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CYTOTOXIC EVALUATION OF CERTAIN INSECT GROWTH REGULATORS AND INSECT SEX PHEROMONES IN CHO-K1 CELLS

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ABSTRACT

The cytotoxic effects of diflubenzuron, pyriproxifen as insect growth regulators, ZZ/ZE-7,11-hexadecadienyl acetate, Z-9- hexadecenal and Z-11-hexadecenal as insect sex pheromones were evaluated *in vitro* using Chinese hamster ovary (CHO-K1) cells. Total cellular protein (TCP) content and methyl tetrazolium (MTT) assays were carried out using serum free medium and medium supplemented with fetal calf serum (FCS, 10%), bovine serum albumin (BSA, 1%) and/or metabolic activation system (S9-mix). All the tested compounds displayed cytotoxic effects that rise with time exposure. TCP assay exhibited higher sensitivity than MTT assay with all the tested compounds. In presence of the added extracellular proteins and/or metabolic activation system (S9-mix), the cytotoxic effects significantly decreased which indicate that the tested agents may be binding non-specifically with protein and extensive metabolized to less toxic metabolites.

Key words: Cytotoxicity, Insect Growth Regulators, Sex pheromones, CHO-K1.

INTRODUCTION

Integrated Pest Management (IPM)-based agrochemicals include natural and synthetic compounds, i.e. insect growth regulators such as diflubenzuron and pyriproxyfen which acting as disrupters of insect molting process and insect sex pheromones which causing mating disruption of insect sexual communication behavior (Tada *et al.* 1986, Dhadialla *et al.* 1998 and Gaston *et al.* 1977). The toxicity of these compounds is quite different between target pests, non-target organisms and mammals. In case of diflubenzuron, it has been reported that such compound is safe in all acute, chronic studies conducted on experimental animals (Young *et al* 1986) and the *in vitro* genotoxic (including carcinogenic) studies (Perocco *et al* 1993). In addition, diflubenzuron, like other benzoylphenylurea derivatives, has proved to have a powerful antitumoral effect against B16 malignant melanoma (Hofs and McVie 1991) and AKR skin carcinoma in mice (Jenkins *et al.* 1984).

Unlike diflubenzuron, little is known about the toxic effects of the juvenile hormone analogue pyriproxifen. In a 6-moth chronic study, rats were found to suffer a clear weight loss, accompanied by alteration in blood parameters and enlargement of the liver, kidney and thyroid gland, although no death were reported at even the largest concentration assayed (10.000 ppm) in the study (Koyama *et al.* 1989). For insect sex pheromones, ZZ/ZE-7,11-hexadecadienyl acetate [7,11-HDDA (PB-ROPE®)] is successfully used against *Pectinophora gossypiella* (pink bollworm) in cotton field and both of Z-9-hexadecenal [Z-9-HDAL (HP-ROPE®)] and Z-11-hexadecenal [Z-11-HDAL (Virelur®)] are effective against *Heliothis armigera* (American bollworm) and *Heliothis virescens* (Tobacco budworm), respectively. In fact, it was reported that pheromones are generally high species-specific and are not likely to pose hazards to non-target organisms in the environment. Toxicological studies indicated that these pheromones are low in toxicity to mammals, birds and fish, but adequate toxicological data are necessary before they can be registered for use in insect control (Knipling, 1976). The acute toxicity of most of these compounds in experimental animals indicates that these substances can be safely managed (Shin-Etsu Chemical Co., 1995). Nevertheless the studies on the cytotoxicity of these compounds and analogues are lacking in the scientific literature.

From another point of view, today the toxic evaluation process for chemical agents using live animals is becoming less used for ethical, scientific and economical reasons which led to replacement of *in vivo* test with *in vitro* one (Balls, 1994). Now a day, *in vitro* toxicity tests using animal cell cultures have become very useful tools in the initial screening of

environmental contaminants, safety assessment and risk evaluation for their several advantages as described by several authors (Spielmann and Goldberg, 1999).

