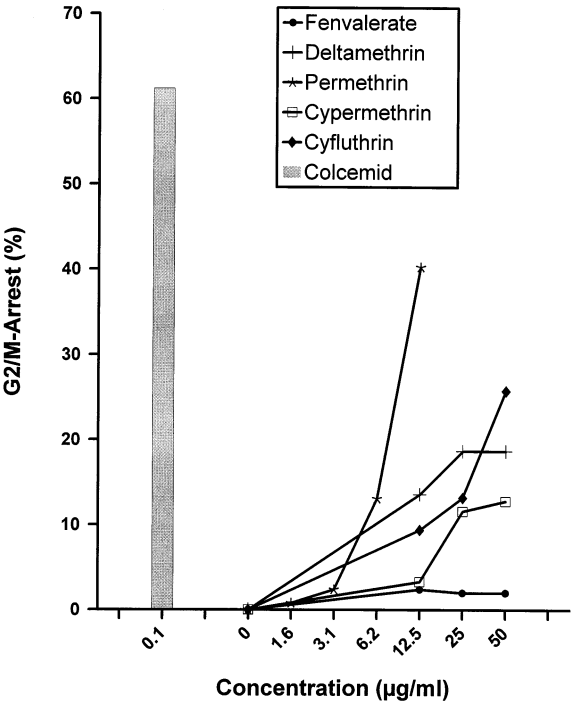


Fig. 2. G2/M-arrest of synchronized V79 cells by different pyrethroids as measured by flow cytometry.

under the light microscope. Therefore, mitotic arresting activity was evaluated by the mitotic index (Fig. 3). With the exception of fenvalerate all tested pyrethroids led to a dose-dependent increase of the mitotic index indicating inhibition of mitosis. From these results it can be concluded that pyrethroids inhibit cell cycle progression during mitosis by interfering with the mitotic spindle apparatus. This leads to mitotic arrest and as a consequence of that also to reduction of the colony forming ability.

In order to know in which stage of the mitosis pyrethroids are active, the evaluation of a mitotic profile is necessary, which is exemplary shown for permethrin in Fig. 4. As can be seen, permethrin leads to a concentration-dependent increase of metaphases, while following mitotic stages as ana- and telophases decrease. That leads to the conclusion that the arrest of cells can exclusively be attributed to the metaphase stage which is also clearly demonstrated by the strong reduction of the ratio of ana/telophases to metaphases.

The arrest at metaphase stage could be attributed to the occurrence of abnormal metaphases, such as initial and full C-metaphases (Fig. 5). For permethrin up to 60% abnormal metaphases at the highest tested concentration of 12.5 μg/ml were observed. From these about 80% belonged to the type of initial C-metaphases. Such initial C-metaphases are the result of partial spindle disturbances, which might lead to aneuploidy in subsequent cell division. Aneuplody or events that lead to aneuploidy may be of considerable concern with regard to somatic and genetic consequences (Hofman et al., 1986; Oshimura and Barrett, 1986; Melnick et al., 1996).

An interesting point is that the increase of mitotic arrest as shown by flow cytometry and light microscopy is in good agreement with the reduction of the CFA. Therefore, it is assumed that the cytotoxic effect of pyrethroids is associated with the mitotic arresting activity. Interestingly, when plotting cytotoxic activity against the degree of impurity a rather good association could be obtained using different pyrethroids (Fig. 6). This might reflect an association between

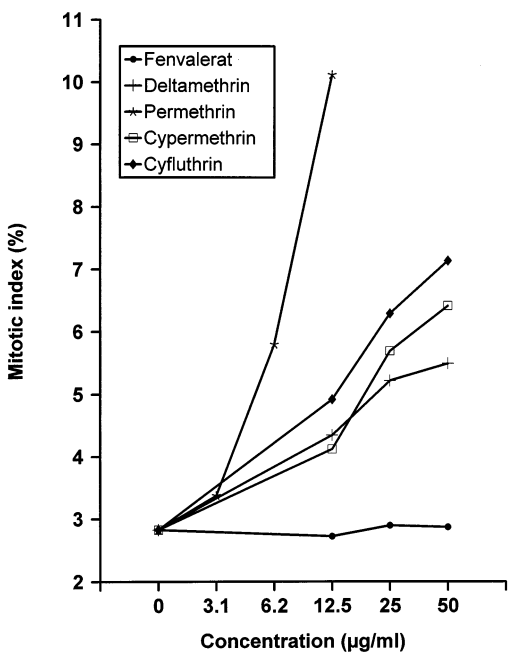


Fig. 3. Mitotic index of V79 cell cultures after treatment with different pyrethroids.

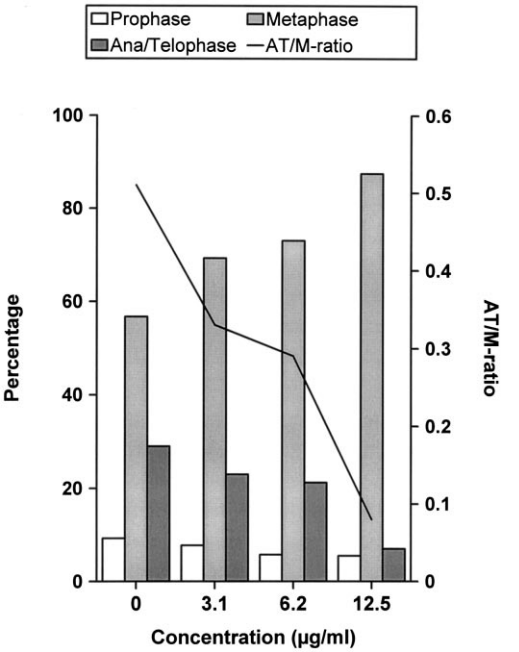


Fig. 4. Mitotic profile of V79 cell cultures after treatment with permethrin.

the degree of chemical impurity of pyrethroids and the reduction of the colony forming ability as well as mitotic arrest. Therefore, it is assumed, that pyrethroids per se do not contribute to the cytotoxic and mitotic arresting activity. The spindle disturbing effect might be the result of present impurities of the pyrethroids. Such effects has already been described for other pesticides such as pentachlorphenol (Kerkfliet et al., 1982). Whereas the technical grade (86%) pure pentachlorphenol led to depressed T cell cytolysis and enhanced tumor formation in mice, the 99% pure form was without effects.

Whether there might be additional factors responsible for cytotoxic and cytogenetic effects, two lots of cypermethrin standards (Ehrenstorfer) differing in their chemical purity (99%, 91%) had been tested by CFA and compared with a cypermethrin obtained from Promochem declared with a chemical purity of 99% (Fig. 7). Both cypermethrin standards from Ehrenstorfer showed different cytotoxic effects, whereby the cytotoxic effect was distinctly stronger for that cypermethrin with

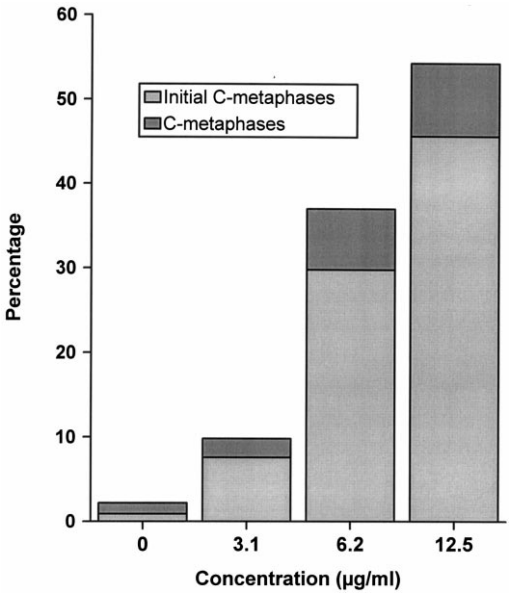


Fig. 5. Percentage of abnormal metaphases in V79 cell cultures after treatment with permethrin.