As a toxicity procedure for *in vitro* screening, several methods were established at different cellular endpoints as biological markers. For example, Total Cellular Protein content (TCP) assay as a marker of cell proliferation assay using different dyes, e.g., trypan blue, methylene blue and/or kenacid blue (Wataha *et al.* 1991). Also, it was reported the validity of the cellular mitochondria as another cellular endpoint which could be considered as a metabolic marker using Methyl Tetrazolium (MTT) assay (Bertheussen *et al.* 1997).

According to the mentioned facts, the present study aimed to evaluate the cytotoxic effects of two insect growth regulators, diflubenzuron, pyriproxifen and the insect sex pheromone, ZZ/ZE-7,11-hexadecadienyl acetate, Z-11-hexadecenal and Z-9-hexadecenal on the mammalian cellular model, CHO-K1. All the tested compounds were evaluated using total cellular protein (TCP) content and methyl tetrazolium (MTT) assays during 24, 48 and 72 h of exposure period in serum free medium and medium supplemented with fetal calf serum (FCS, 10%), bovine serum albumin (BSA, 1%) and/or metabolic activation system (S9-mix).

MATREALS AND METHODS

1. Reagents and Chemicals Used

Reagent grade chemicals and cell culture components, culture medium HAM F-12, antibiotics, trypsin/EDTA solution, HEPES buffer, methylene blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye), bovine serum albumin (BSA), NADP, glucose-6-phosphate and dimethyl sulfoxide (DMSO), were obtained from Sigma Chemical Co., (St Louis, MO USA). Fetal calf serum (FCS) was obtained from Boehringer Ingelheim GmbH (Germany).

2. Compounds Used

Five compounds were tested in form of active ingredient, i.e. diflubenzuron (Dimilin®) [1-(4-chlorophenyl)-3-(2,6-diflurobenzoyl) urea] obtained from Dr. Ehrenstorfer Reference Materials (Scharlau, Sl. La Jota, Barcelona, Spain), pyriproxifen (Admiral®) [4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether] purchased from Sumitomo Chemical Co. (Osaka, Japan), 7, 11-HDDA (PB-ROPE®) [ZZ/ZE-7,11-hexadecadienyl acetate (pheromone for *Pectinophora gossypiella*) obtained from Shin-Etsu Co. (Osaka, Japan), Z-11-HDAL (Virelur®) [Z-11-hexadecenal], (pheromone for *Heliothis virescens*) and Z-9-HDAL (HP-ROPE®) [Z-9-hexadecenal] (pheromone for *Heliothis armigera*) were obtained from Sigma Chemical Co., (St Louis, MO USA).

The primary stock solution of each compound was prepared by dissolving in sterilized DMSO, but for preparation of the subsequent dilutions, i.e. 5, 10, 50, 100, 500, 1000, 2000, 2500, 5000 and 10.000 µM, the serum free culture medium as a diluting solution was used.

3. Cell line

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, CCL61). The original of cell line belongs to the ovary of an adult Chinese hamster, *Mesocricetus auratus*. Cells were plated at 10^4 cells/cm^2 as a monolayer in 22 cm^2 polyestyrene tissue culture dishes with HAM F12 medium supplemented with 25 mM HEPES buffer (pH 7.4) and 10 % heat-inactivated fetal calf serum (FCS) and gentamycin (30 µg/ml). Cells were subcultured routinely twice a week to maintain the genetic homogeneity. Cells were counted in an improved Neubauer haemocytometer and cell viability was determined by exclusion intake of Trypan Blue dye (Freshney, 1987).

4. Cytotoxicity assays

The cytotoxicity testing of the selected compounds was carried out during 24, 48 and 72 h of exposure periods in different conditions, i.e. serum free medium, medium supplemented with fetal calf serum (FCS, 10%), bovine serum albumin (BSA, 1%) and/or metabolic activation system (S9-mix) at different concentrations.

4.1. Total cellular protein content (TCP) assay

Total cellular protein content was measured according to the method described by Pelletier *et al.* (1988) which depends on staining of the cellular proteins by methylene blue. After culturing CHO-K1 cells in a 96-well tissue culture microtiter plates at a 65% confluence (mid-log phase of growth), the medium was replaced with serum free medium containing the tested concentrations of the selected compounds. After 24, 48 and 72 h of exposure, the medium was removed and each well washed twice with 200 µl of phosphate buffer saline (PBS). Cells were fixed at room temperature by adding 200 µl of 10 % v/v formaldehyde in PBS to each well. After 10 min, the formaldehyde was removed and the cells washed twice with 200 µl of borate buffer (0.01 M, pH 8.4). The cells were stained by adding 100 µl of methylene blue (1% w/v in borate buffer) to each well. After 10 min, the methylene blue was removed and the cells were washed extensively with borate

buffer at least five times to remove the excess dye. The plates were left to dry at room temperature for 3 h. To solubilize the stain, 200 µl of 0.1 N HCl was added to each well and the plates were shaken for 15 min at room temperature. Optical density was measured using an ELISA microtiter plate reader (Merck/Mios) at 660 nm.

4.2. Methyl tetrazolium (MTT) assay

The protocol was based on a method described by Plumb *et al.* (1989) using MTT dye. This assay is depends on the cellular reduction of MTT dye by the mitochondrial dehydrogenase of viable cells to blue formazan product which measured spectrophotometrically. After culturing CHO-K1 cells in a 96-well tissue culture microtiter plates at a 65% confluence, the medium was replaced with serum free medium containing the different concentrations of the tested compounds. After 24, 48 and 72 h of exposure, the medium was removed and immediately 50 µl of MTT dye in PBS at a concentration 5 mg/ml was added to each well. The plates were wrapped in foil and incubated for 4 hours at 37°C, the solution of MTT in PBS was removed and 200 µl DMSO per well was rapidly added, followed by 25 µl of Sorensen`s glycine buffer (0.1 M glycine plus 0.1 M NaCl equilibrated to pH 10.5 with 0.1 M NaOH). After rapid agitation of the plates for 15 min at room temperature, the optical density of the blue formazan product was measured using an ELISA microplate reader (Merck/Mios) at 570 nm.

4.3. Preparation of metabolic activation system (S9-mix)

S9-mix (microsomal fraction) was prepared on the day which the assay is to be performed as described by Venitt *et al.* (1984). Three separate concentrations of S9-mix were used, 4, 10 and 30% which construct a final concentration of 10% in the used serum free medium. Each S9-mix concentration contained 1 M KCl, 0.25 M $MgCl_2$, 0.2 M glucose-6-phosphate, 0.04 M NADP, 0.2 M $NaHPO_4$, deionized water and S9 fraction, derived from homogenized rat livers (Sprague-Dawley rats, 6-8 weeks old weighing 200 ± 15 g) after their treatment with Aroclor 1254 (Sigma Chemical Co.) at dose level 500 mg/kg body weight using an peanut oil as solvent. One ml of freshly prepared S9-mix was incubated with the stock concentration of each compound (freshly prepared using a serum free medium) during a period of 24 h at 37°C before starting the TCP assay.