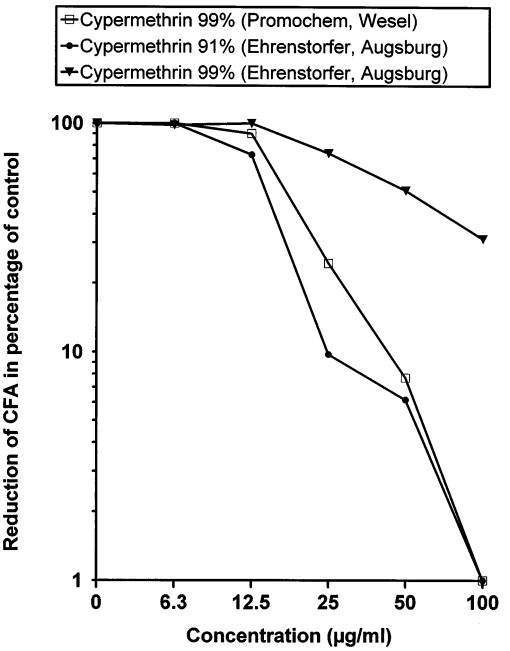


Fig. 7. Reduction of CFA of V79 cells by cypermethrin.

a chemical purity of 91%. This is in good agreement with above assumption of an association between the degree of chemical impurities and cytotoxic effects. On the contrary, that cypermethrin obtained from Promochem and declared with a chemical purity of 99% showed a similar strong cytotoxic effect as the cypermethrin from Ehrenstorfer with only 91% chemical purity.

These results indicate that besides the chemical impurities also the source of the pyrethroids seems to be of considerable concern in view of cytotoxic effects and might strongly support the assumption of Surrallés et al. (1995). Therefore, with regard to toxicological valuation of pyrethroids additional factors such as chemical impurities, source as well as manufacturing process and isomer composition should be taken into consideration.

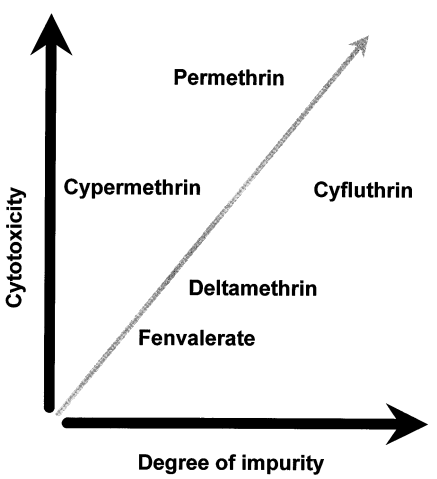


Fig. 6. Correlation between the degree of chemical impurity of pyrethroids and cytotoxic activity.

## References

Agarwal, D.K., Chauhan, L.K.S., Gupta, S.K., Sundararaman, V., 1994. Cytogenetic effects of deltamethrin on rat bone marrow. Mutation Res. 311, 133–138.

Barrueco, C., Herrera, A., Caballo, C., de la Peña, E., 1992. Cytogenetic effects of permethrin in cultured human lymphocytes. Mutagenesis 7, 433–437.

Barrueco, C., Herrera, A., Caballo, C., de la Peña, E., 1994. The induction of structural chromosomal abberations in human lymphocytes and CHO cells by permethrin. Teratog. Carcinog. Mutag. 14, 31–38.

Bolognesi, C., Parrini, M., Merlo, F., Bonassi, S., 1993. Freuqency of micronuclei in lymphocytes from a group of floriculturists exposed to pesticides. J. Toxicol. Environ. Health 40, 405–411.

Carbonell, E., Puig, M., Xamena, N., Creus, A., Marcos, R., 1989. Mitotic arrest induced by fenvalerate in human lymphocyte cultures. Toxicol. Lett. 48, 45–48.