4.4. Determination of cytotoxicity values and the statistical analysis

To evaluate the cytotoxicity of the tested compounds, 10 concentrations of each compound was prepared, i.e. 5, 10, 50, 100, 500, 1000, 2000, 2500, 5000 and 10.000 µM. Four separate experiments and four replicates were performed for each compound. The determination of the cytotoxicity values were depended on the obtained absorbance of the extracted dye, i.e. formazan from the mitochondria or methylene blue from the cellular proteins, which in turn could be quantified the number of surviving, undamaged, viable cells and then could be estimate the percent of cellular mortality. The percentages of cellular mortality corresponding to the absorbance values obtained were used to plot the toxicity regression lines in the form of a probit/logarithmic relationship. The midpoint cytotoxicity values, [LC_{50}, and/or MTT_{50}] which reflect the percent of cellular mortality were defined as a concentration of test agent needed to reduce the absorbance of extracted dye by 50%, as compared to control value. The differences between the cytotoxicity values at 48 and 72 h were compared with the values obtained at 24 h and statistically analysed by student *t*-test using the computer program Sigma Plot for Windows, (Version 2.0).

RESULTS

Data in Table (1) shows the midpoint cytotoxicity values of diflubenzuron, pyriproxifen and ZZ/ZE-7,11-hexadecadienyl acetate which were evaluated using total cellular protein content (TCP) and MTT assays in CHO-K1 cells. Through TCP assay, diflubenzuron was the most toxic compound than ZZ/ZE-7,11-hexadecadienyl acetate while pyriproxyfen was the less toxic substance meanwhile by MTT assay diflubezuron was the less toxic compound than the two other agents. However, the cytotoxicty of the tested compounds was significantly a time-dependent. For example, after 48 h the toxicity of diflubenzuron, pyriproxyfen and ZZ/ZE-7,11-hexadecadienyl acetate as determined by TCP assay significantly increased by 244, 9.76 and 4.10-folds while throughout MTT assay, the toxicity significantly elevated by 16.87, 8.08 and 3.40-folds, respectively.

In addition, TCP assay exhibited the higher sensitivity than MTT assay with the three tested compounds after 24, 48 and 72 h by 63.90, 9.23 and 45.59-folds for diflubenzuron, 2.10, 2.53 and 1.85-folds for pyryproxyfen and 3.06, 3.69 and 3.16-folds for ZZ/ZE-7,11-hexadecadienyl acetate, respectively. On the ground of the high reliability of TCP assay, LC_{50} values of the tested compounds were used to analyze the effect of 10% FCS and 1% BSA on the sensitivity of CHO-K1 cells to those compounds.

After the extracellular proteins, i.e. 10% FCS and 1% BSA were added in the incubation medium as a manner to study the binding of the tested substances with proteins, the cytotoxicity of the mentioned agents exhibited a time-dependent relation but in form of significant reduction of their toxic effects in CHO-K1 cells at all the mentioned exposure periods (Table 2).

Such reduction caused by 10% FCS and 1% BSA after 24 h could be calculated by 20.58, 57.72-folds for diflubenzuron, 1.91 and 2.59-folds for pyriproxyfen and 1.88 and 3.96-folds for ZZ/ZE-7,11-hexadecadienyl acetate, respectively. The same trend but to lower extent was observed after 48 and 72 h. However, in spite the cytotoxic reduction caused by adding the extracellular proteins, diflubenzuron showed the lower cytotoxic compounds followed by pyriproxyfen and ZZ/ZE-7,11-hexadecadienyl acetate.

Moreover, it is clear that 1% BSA caused higher cytotoxic reduction than 10% FCS which calculated after 24, 48 and 72 h by 2.80, 4.37 and 4.57-folds for diflubenzuron, 1.36, 7.61 and 5.72-folds for pyriproxyfen and 2.11, 3.30 and 2.69-folds for ZZ/ZE-7,11-hexadecadienyl acetate, respectively.

When the rat liver microsomal (S9) fraction was added at different concentrations, i.e. 4, 10 and 30% to the tested substances in serum free medium before exposing to CHO-K1 cells, the cytotoxicity was significantly reduced and diflubenzuron exhibited the lower toxic compound (Table 3). According to the mentioned conditions related to the incubation the tested compounds with S9 mix, protection against these agents was found to be higher than 1, 2 or 5 mM for diflubenzuron after 24 h as effect of the added 4, 10 and 30% of S9 fraction, respectively. The same trend was observed for pyriproxyfen and ZZ/ZE-7,11-hexadecadienyl acetate but to lower extent when the midpoint cytotoxicity values were higher than 1 mM.

To differentiate between the cytotoxicity of the two isomers of hexadecenal pheromones, i.e. *cis*-9- and *cis*-11-, it was determined the midpoint cytotoxicity values of these isomers in serum free medium and in presence of the higher concentrations of rat liver microsomal S9 fraction, i.e. 30% (Table 4). The obtained results indicate that the tested S9 fraction significantly reduced the toxic effect of the tested isomers to be 7.76, 5.40 and 3.75-folds lower toxicity for *cis*-9-hexadecenal and 5.79, 8.47 and 9.23-folds lower toxicity for *cis*-11-hexadecenal after 24, 48 and 72 h, respectively.

Table (1): Cytotoxicity of diflubenzuron, pyriproxyfen and ZZ/ZE-7,11-hexadecadienyl acetate in CHO-K1 cells.

Compound	Time of Exposure (Hour)	Midpoint Cytotoxicity Values (μM)		Published Acut oral LD_{50} (mg/kg) rats
		TCP	MTT	
		LC_{50}	MTT_{50}	
Diflubenzuron	24	48.51 ± 5.01	$3100.00^{\#\#\#}$ ± 90.00	> 4640 Tomlin (1997)
	48	19.89^{**} ± 2.69	$183.70^{***\ \#\#\#}$ ± 4.23	
	72	5.65^{**} ± 0.57	$257.60^{***\ \#\#\#}$ ± 7.81	
Pyriproxyfen	24	401.99 ± 18.36	$844.79^{\#\#}$ ± 13.52	> 5000 Tomlin (1997)
	48	41.15^{**} ± 0.81	$104.50^{**\ \#\#}$ ± 2.88	
	72	66.38^{**} ± 10.47	$122.75^{**\ \#\#}$ ± 1.13	
ZZ/ZE-7,11-hexadecadie-nyl acetate	24	280.59 ± 22.57	$859.83^{\#\#}$ ± 6.91	5000 Shin-Etsu Chemical Co., (1995)
	48	68.34^{**} ± 5.85	$252.35^{**\ \#\#}$ ± 2.32	
	72	63.80^{**} ± 1.69	$211.53^{**\ \#\#\#}$ ± 8.76	

Each midpoint cytotoxicty value is an average of four experiment by triplicates ± S.D.
TCP: Total Cellular Protein Assay, **MTT**: Methyl Tetrazolium Assay. Comparing to values obtained after 24 h, ***: Highly significant ($p \leq 0.001$), **: Moderately significant ($p \leq 0.01$). Comparing to values obtained by TCP assay, ###:Highly significant ($p \leq 0.001$), ## Moderately significant ($p \leq 0.01$) (student *t*-test).

Table (2): Effect of 10% FCS and 1% BSA on the cytotoxicity (LC_{50}) of diflubenzuron, pyriproxyfen and ZZ/ZE-7,11-hexadecadienyl acetate in CHO-K1 cells.

Compound	Medium Content	LC_{50} (µM)		
		24 hr	48 hr	72 hr
Diflubenzuron	Serum Free	48.51 ± 5.01	19.89 ± 2.69	5.65 ± 0.57
	FCS 10 %	998.33^{***} ± 1.66	405.46^{***} ± 18.86	244.29^{***} ± 5.74
	BSA 1 %	$2800.00^{***###}$ ± 18.17	$1772.26^{***###}$ ± 18.36	$1117.14^{***###}$± 17.82
Pyriproxyfen	Serum Free	401.99 ± 18.36	41.15 ± 0.81	66.38 ± 10.47
	FCS 10 %	768.44^{*} ± 88.46	128.54^{***} ± 1.36	117.77^{**} ± 10.56
	BSA 1 %	$1042.83^{***###}$ ± 49.00	$978.13^{***###}$ ± 22.35	$673.10^{***###}$ ± 46.99
ZZ/ZE-7,11-hexadecadie-nyl acetate	Serum Free	280.59 ± 22.57	68.34 ± 5.85	63.80 ± 1.69
	FCS 10 %	527.93^{**} ± 12.41	183.08^{**} ± 2.20	139.05^{**} ± 4.17
	BSA 1 %	$1113.48^{***###}$ ± 6.67	$606.76^{***###}$ ± 4.03	$375.00^{***###}$ ± 3.49