Chauhan, L.K.S., Dikshith, T.S.S., Sundararaman, V., 1986. Effect of deltamethrin on plant cells 1. Cytological effects on the root meristem of *Allium cepa*. Mutation Res. 171, 25–30.

Gandhi, G., Chowdhury, J.B., Sareen, P.K., Dhillon, V.P.S., 1995. Genotoxic effects of deltamethrin in the mouse bone marrow micronucleus assay. Mutation Res. 346, 203–206.

Hader, C., Hadnagy, W., Seemayer, N.H., 1996. A rapid method for detection of nongenotoxic carcinogens of environmental pollutants using synchronized V79 cells and flow cytometry. Toxicol. Lett. 88, 99–108.

Hadnagy, W., Seemayer, N.H., 1986. Induction of C-type metaphases and aneuploidy in cultures of V79 cells exposed to extract of automobile exhaust particulates. Mutagenesis 1, 445–448.

Hadnagy, W., Sust, H., Seemayer, N.H., 1997. Induction of cell division disturbances in Chinese hamster lung cell line V79 by a complex mixture of polycyclic aromatic hydrocarbons and aza-heterocyclic compounds. Toxicol. Vitro 11, 703–709.

Hofman, G.R., Dellarco, V.L., Voytek, P.E., 1986. A review of the symposium on aneuploidy: etiology and mechanisms. Environ. Mutagen. 8, 643–651.

IARC, 1991. IARC Monograph on the Evaluation of Carcinogenic Risks to Humans. Vol. 53: Occupational Exposures in Insecticide Application, and Some Pesticides. Lyon, France.

Kerkfliet, N.I., Baecher-Steppan, L., Schmitz, J.A., 1982. Immunotoxicity of pentachlorphenol (PCP): increased susceptibility to tumor growth in adult mice fed technical PCP-contaminated diets. Toxicol. Appl. Pharmacol. 62, 55–64.

Melnick, R.L., Kohn, M.C., Portier, C.J., 1996. Implications for risk assessment of suggested nongenotoxic mechanisms of chemical carcinogenesis. Environ. Health Perspect. 104 (Suppl. 1), 123–134.

Oshimura, M., Barrett, C., 1986. Chemically induced aneuploidy: mechanism and biological significance in cancer. Environ. Mutagen. 8, 129–159.

Páldy, A., Puskás, N., Vincze, K., Hadházi, M., 1987. Cytogenetic studies on rural populations exposed to pesticides. Mutation Res. 187, 127–132.

Pluijmen, M., Devron, C., Montesano, R., Malaveille, C., Hautefeille, A., Bartsch, H., 1994. Lack of mutagenicity of synthetic pyrethroids in *Salmonella typhymurium* strains and V79 Chinese hamster cells. Mutation Res. 137, 7–15.

Puig, M., Carbonell, E., Xamena, N., Creus, A., Marco, R., 1989. Analysis of cytogenetic damage induced in cultured lymphocytes by the pyrethroid insecticides cypermethrin and fenvalerate. Mutagenesis 4, 72–74.

Schmid, E., Bauchinger, M., Ziegler-Skylakakis, X., Andrae, U., 1989. -Chlorobenzylidene (CS) causes spindle disturbances in V79 Chinese hamster cells. Mutation Res. 226, 133–136.

Surrallés, J., Carbonell, E., Puig, M., Xamena, N., Creus, A., Marcos, R., 1990. Induction of mitotic micronuclei by the pyrethroid insecticide fenvalerate in cultured human lymphocytes. Toxicol. Lett. 54, 151–155.

Surrallés, J., Xamena, N., Creus, A., Catalán, J., Norppa, H., Marcos, R., 1995. Induction of micronuclei by five pyrethroid insecticides in whole-blood and isolated human lymphocyte cultures. Mutation Res. 341, 169–184.