Each midpoint cytotoxicty value is an average of four experiment by triplicates ± S.D. **FCS**: Fetal Calf Serum, **BSA**: Bovine Serum Albumin. Comparing to values obtained in serum free medium, ***: Highly significant ($p \leq 0.001$), **: Moderately significant ($p \leq 0.01$). Comparing to values obtained after 24 h, ###: Highly significant ($p \leq 0.001$), ##: Moderately significant ($p \leq 0.01$) (student *t*-test).

Table (3): Effect of different concentrations of rat liver microsomal S9 fraction on cytotoxicity of diflubenzuron, pyriproxyfen and ZZ/ZE-7,11-hexadecadie-nyl acetate determined by TCP assay in CHO-K1 cells.

Compound	S9-mix	LC_{50} (µM)		
		24 hr	48 hr	72 hr
Diflubenzuron	No	48.51 ± 5.01	$19.89^{\#}$ ± 2.69	$5.65^{\#\#}$ ± 0.57
	4%	1680.16^{***} ± 119.83	$978.59^{***\#}$ ± 21.41	$743.46^{***\#}$ ± 47.80
	10%	2755.00^{***} ± 25.50	$1148.24^{***\#\#}$ ± 56.8	$1091.51^{***\#\#}$ ± 87.86
	30%	5950.00^{***}± 18.50	5450.00^{***} ± 50.00	$834.82^{***###}$ ± 39.16
Pyriproxyfen	No	401.99 ± 18.36	$41.15^{\#\#}$ ± 0.81	$66.38^{\#\#}$ ± 10.47
	4%	1340.04^{**} ± 15.42	$789.95^{***\#\#}$ ± 10.30	$628.02^{***\#}$ ± 89.76
	10%	1514.90^{**} ± 40.86	$690.46^{***\#}$ ± 66.73	$535.67^{***\#}$ ± 34.485
	30%	4400.00^{***} ± 15.00	$1404.19^{***###}$ ± 73.81	$1667.97^{***###}$ ± 32.03
ZZ/ZE-7,11-hexadecadie-nyl acetate	No	280.59 ± 22.57	$68.34^{\#\#}$± 5.85	$63.80^{\#\#}$ ± 1.69
	4%	1184.21^{***} ± 24.96	891.92^{***} ± 69.68	934.13^{***} ± 75.12
	10%	773.53^{**} ± 33.48	639.71^{***} ± 74.77	613.31^{***} ± 17.65
	30%	924.64^{***} ± 18.09	$265.38^{***\#\#}$ ± 27.71	$279.16^{***\#\#}$ ± 85.55

Each midpoint cytotoxicty value is an average of four experiment by triplicates ± S.D. Comparing to values obtained in serum free medium, ***: Highly significant ($p \leq 0.001$), **: Moderately significant ($p \leq 0.01$). Comparing to values obtained after 24 h, ###: Highly significant ($p \leq 0.001$), ##: Moderately significant ($p \leq 0.01$) (student *t*-test).

Table (4): Cytotoxicity of *cis*-9-hexadecenal and *cis*-11-hexadecenal determined by TCP assay in CHO-K1 cells in absence of protein and in presence of 30% rat liver microsomal S9 fraction.

Compound	Medium Content	LC_{50} (μM)		
		24 hr	48 hr	72 hr
cis-9-hexadecenal	Serum free	123.09 ± 19.11	108.95± 8.26	153.32 ± 24.50
	S-9 30 %	955.04*** ± 7.618	588.44** ± 5.86	575.68** ± 3.46
cis-11-hexadecenal	Serum free	311.93 ± 51.14	139.82 ± 3.17	128.14* ± 17.42
	S-9 30 %	1805.91** ± 14.64	1184.57*** ± 14.64	1183.14*** ± 11.01

Each midpoint cytotoxicty value is an average of four experiment by triplicates ± S.D. Comparing to values obtained in serum free medium, ***: Highly significant ($p \leq 0.001$), **: Moderately significant ($p \leq 0.01$) (student *t*-test).

DISCUSSION

The obtained midpoint cytotoxicity values of TCP assay showed that diflubenzuron is more cytotoxic than ZZ/ZE-7,11-hexadecadienyl acetate, while pyriproxifen was the less toxic compound to CHO-K1 cells. In contrarily, MTT assay exhibited that either pyriproxifen and/or ZZ/ZE-7,11-hexadecadienyl were significantly more toxic than diflubenzuron. It was found that the determined midpoint cytotoxicity values of TCP assay were in harmony with the published *in vivo* acute oral LD_{50} which confirmed that diflubenzuron is more toxic than ZZ/ZE-7,11-hexadecadienyl acetate, and/or pyriproxifen (Tomlin 1997, Shin-Etsu Chemical Co., 1995).

In addition it was observed that TCP is more sensitive assay than MTT. This sensitivity may due to the fact that the cell protein content increases exponentially in actively growing cultures, so that, growth inhibition is indicated by a lack of increase of cell protein. In addition, with cells that grow attached to a substratum, cell death is typically accompanied by detachment, thus loss of cell protein can serve as a quantitative indicator of cell death. Furthermore, cell protein synthesis is a continuous function in actively growing cultures. Because it requires complex cellular machinery, it is subject to inhibition by a wide variety of toxic process, and thus is a sensitive and general indicator of cell damage (Shopsis and Eng, 1985). Moreover, inhibition of cell protein synthesis has been demonstrated to be reproducible toxic endpoint in a large interlaboratory *in vitro* toxicity research project (Balls and Bridges, 1984). Also, when several methods have been used to measure the cytotoxicity of a variety of chemicals, it was reported that the determination of total cellular protein content was the method chosen, since it was considered to be rapid, reproducible, did not require radioactive chemicals and because a high degree of automation seemed feasible (Knox *et al.* 1986).

On the other hand, the lower sensitivity of MTT assay to the tested substances, may be due to several factors, e.g. the lower and poor solubilization of the resulted formazan product (Denizote and Lange, 1986). In addition, since MTT assay does not include a fixation step, cells tend to detach from the surface of the culture plates during the formazan solubilization procedure. Also, Petty *et al.* (1995) found that formazan production by cells was very little to de detected spectrophotometrically when the cell number in each well of the microtiter plate was less than 25000 cell. Also, a high possibility that the mitochondria could be malformed as a side effect to the tested compounds which may lead to a false positive results (Walton and Buckley, 1975). Finally, the MTT assay is still in the developmental stages, where various modifications being recommended for formazan solubilization. For example, the suggested solvents include HCl-isopropanol (Mosmann, 1983), ethanol (Denizote and Lange, 1986), DMSO-mineral oil (Charmichael *et al.* 1987).

Comparing between the cytotoxicity values of the tested compounds after adding extracellular proteins (10 % FCS and/or 1 % BSA) in the incubation medium, it was observed a protection role of proteins, especially albumin against the tested compounds.

Such protection may be attributed to non-specific binding of the tested compounds with protein as mentioned by Hayes (1975) which demonstrated that many foreign compounds are bound to proteins especially albumin. Also, it was reported that nonspecific binding protein in whatever tissue, reduces the concentration of compound available for reaction at a specific site. So that, the protection conferred by extracellular proteins (FCS or BSA) may explain the low toxicity of diflubenzuron *in vivo*. Binding to serum or tissue proteins is known to afford protection from toxic compounds by diminshing the interaction between the free active fraction and its molecular targets. The results observed here provide an explanation for the low acute toxicity of diflubenzuron in the experimental animal and the large doses required to reduce experimental B16 melanomas or skin tumors (800 mg/kg) in mice (Jenkins *et al.* 1984). Moreover, 24 h incubation of CHO-K1 cultures with the products resulting from of phase I metabolism was observed to give rise to significantly less toxic products that effectively protect non-target animals against the presence of diflubenzuron in the environment (Bayoumi *et al.* 2003).

From another viewpoint, the observed reduction in the cytotoxic parameters of the tested compounds after their incubation with different concentrations of the metabolic activation system (microsome fraction or S9-mix), may be due to the degradational enzymatic reaction caused by the cytochrome P450/448 included in the tested S9 fraction. Such interpretation is supported by the obtained results, which indicated that the cytotoxicity dropped as the presence and/or percentage of S9-fraction rose. The same observation was reported by Perocco *et al.* (1993) who demonstrated that diflubenzuron and other pesticides exhibited their cytotoxic effects to BALB/c 3T3 cells in the absence of S9-mix following colonies scoring & counting and cell transforming techniques. Similarly, In an i*n vitro* studies of pyriproxifen metabolism using housefly microsome, it was found that resistant strains accumulate more pyriproxifen hydroxyl derivatives than the wild type or susceptible houseflies, suggesting oxidative deactivation of the parent compound by the microsomal cytochrome P450 monooxygenase (Zhang *et al.* 1998). Moreover, the lower cytotoxic effect of pyriproxifen may be due to its role in increasing the cellular protein, e.g. albumin and alpha globuline as described by the *in vivo* study of Koyama *et al.* (1989).

In case of the tested lepidopteran pheromones, the cytotoxic effect is both dose- and time-dependent and the presence of the added proteins in the culture medium significantly reduced the lethal effects of the tested pheromone. No toxicokinetic studies have been performed in experimental animals to date, but the binding to serum proteins after absorption may be one of the reasons for their low toxicity after acute or chronic exposure (Abdel-Ghani *et al.* 2004). In addition, in an *in vitro* study, it was observed a conversion of pheromonal aldehydes (Z)-11-hexadecenal to carboxylic acid in tissue extracts of *Heliothis virescens* which is catalyzed by both aldehyde dehydrogenase and aldehyde oxidase enzymes (Tasayaco and Prestwich 1990, Bayoumi *et al.* 2002). Furthermore, due to the long hydrophobic aliphatic chain of the majority of insect sex pheromone, resembling normobiotic fatty acids, these compounds can be carried and/or distributed by serum albumin, reducing undesirable effects. In addition, the preincubation of such pheromone with microsomal enriched rat liver S9 fraction, originated non-cytotoxic metabolites (concentration dependent), thus suggesting hepatic degradation of these substances, which would explain their organic safety. The hydrolysis and oxidation of functional groups, i.e. acetate or aldehyde, were assayed *in vitro*, incubating ZZ/ZE-7,11-hexadecadienyl acetate with a carboxylesterase (Vogt *et al.* 1985) and a yeast aldehyde dehydrogenase (Blatter *et al.* 1990) respectively, 30 min before incubation with cells. After effective hydrolysis or oxidation of these functional groups the cultures suffered no-lethal consequences, indicating that the pheromone in this form has less adverse effects on the cells.

In conclusion, the obtained results indicate the utility and high sensitivity of total cellular protein content assay using CHO-k1 cells in presence of the extra-cellular proteins and the metabolic activation system (S9-mix) in the rapid screening procedures of xenobiotic.

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